PREPARATION OF MAHUWA *(Madhuca longifolia)* FLOWER WINE FROM ISOLATED NATIVE YEAST

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Approval Letter

This ***dissertation*** entitled ***Preparation of Mahuwa (Madhuca longifolia) Flower Wine from Isolated Native Yeast*** presented by **Aadarsha Paudel** has been accepted as the partial fulfillment for the **B. Tech. degree in Food Technology**

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Abstract

Mahuwa (*Madhuca longifolia*) flowers and *murcha* samples from Gadhawa Gaunpalika, Ward No. 08 (followed by a brief survey) were screened for fermentative yeast to study the effect of different yeasts and pH levels on the chemical and sensory quality of mahuwa wine. Yeasts were screened by culturing on MYPG and molasses agar media at 30°C for 2-3 days. The isolates that could ferment molasses broth at a low pH were utilized for must fermentation, while the others were rejected. Yeast isolates were characterized based on colony morphology, microscopic characteristics, fermentative capacity. Furthermore, mahuwa juice was subjected to a 21-day of the fermentation period at room temperature (25±2°C), nine (9) treatment combinations were used, each including three varieties of yeasts, viz., Fy_1 (mahuwa flower yeast), My_2 (*murcha* yeast), and Wy_3 (commercial wine yeast) at three pH values (3, 3.5, and 4). Fermentation kinetics for total soluble solids (TSS), titratable acidity and yeast growth were also studied. The quality of wines prepared using Fy_1, My_2, Wy_3 were assessed and compared by chemical analyses (ethanol, higher alcohols, esters, aldehydes, total phenolic content, and acidity) and sensory evaluation (7-point hedonic rating for the attributes appearance, odor, mouthfeel, finish, and overall acceptance).

The survey showed engagement of 53% of the households of Gadhawa Gaunpalika, Ward No. 08 in mahuwa spirit production, with an annual income of ~ NRs 40000.0 per household. Variations in yeast strain and pH of mahuwa must had a considerable ($p \leq 5\%$) effect on wine quality and fermentation kinetics. After 21 days of fermentation of mahuwa flower juice, sample E (fermented using isolated *murcha* yeast at pH 3.5) wine was found to have the best sensory quality. Upon physicochemical analysis, the best sample had the following characteristics: ethanol (12.13±0.23% abv), total titratable acidity (0.97±0.07% as lactic acid), volatile acidity (0.083±0.006% as acetic acid), fixed acidity (0.84±0.08% as lactic acid), total phenolic content (250.7±5.3 mg GAE/L) of wine), esters (236±6.7 g ethyl acetate/100 L), aldehyde (148.8±4.3 g aldehyde /100 L) and higher alcohol 181.7±6.6 mg/L. It can be concluded that all fermentative yeast types can be used to prepare mahuwa wine, but a pH of 3.5 appears to be the best condition. Further, the physicochemical properties (ethanol, methanol, esters, aldehydes, etc., in particular) either do not necessarily relate or are too complex to relate to the sensory quality of mahuwa wines.

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List of abbreviations

Abbreviation	Full form
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
AWRI	Australian Wine Research Institute
GAE	Gallic acid equivalent
DMAB	*p*-dimethylaminobenzaldehyde
TPC	Total phenolic content
TSS	Total soluble solids

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PART I

Introduction

1.1 General introduction

Mahuwa (*Madhuca longifolia*), also spelt mahua, is a multipurpose tropical tree native to South Asia that is primarily cultivated or harvested in the wild for it edible flowers and oil seeds (Gedela *et al.*, 2016) and can reach a height of 16-20 m. Flowers are borne on green or pink furry bunches with 12 fragrant cream-colored flowers in each bunch. The flowers only live for one night before dying and falling to the ground. Pollinated flowers produce a fleshy, greenish ovoid fruit with 1-4 shiny, oily brown seeds (Heuzé *et al.*, 2016)

Mahuwa is indigenous to Nepal, Sri Lanka, India and Myanmar. It is a frost-resistant species that can grow in the margins of dry tropical and subtropical forests at elevations ranging from 1200 to 1800 m. It can be found in pastures, crop fields, and river banks in semi-evergreen forests in central India (Heuzé *et al.*, 2016). It thrives in climates with annual rainfall ranging from 500 mm to 1500 mm and temperatures ranging from 2 to 46°C (Orwa *et al.*, 2009). In Nepal, mahuwa is abundantly found in the Terai region of Province 2 (renamed Madhesh Province), Gandaki, Lumbini Some major districts are Dang, Banke, Kapilbastu, Rupandehi, Nawalparasi, Chitawan, Parsa, Siraha, Saptari, and Dhanusha (Kandel, 2021).

Mahuwa has a variety of chemical compounds that are responsible for its therapeutic effects. Terpenoids, proteins, starch, anthraquinone glycosides, phenolic compounds, mucilage cardiac glycosides, tannins, and saponins are all present. The bark can be used to treat phlegm, itching, swelling, fractures, snake bites, diarrhea, chronic tonsillitis, leprosy, fever, and rheumatism. It has been reported that mahuwa has wound-healing, antibacterial, antioxidant, anti-inflammatory, anticancer, and antidiabetic properties. Its blossoms are tonic, aphrodisiac, astringent, and cooling (Khare *et al.*, 2018). Descriptions on phenolic compounds in relation to alcoholic fermentation are given by Czyzowska and Pogorzelski, (2002), Ginjom *et al.* (2011), Pérez-Gregorio *et al.* (2011), Soni and Dey (2013), and Manoj (2018).

Wine without qualification means the end products of complete or partial fermentation of fresh grape juice. Wines produced by the fermentation of juices of fruits, berries, honey, etc., are required to indicate source (the raw materials) on the label. For example, orange wine, pear wine, apple wine (cider/cyder), etc. (Rai, 2012)

Mahuwa flowers are rich in total sugars (68-72%), out of which maximum proportion is reducing sugars. Sugars identified are sucrose, maltose, glucose, fructose, arabinose and rhamnose. When flowers are mature and ready to fall, there is maximum total sugar content in the flowers. The majority of dried mahuwa flowers are used in the production of country liquor (Singh *et al.*, 2013).

1.2 Statement of the problem

Wine is mostly made from grapes, apple, apricot, pomegranate, and other fruits. Fruit wine is widely produced and consumed in industrialized countries around the world. However, there has been relatively little work done to exploit mahuwa for value addition. The advantages of maximal mahuwa productivity, cheap production costs, and therapeutic characteristics may have a large potential for mahuwa wine preparation in the wine sector (Belkhede, 2019).

Although Nepal's traditional alcoholic beverages have a long history, manufacturing is still limited to small-scale home brewing. One important impediment to their progress is an absence of research in the field (Dangol, 2006). Alcoholic fermentation research can help to eliminate the limitations of existing processes, retain ancestral knowledge through documentation, improve product understanding, and so optimize technology and deliver quality improvement (Dangol, 2014). Mahuwa is solely used to make country liquor (Chaudhary, 2021). Even though it offers several health benefits, it is underutilized. In the context of Nepal, research on mahuwa goods, even in *raksi*, is very scarce. There is limited information available on mahuwa wine preparations.

1.3 Objectives of the study

1.3.1 General objective

The main objective of the present work was to study the effect of different yeast and pH levels on chemical and sensory quality of mahuwa wine.

1.3.2 Specific objectives

The specific objectives were to:

1. Conduct a brief survey on the production of mahuwa spirit by the natives of Gadhawa Gaunpalika (Dang district)
2. Isolate the native fermentative yeast from mahuwa flower.
3. Isolate fermentative yeast from *murcha* indigenous to Gadhawa Gaunpalika.
4. Carry out sensory and physicochemical analysis of the prepared mahuwa wines.

1.4 Significance of the study

The native yeast flora of mahuwa flower can produce brandy of exceptional quality. Wine of good taste can be prepared from mahuwa flowers, but its clarifications is difficult. At the moment, only brandy production seems suitable. If clarification can be achieved by some means, mahuwa flower can be used for commercial wine production. Some basic scientific inputs such as sanitations, use of controlled environment for fermentation, use of pure culture, etc., can improve the traditional technology of brewing of mahuwa wine and brandy (Karmayogi, 2003)

Mahuwa flowers are high in total sugars, with a high amount of reducing sugars. Sucrose, maltose, glucose, fructose, arabinose, and rhamnose are the sugars recognized. It is critical to identify the potential of mahuwa flowers in the food business. A variety of food products, including juice concentrate, jam, jelly, sauce, and mango-mahuwa leather, can be created.

Indigenous fermented food products promote sustainable development in developing countries by providing employment opportunities, thereby improving citizens' livelihoods, poverty alleviation, empowerment initiatives, market improvement through the use of simple, low-cost, traditional food processing techniques, and enhanced food security, which provides producers with consistent income. Fermentation of indigenous fermented foods improves their organoleptic and preservation qualities, hence increasing their nutritional quality (Adesulu and Awojobi, 2014).

Sugar profiling of mahuwa flower revealed that it is a significant source of both reducing and non-reducing sugar (inositol, sorbitol, mannitol, dextrose, fructose, sucrose, raffinose and maltose), demonstrating that mahuwa flower juice can be used as a natural sweetener in the development of various industrial food products (biscuits, cookies, cake, jam, jelly, juice,

squash, and so on). The qualitative study revealed that mahuwa is rich in various minerals such as sodium, potassium, magnesium, calcium, nitrogen, hydrogen and carbon. Mahuwa flowers are also rich in total phenolic content, flavonoids and also antioxidant activity (Singh *et al.*, 2020).

In spite of being a rich source of nutrition and easy availability in the rural areas, these flowers are not very popular as food. Only a small quantity of flowers is consumed raw, cooked or fried in different parts of India (Patel and Naik, 2010) and Nepal. Major quantity of flowers is used in the preparations of distilled liquors. Apart from food and liquors, the flowers are also used as a sources of cattle feed (Patel and Naik, 2010) and as fertilizer as intercrop (Khare *et al.*, 2018). The flowers are used as a tonic, analgesic, and diuretic; the bark is used to treat rheumatism, chronic bronchitis, and diabetes mellitus (Khare *et al.*, 2018).

In India, mahuwa trees can be found in large numbers in the states of Uttar Pradesh, Madhya Pradesh, Orissa, Jharkhand, Chhattisgarh, Andhra Pradesh, Maharashtra, Bihar, West Bengal, Karnataka, Gujarat, and Rajasthan, and the estimated annual production of mahuwa flowers is 45000 million tons during the seasons (Pinakin *et al.*, 2018). The majority of mahuwa flower research has focused on early chemical analysis of dried flowers and sugar syrup. One of the most neglected nutrient sources is mahuwa flowers.

1.5 Limitations of the study

- Variations in wine quality from different mahuwa tree species were not studied.
- Only a few process factors, such as TSS, pH, and culture, were studied.

Part II

Literature review

2.1 Mahuwa

Madhuca longifolia, locally termed as mahuwa or butter nut tree, belongs to family Sapotaceae. These are medium evergreen deciduous tree, distributed widely across India, Nepal, and Sri Lanka. Various parts of mahuwa are used in traditional and folklore system of medicine, due to its various pharmacological properties. Therefore, it is also termed as universal panacea of ayurvedic medicine (Jha and Mazumder, 2018). This tree's flowers are treasured as a source of food by tribal groups across India.

It is a multipurpose tree that meets three basic needs of tribal people: food, fodder, and fuel (Patel *et al.*, 2011). Flowers are edible and have a high nutritional value, having a large number of sugars and a good amount of vitamins, proteins, minerals, and fats (Patel *et al.*, 2011). Because of the greater sugar content, the flowers are used as a sweetener in the preparation of many traditional Indian foods such as *halwa*, *kheer*, *meethi puri*, and *barfi* throughout India's mahuwa producing belt (Madhumita Patel, 2008). *Mahuwa* has many properties such as analgesic activity, antibacterial activity, hepatoprotective activity and antioxidant activity (Madhumita Patel and Naik, 2010).

Mahuwa leaves are coriaceous, elliptic, acuminate, and base cuneate, and are grouped at the ends of the branches. Berries are ovoid, meaty, green berries with seeds. Mahuwa flowers are abundant and drop on pedicels near the tips of the branches. Coriaceous, thickly coated rusty tomentum calyx (Yadav *et al.*, 2011). When ripe, the corolla is tubular, fleshy, cream-colored or pale yellow, and scented. The dried blossom has a dark reddish brown color and shrinks in size, similar to raisins (Belkhede, 2019).

2.2 Sugar profile

Dextrose, levulose, maltose, sucrose, pentoses, and cellulose are found at various stages of flower development. The total sugar level rises as the flowers develop and reaches its peak when the flowers are ready to drop. Levulose is always present in greater amounts than dextrose during the developing stages. The quantities approximate each other at the last stage, but they do not become equal. The percentage of sucrose grows till the corolla is shed.

After that, and during storage, the proportion of sucrose to invert sugar decreases (Fowler *et al.*, 1920)

Fresh mahuwa flowers contain 73.6% moisture and 18.5-19.8% sugar. The dried form contains 22.7% moisture and 72-83% sugar (Belkhede, 2019). Total reducing sugar (TRS) content was in the range of 57.09 to 70.29%. An average 40-72% of TRS was reported by different researchers from different geographical regions. Reducing Sugar (RS) ranges from 36.04 to 47.34% and Non-Reducing Sugar (NRS) from 17.11 to 27.56% in mahuwa flowers. Similarly, RS 48-57% and slightly lower NRS 3-18% have been previously reported (Mokat, 2020).

2.3 Chemical composition/ constituents

The biochemical content of mahuwa flowers changes depending on the conditions under which they are maintained, such as fresh, sun-dried, or stored flowers (Belkhede, 2019). Mahuwa can be a good source of nutrients. Mahuwa flower was found comparatively more potent than fruit in terms of nutritional quality. Qualitative study has revealed that mahuwa is rich in various minerals such as sodium, potassium, magnesium, and calcium (Singh *et al.*, 2020). An excellent review of chemical constituents of mahuwa can be found in Mohd *et. al.* (2020). The nutritional components of fresh and dry mahuwa flowers are given in Table 2.1. The essential amino acid profile of the flowers is shown in Table 2.2. The pH of mahuwa flowers is 4.6.

2.4 Ethnomedical uses of different parts of mahuwa

Mahuwa plant has many ethnomedicinal values, some of which have been described in the following paragraphs.

Mahuwa flower: The edible corollas of mahuwa flowers, are a staple of traditional diets. They can be eaten raw or cooked, and syrups can be made for medicinal purposes. They're also extensively employed in the manufacturing of country liquor (Patel and Naik, 2010). After drying the flowers in the sun, they are boiled with *Tamarindus indicus* and *Shorea robusta* seeds. This is primarily stored by the impoverished as a grain replacement. The blossoms were fermented and made into alcoholic beverages. The dried flower powder can be made into *halua* (a sweet dish). Mahuwa flowers are used to treat eye disorders and impotency. Analgesic, diuretic, aphrodisiac, demulcent, astringent, and cooling properties

are all found in the blooms. The juice of the mahuwa flower is effective in treating skin conditions. Unmarried girls and boys may be able to marry soon if they dance around the tree, according to traditional beliefs (Khare *et al.*, 2018).

Table 2.1 Mahuwa flower nutritional components

Constituents	Fresh flowers	Dry flowers
Moisture	73.6-79.82 (%, d. b.)	11.61-19.8 (%, w. b.)
Starch (g/100 g)	0.94	-
Total sugar (g/100 g)	47.35-54.06	41.62
Reducing sugar (g/100 g)	36.3-50.62	28.12
Total inverts (%)	54.24	-
Sucrose (%)	3.43	-
Protein (%)	6.05-6.37	5.62
Fat (%)	1.6	0.09-0.06
Fiber (%)	10.8	-
Calcium (mg/100 g)	45	0.14-8
Phosphorus (mg/100 g)	22	0.14-2
Carotene (µg/100 g)	307	-
Vitamin-C (mg/100 g)	40	7

Sources: Hiwale (2015), Johar and Kumar (2020), and Swain *et al.* (2007)

Table 2.2 Essential amino acid pattern of mahuwa flower

Amino acid	g/100 g protein
Isoleucine	7.91
Leucine	12.98
Lysine	4.67
Phenylalanine	6.4
Tyrosine	3.94
Cystine	3.35
Methionine	1.8
Threonine	5.86
Valine	7.25

Source: Jayasree *et al.* (1998)

Mahuwa fruits: The inner part of the fruit is used to make cakes, while the outer part is eaten raw. The fruits are used as an astringent, in the treatment of tonsillitis, and as a lotion for chronic ulcers. Mahuwa oil is used in cooking, making margarine, hair oil, soap, and lighting lamps (Khare *et al*., 2018).

Mahuwa seed: *M. indica* and *M. longifolia* are prized for their seeds, which produce mahuwa butter/mahuwa fat, a fatty oil. This is also known as illipe butter, which has caused some confusion because illipe is also the name of a widely traded and truly valued confectionary fat derived from *Shorea stenopiter* (Patel and Naik, 2010).

Mahuwa stem barks: Diabetes mellitus can be healed with a stem bark decoction. Its paste can be used as a scorpion sting antidote. It's also used to treat ulcers and tonsillitis (Prashanth *et al*., 2010). The wood is used to build houses, cartwheels, and doors. It's an excellent source of nitrogen fixation. The tree's various parts are utilized as cattle fodder, fertilizer, and intercrop. As a result, mahuwa tree reduces soil erosion and also aids with nitrogen fixation (Ekka and Ekka, 2014). Mahuwa flowers have been used as a cooling agent, aphrodisiac, astringent, tonic, demulcent, and for the treatment of acute and chronic tonsillitis, pharyngitis bronchitis, and for the treatment of acute and chronic tonsillitis. *Latta* is a Bihar (India) dish made with mahuwa flowers and roasted maize grain to treat arthritis pain. Debility, diabetes, snake bite, arthritis, tuberculosis, cholera, paralysis, low semen count, tonsillitis, influenza, piles, and sinusitis are all treated using mahuwa stems. Flowers work as a tonic and a cooling agent. Chronic bronchitis, Cushing's disease, and eczema can all be treated using the leaves. Mahuwa bark is used to treat itching, inflammation, diabetes, as well as a snake bite antidote and blood purifier (Khare *et al*., 2018).

2.5 Products from mahuwa

2.5.1 Mahuwa oil

Mahuwa seeds contain around 40% pale yellow semi-solid fat. The seed oil is commonly referred to as "mahuwa butter." The oil content of the seed ranged from 33 to 43% of the kernel's weight. Mahuwa oil is by far the most significant tree seed oil for the tribal communities of India. Fresh mahuwa oil from properly preserved seeds is yellow in color and has a mild flavor. The oil is utilized as cooking oil by the majority of tribal. The oil is edible and can be used in cooking. It is one among the ingredients of hydrogenated oil (termed *vanaspati* in India). The oil is also utilized in the production of soaps, particularly

laundry chips. In the rural areas near the manfacturing cities, the oil is used as an illuminant and hair oil (Sunita and Sarojini, 2013).

2.5.2 Sugar syrup

There have been numerous reports on the preparation of sugar syrup from dried mahuwa flowers. Before concentrating the water extract of dried flowers to the desired concentration, it was decolored with various decolorization agents such as slacked lime and activated charcoal. Activated charcoal at a concentration of 3.5-5% was found to be the best decolorization agent for mahuwa sugar syrup preparation (Chand and Mahapatra, 1983).

2.5.3 Fermented product

Because of their high sugar content, dried mahuwa flowers are an appealing source of fermented products. It has also been reported that mahuwa wine can be made from fresh flowers (Preeti *et al.*, 2009). The dry mahuwa flowers have been used to make alcohol, brandy, acetone, lactic acid, and other fermented goods (Patel and Naik, 2010).

2.5.4 Biodegradable plastic

Mahuwa flowers have been used in the synthesis of Poly(hydroxybutyrate-co-hydroxy valerate) (Kumar *et al.*, 2007).

2.5.5 Other products

Mahuwa concentrate as a liquid sweetener can be used in bakery and confectionery goods (candy, biscuits, and cake). Jam and jelly can also be prepared using mahuwa flowers (Patel and Naik, 2010).

2.6 Pharmaceutical actions

2.6.1 Anthelmintic activity

Katiyar e*t al.* (2011) studied the ethanol and methanol extracts of mahuwa flowers for their potential anthelmintic action in *Pheretima posthuma* (Indian earth worm). It was noticed that aqueous and methanolic extracts (20,40 and 60 mg/ml each) of mahuwa flowers were potent anthelmintic agents, comparable to standard mebendazole dose. The anthelminthic activity of mahuwa leaf extract (methanolic) against adult Indian earthworm (*Pheritima posthuma*)

was found to be very potent at 60 mg/ml, comparable to standard albendazole (60 mg/ml) (Khare *et al.*, 2018).

2.6.2 Antibacterial activity

Verma *et al*. (2010) observed antibacterial action of mahuwa flowers (aqueous and methanolic) against *Bacillus subtilis* and *Klebsiella pneumonia*. For both bacteria, aqueous extract outperformed methanolic extract. The flower possesses antibacterial properties against *Escherichia coli* and is resistant to rice pest illness (Verma *et al.*, 2010). The antimicrobial action of methanolic extracts of mahuwa flowers was also shown by Nimbekar *et al*. (2012).

2.6.3 Antifungal activity

By defatting a cake of mahuwa oil seeds, which includes saponins, a by-product is formed, and these saponins are responsible for eliciting antifungal action. The inhibitory values for plant pathogenic fungi were found to be in the 500 to 2000 ppm range. Saponins work by forcing cell contents to seep out, causing the *Trichoderma viride* fungus to be killed.

2.6.4 Antioxidant activities

Mahuwa leaf methanolic extract has antioxidant properties. Mahuwa leaves were shown to have antioxidant activity equivalent to butylated hydroxyl anisole. Reducing power test, super oxide radical scavenging activity, and hydroxyl radical scavenging activity were used to assess antioxidant activity. The antioxidant effect of mahuwa was attributed to the active component madhucic acid (Inganakal *et al*., 2012).

2.6.5 Analgesic activity

Both the aqueous and alcoholic extracts of mahuwa flowers showed dose-dependent analgesic properties when experimented on mice or rats at doses ranging from 4.0 to 64.0 mg/kg (Dinesh, 2001).

2.6.6 Antipyretic activity

The raised body temperature (pyrexia caused by brewer's yeast in male Wistar rats) was reduced by 50 and 100 mg/kg body weight of mahuwa methanolic extract after 60 min, and 200 mg/kg body weight for 30 min (Shekhawat and Vijayvergia, 2010).

2.6.7 Hepatoprotective activity

The methanolic extract of mahuwa flowers demonstrates hepatoprotective efficacy against paracetamol-induced hepatotoxicity. Two dosages of methanolic extract of mahuwa (100 and 200 mg/kg) were given orally to rats suffering from hepatotoxicity caused by paracetamol (2 g/kg). In the selected model, the methanolic extract significantly reduced serum levels of various biochemical parameters such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), serum alkaline phosphatase (ALP), and total bilirubin while increasing serum levels of total protein and albumin (Sinha *et al.*, 2017; Umadevi *et al.*, 2011).

2.6.8 Anti-inflammatory activity

The ethanol extract and saponin combination of mahuwa seeds showed a strong anti-inflammatory efficacy in cotton pellet-induced granuloma (Gaikwad *et al*., 2009). The flowers are used to treat tonsillitis, helminths, pharyngitis, bronchitis, impotency, inflammation, and eczema, as well as a cooling agent, aphrodisiac, astringent, and demulcent. Flowers are used to treat eye disorders (Bhaumik *et al.*, 2014).

2.6.9 Antihyperglycemic activity

The ethanolic extract of mahuwa seeds was efficient in lowering plasma glucose levels in normal albino rats in a dose-dependent manner, causing a hypoglycemic effect via stimulating insulin release from -cells and/or boosting glucose absorption from the plasma (RD, 2007).

2.6.10 Antiulcer activity

The anti-ulcer efficacy of crude alkaloid extract and ethanolic extract of mahuwa seeds was investigated. According to the findings, ethanolic extract was considerably efficient in preventing pylorus ligation-induced stomach ulcers. The ethanolic extract at a dosage level of 10 mg/kg showed a substantial decrease in the ulcer index when compared to vehicle, and was comparable to lansoprazole at a dose level of 40 mg/kg, but crude alkaloid extract had no significant gastroprotective effect (RD, 2007).

2.6.11 Anticancer activity

Various extracts of mahuwa fruit-seeds exhibit very good to moderate anticancer activity (Bhaumik *et al.*, 2014). The ethanolic extract of mahuwa leaves has antitumor action. In mice, it was shown to be effective against Ehrlich Ascites Carcinoma (EAC). According to the study, the mahuwa leaves extract significantly reduced tumor volume, tumor weight, tumor cell count, and increased mean survival time. Its activity was equivalent to that of 5-fluorouracil (standard drug) (Sangameswaran *et al.*, 2012).

2.7 Yeast

Yeasts are found all over the place in nature. They can be found in soil, plants, berries, fermenting foods, and even animals. However, only a few yeast species have been linked to fermented and microbial foods. *Saccharomyces*, *Schizosaccharomyces*, *Candida*, and other yeasts have privileged role in the food and fermentation sectors. There are currently 86 yeast genera and 597 yeast species. The most common yeast in alcoholic fermentation, *Saccharomyces cerevisiae*, has over 125 variants (Rai and Subba, 2016). Yeasts are single-celled microorganisms of the Kingdom Fungi. Although yeasts are unicellular organisms, their cellular arrangement is comparable to that of higher organisms. Their genetic material, in particular, is contained within a nucleus (Thapa *et al.*, 2015). *Saccharomyces cerevisiae* are larger, oval in shaped (Rosini, 1984). In general, yeast can reproduce sexually as well as asexually. Asexual mode consists of (i) budding, (ii) pseudomycelia formation, (iii) fission, (iv) bud-fission, (v) budding and fission, and (vi) clamp connection. So, for the identification of *Saccharomyces cerevisiae* among the genera by the budding is ellipsoidal to spheroid followed by multilateral budding. But other budding patterns are *Trigonopsis* (triangular); tripolar budding, *Pityrosporum* (flask-shaped); budding at one pole, *Candida* (pseudomycelia); budding at shoulders, *Hanseniaspora* (apiculate shape); bipolar budding (Rai, 2012).

Saccharomyces also has subcellular organelles that are found in higher eucaryotic species. There is a membrane-bound nucleus, as well as endoplasmic reticulum, golgi bodies, and secretory vesicles. Mitochondria can also be found. Because of their fermentative nature, *Saccharomyces* strains can live without mitochondria, resulting in petite or respiratory-deficient mutants. Petite strains are unable to thrive on non-fermentative substrates such as ethanol, pyruvate, and lactate, but can grow fermentatively on sugars. During anaerobic

fermentation, mitochondrial activity is reduced and electron transport chain enzymes are not produced. Aside from respiration, many oxidative processes are limited to the mitochondrion and hence are inactive during fermentation. These include tricarboxylic acid (TCA) cycle processes, proline oxidation, and fatty acid production. Because of their vast membranous nature, mitochondria also serve as a lipid store that can be cannibalized and utilized for plasma membrane production during anaerobic development (Boulton *et al.*, 1999).

Yeasts of the genera *Kluyveromyces*, *Saccharomyces*, and *Zygosaccharomyces*, for example, ferment, at least, glucose vigorously, whereas others, such as *Rhodosporidium* and *Sterigmatomyces*, do not noticeably ferment any sugars. Species ranging from non-fermentative to strongly fermentative are found in other genera (Thapa *et al.*, 2015).

Alcoholic beverages (e.g., fruit-, palm-, and rice wines), cereal-based leavened items (e.g., sourdough and *idli*), milk products (e.g., cheese and *dahi*), and condiments such as soy sauce and *papads* are examples of foods in which yeasts predominate. Yeasts are either dominant alone or in combination with lactic acid bacteria or mycelial fungus in natural food fermentation. Many yeast strains have been isolated from natural fermentation and used successfully as starting cultures in industrial food preparation. They have a substantial impact on food quality by improving taste, flavor, texture, nutritive qualities, lowering anti-nutritional components, and increasing functioning (health promoting properties) (Rai and Jeyaram, 2017). Reviews on wine flavor and its relation to yeast, temperature, pH, etc., have also been given by Rapp and Mandery (1987), Vidrih and Hribar (1999) and Suomalainen and Lehtonen (1979)

2.7.1 Indigenous yeast used in wine

Although several microbial species can participate in alcoholic fermentation and contribute to the sensory aspects of the end products, the yeast *S. cerevisiae* consistently predominates in the final stages of fermentation. *S. cerevisiae* outcompetes other organisms during alcoholic fermentation processes due to its high fermentative strength and ability to endure difficult environmental circumstances such as high levels of ethanol and organic acids, low pH values, restricted oxygen availability, and nutrient depletion (Albergaria and Arneborg, 2016).

Wine production entails complex metabolic events that take place in the presence of complex microbiological organisms. Wine chemistry is the outcome of the total microbiota

(yeast, bacteria and filamentous fungi) contribution; however, yeasts receive special attention because 'modern' winemaking is primarily carried out by selected *Saccharomyces cerevisiae* strains (Capozzi *et al.*, 2015).

2.7.2 Non-*Saccharomyces* yeast

Non-*Saccharomyces* yeast produce a number of secondary chemicals that were undesirable even at low concentrations, including acetic acid, acetaldehyde, acetoin, and ethyl acetate. They are also responsible for the occurrence of off-odors such as ethyl and vinyl phenols, which are often produced by *Brettanomyces*/*Dekkera* spp. (Chatonnet *et al.*, 1995). Furthermore, the majority of non - *Saccharomyces* strains have poor fermentative properties, such as low SO_2 resistance, power, and rate of fermentation (Fleet, 2008). Collectively, risk of using Non-*Saccharomyces* yeast in fermentations despite of some oenological benefits are shown in Table 2.3.

Non-*Saccharomyces* strains as components of mixed and multi-starter formulations with *S. cerevisiae* strains are thought to have an important role in the development of wine aroma complexity. The development of a non-*Saccharomyces*/*Saccharomyces* multi-strain starter could be a reliable alternative to uncontrolled spontaneous fermentations (Capozzi *et al.*, 2015).

2.7.3 Wild strains of *S. cerevisiae*

Scientific data has shown that using autochthonous *S. cerevisiae* strains can result in the development of wine products that promote and relate to the distinct sensory expression of a certain place, resulting from diverse biogeographical patterns (Lappa *et al*., 2020). The authors suggested that one isolated strain may be used as a viable wild starting culture because beneficial features such as ethanol tolerance, resistance to SO_2, and even safety attributes of negative biogenic amines activities were detected. A wild strain of *Saccharomyces cerevisiae* is more adaptable in the formation of a wine with a more desired sensory profile (Lappa *et al*., 2020). *S. cerevisiae* wild strains have been isolated from a range of natural sources and have commonly been discovered in conjunction with oak tree exudates, bark, and soil (Naumov *et al*., 1998).

Table 2.3 Yeast genera and their effects in wine

Yeast genera	Oenological properties	Negative effect
Torulaspora	Low concentration of acetic acid	Slower fermentation rate, Production of sulfur compounds
Metchinkowia	High concentration of esters, Increase wine flavor and aroma, Antimicrobial activity (pulcherrimin)	Delays in fermentation due to antimicrobial activity
Hanseniaspora	Increased amounts of 2- phenyl-ethyl acetate, higher alcohols, acetate, ethyl esters and medium-chain fatty acids, Reduced level ochratoxin A	Negative compounds (volatile acidity, sulfur compounds, etc.), Biogenic amine production, Production of acetoin, Sluggish or stuck fermentation
Candida	High glycerol producer (up to 14 g/L), Low acetic acid concentration, Increased concentrations of terpinol, Decreased concentrations of aldehydes and acetate esters	Production of sulfur compounds, Slower kinetics rate (low ethanol concentration)
Kluyveromyces	Enhancement of aroma and flavor Increased concentrations of lactic acid, glycerol and 2-phenylethanol	Higher “spicy” and “acidity” attributes

Source: Capozzi *et al.* (2015)

A wild strain of *S. cerevisiae* may include wine aroma and flavor, which have long been of interest to winemakers. Yeast metabolites are known to affect wine sensory characteristics by producing esters, higher alcohols, carbonyl compounds, volatile acids, volatile phenols, and sulfur compounds (Swiegers and Pretorius, 2005b). It has also been demonstrated in some circumstances that individuals can distinguish between wines produced with various strains of *S. cerevisiae* (Callejon et al., 2010).

The yeast strain had a considerable influence on the concentrations of the majority of the volatile chemicals. According to the sensory investigation, autochthonous yeast generated wines of greater organoleptic quality. Autochthonous yeast strains could be used instead of commercial yeast strains, increasing biodiversity. Autochthonous yeast strains can yield wines with a variety of volatile characteristics. Some of them have made wines high in

alcohol, while others have made wines high in fruity esters. Depending on the desired wine style, any of these could be ideal for winemaking (Callejon *et al.*, 2010).

The autochthonous *S. cerevisiae* strains used in this study presented good fermentative ability. The autochthonous *S. cerevisiae* were able to produce a higher concentration of ethyl esters and acetates, which are responsible for fresh/fruit attributes. Scientific data has shown that using autochthonous *S. cerevisiae* strains can result in the development of wine products that encourage and relate to the distinct sensory expression of a certain place, resulting from diverse biogeographical patterns (Çelik *et al.*, 2019).

2.8 Yeast characteristics useful for wine fermentation

Wine yeasts require fast, aggressive, and full fermentation of grape juice sugars to high ethanol concentrations (greater than 8% v/v). The yeast should be tolerant of sulfur dioxide concentrations provided to the juice as an antioxidant and antibacterial, have uniform dispersion and mixing throughout the fermenting juice, create minimum froth, and sediment fast from the wine at the end of fermentation. These processing qualities should be well expressed at low temperatures (for example, 15°C) for white wine fermentations and at higher temperatures (for example, 25°C) for red wine fermentations. It is critical that the yeast does not produce a slow, sluggish, or stuck fermentations (Bisson, 1999).

In terms of wine quality and character, any yeast must create a balanced array of flavor metabolites while avoiding unwanted excesses of volatiles such as acetic acid, ethyl acetate, hydrogen sulfide, and sulfur dioxide. It should not produce unpleasant autolytic flavors after fermentation. It should not have an unfavorable effect on the color or tannic qualities of the wine. In essence, the yeast must produce a wine with a good clean flavor that is devoid of sensory flaws and allows the consumer to perceive the grape varietal character (Bisson, 2004; Swiegers and Pretorius, 2005a, 2005b).

Technologies are well established for the selection, development and production of native yeasts for wine with good basic enological criteria. These developmental targets can be broadly described under 5 categories (Fleet, 2008), viz.:

1. Improved fermentation performance (e.g., yeasts with greater efficiency in sugar and nitrogen utilization, increased ethanol tolerance, decreased foam production).

2. Improved process efficiency (e.g., yeasts with greater production of extracellular enzymes such as proteases, glucanases and pectinases to facilitate wine clarification; yeasts with altered surface properties to enhance cell sedimentation, floatation and flor formation, as needed; and yeasts that conduct combined alcoholic-malolactic fermentations).
3. Improved control of wine spoilage microorganisms (e.g., yeasts producing lysozyme, bacteriocins and sulfur dioxide that restrict spoilage bacteria).
4. Improved wine wholesomeness (e.g., yeasts that give less ethanol, decreased formation of ethyl carbamate and biogenic amines, increased production of resveratrol and antioxidants).
5. Improved wine sensory quality (e.g., yeasts that give increased release of grape terpenoids and volatile thiols, increased glycerol and desirable esters, increased or decreased acidity and optimized impact on grape phenolics).

2.9 Biochemistry of alcohol fermentation by yeasts

When yeast begins to absorb glucose, a considerable amount of carbon dioxide is produced. The emission of carbon dioxide displaces oxygen, resulting in semi anaerobic conditions favorable to fermentation. *Saccharomyces cerevisiae* will not ferment if the sugar concentration is greater than 9 g/L, even in the presence of oxygen. Crabtree initially observed this behavior, known as the Crabtree effect, catabolic repression by glucose, or the Pasteur opposite effect, in 1929. *Saccharomyces cerevisiae* degenerate when grown in high sugar concentrations, such as those found in grape juice. The enzymes of the Krebs cycle and the components of respiratory chains are both suppressed at the same time. As a result, *Saccharomyces cerevisiae* can only ferment carbohydrates under wine fermentation conditions. When the sugar concentration is extremely low and oxygen is present in the medium, *Saccharomyces cerevisiae* can utilize respiration. These conditions are employed in the industrial manufacture of dry yeast (Zamora, 2009). *Saccharomyces cerevisiae* primarily directs the production of ethanol from pyruvate in order to restore the NAD^+ used by glycolysis; this process is known as fermentation (Boulton *et al.*, 1999).

Pyruvate decarboxylase first decarboxylates pyruvate producing ethanal (acetaldehyde) (Fig. 2.1). As cofactors, this enzyme requires magnesium and thiamine pyrophosphate. Following that, alcohol dehydrogenase converts ethanal to ethanol, recycling the NADH to NAD^+. In *Saccharomyces cerevisiae*, there are three isoenzymes of alcohol dehydrogenase,

although isoenzyme I is primarily responsible for converting ethanal to ethanol. Zinc is used as a cofactor by alcohol dehydrogenase. Simple diffusion transports both the ultimate products of alcoholic fermentation, ethanol & carbon dioxide outside the cell (Zamora, 2009).

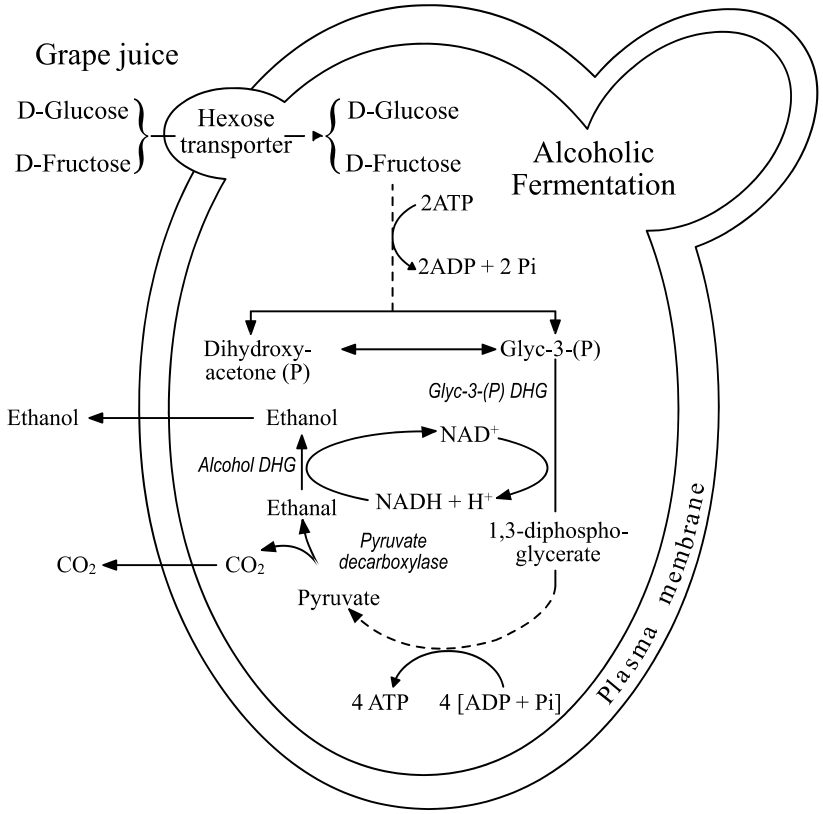

Fig. 2.1 Biochemistry of alcohol fermentation by yeasts

Source: Zamora (2009)

2.10 Other alcoholic fermentation byproducts

Alcoholic fermentation is more than just the simple conversion of sugars to ethanol. On the contrary, it is a pretty complicated process that results in a very pleasant beverage. It refers to the conversion of sugars into ethanol, as well as other byproducts that can affect sensory quality positively or negatively. A greater grasp of the regulation of all routes necessitated a better understanding of the process and the development of methods to improve the product's quality (Moreno-Arribas and Polo, 2005). During fermentation, the by-product formed other than ethanol which imparts complexity in wine are diacetyl, acetoin, 2,3-butanediol, acetic acid, ethanal, higher alcohols, esters, and succinic acid (Fig. 2.2) (Lambrechts and Pretorius, 2000).

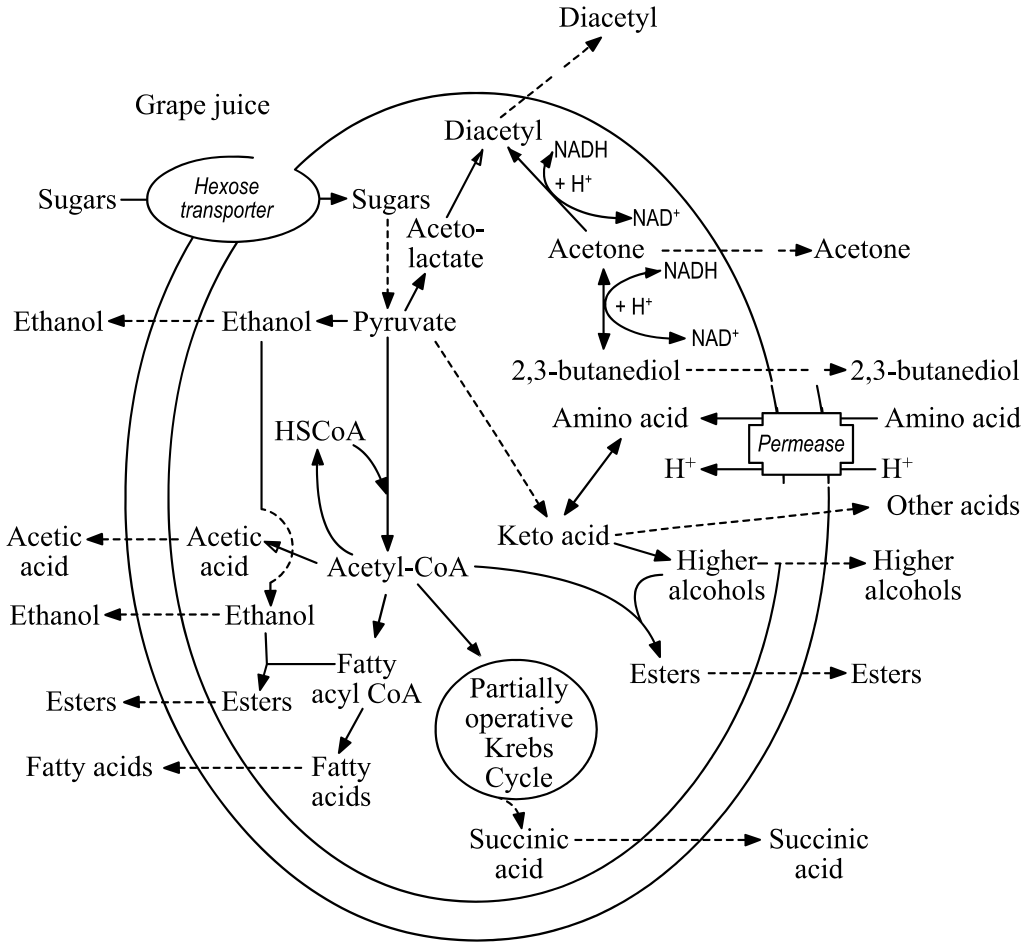

Fig. 2.2 Alcoholic fermentation byproducts

Source: Zamora (2009)

2.11 *Murcha*

Murcha, an amylolytic starter cake indigenous to Nepal, is extensively used as a source of fermenting yeasts and amylolytic molds in the production of traditional, cereal-based alcoholic beverages, namely *jand* (undistilled) and *raksi* (distilled). *Murcha* functions both as a substrate and carrier for the indigenous yeasts, mold, and bacteria. From this perspective, *murcha* can also be considered as a simple stock culture (Rai and Subba, 2016).

Murcha, like other mixed-culture beginnings like *ragi* from Indonesia, *bubod* from the Philippines, *chu* from China, *loogpang* from Thailand, and *nuruk* from Korea, is typically produced by wrapping the cakes with fern fronds with the fertile side contacting. This could be owing to a scarcity of paddy straw, but also to an abundance of ferns in high altitude places. In cold areas, germination of spores in sori may aid to preserve the warmth of the

fermenting mass. The remaining stages in preparing murcha are similar to those in preparing other starting cultures. Spices and wild plant parts added to starter doughs limit the growth of unwanted microorganisms (Tamang and Sarkar, 1995).

2.12 pH influence on wine

Saccharomyces cerevisiae is the most important microbe in fermentation, particularly in winemaking. Most *S. cerevisiae* strains grow at pH values ranging from 2.50 to 8.50, however they are acidophilic organisms that thrive in acidic environments (Carmelo *et al.*, 1996).

The ideal pH range for yeast development can range from 4.00 to 6.00, depending on temperature, oxygen present, culture, and yeast strain(Narendranath and Power, 2005). *S. cerevisiae* is constantly confronted with adverse environmental circumstances during the winemaking process. For example, during the start of fermentation, yeast cells are subjected to osmotic stress due to high sugar concentrations and low pH(Cardona *et al.*, 2007).The initial pH of the must had an effect on yeast growth and fermentation performance. Except in a few cases, low starting pH lengthened the yeast lag phase, restricted yeast growth, lowered fermentation pace, raised final acetic acid and glycerol content, and decreased final ethanol and succinic acid content. A decrease in the initial pH value of must prevented the metabolic activities of contaminated bacteria during wine fermentation, allowing wine yeast to flourish. However, a pH value that is too low may limit the growth of wine yeast (Liu *et al.*, 2015).There are also other research on the influence of grape starting pH on *S. cerevisiae*, however they generally looked at pH values over 3 (Ough, 1966; Yalcin and Ozbas, 2008). The monitoring of acidity in wine was studied by Thoukis *et al.* (1965) , Peng *et al*. (2016), and Singh *et al.* (2013).

2.13 Effect of starting pH on *S. cerevisiae* fermented wine

Various factors can be controlled to improve ethanol fermentation activity. pH is a major component that impacts ethanol fermentation in addition to yeast, temperature, and substrate concentration. The enological parameters ethanol, glycerol, acetic acid, and succinic acid are affected by the initial pH (Gunasekaran and Raj, 1999; Lin *et al.*, 2012; Liu *et al.*, 2015). Yang (1973) and Charoenchai *et al.* (1998) also made study on pH and its relation to wine fermentation.

Studies on the generation of volatile acidity by *S. cerevisiae* under winemaking circumstances revealed that this acid is mostly produced during the start of alcoholic fermentation and is influenced by a variety of parameters such as yeast strain, anaerobiosis, extremely low pH (3.1), or very high pH (>4) (Ribéreau-Gayon *et al.*, 2006). The pH 3.9 at 20°C (5.95%, v/v) and 30°C (5.56%, v/v) produced more ethanol than pH 3.1 (5.25 and 5.01 %, v/v). Regardless of temperature, pH 3.1 produced much more isobutyl alcohol and isoamyl alcohol, up to 64.52±6.39 and 56.27±3.00 mg/L, respectively, than pH 3.9 (Lu *et al.*, 2017). According to Gao and Fleet (1988), low pH may cause stress to yeast cells' development by increasing their susceptibility to ethanol.

Changes in the operational pH of the ethanol production process may have influenced the major fermentation route. As a result, maintaining a pH value in the range of 4.0-5.0 is critical. Beyond this range, the development of byproducts such as acetic acid and butyric acid may have consumed some of the substrate, lowering the efficiency of ethanol fermentation (Lin *et al.*, 2012).

PART III

Materials and methods

3.1 Materials

3.1.1 Collection of samples

During the month of Baisakh, 2078 (May 2021), fully ripened and well dried mahuwa flowers were collected from Gadhawa Rural Municipality Ward No. 08, Koilabas of Dang Deukhuri District in Lumbini Province, south-western Nepal (Color plates P 1, and P2 in the Appendix). The flowers were brought to the Central Campus of Technology's Department of Food Technology laboratory. Ward No. 08 has 187 households, which represents 2.6% of the total population of Gadhawa Rural Municipality (Karki, 2019).

3.1.2 Survey for sample collection

A questionnaire was prepared (Appendix A) for conduction a brief survey on mahuwa during sample collection. The aim of the survey was to collect data on many elements such as flower collecting technique, use, value, market, risks, and so on. The study was carried out at the Gadhawa Rural Municipality, Ward No. 08, Dang districts. Twenty one respondents who were experienced with collecting, consumption, and marketing were chosen for survey (Color plates P 3 in the Appendix). Of the respondents, 12 were male, with the remainder being female. 66% of those polled were of age from 20 to 50, with the remainder 50 to 60 or older.

3.2 Chemicals

Chemicals used for this study were of analytical grade and obtained from laboratory, Department of Food Technology, Central Campus of Technology. The chemicals used for the analysis were: Mixed indicator solution, Boric acid, Phenolphthalein, Conc. H_2SO_4, NaOH, HCl acid, Ethanol, standard higher alcohol (isoamyl and butyl alcohol), Ammonia solution, Methylene blue, Saturated ammonium oxalate, Potassium Iodide, sodium carbonate, Sodium thiosulphate, Sodium bisulphite, starch indicator, Potassium permanganate, DMAB (Ehrlich's Reagent) *p*-Dimethylaminobenzaldehyde, Folin-Ciocalteau reagent, Gallic acid, Citric acid, and Dextrose.

3.3 Glassware

Petri dish, burette, pipette, test tubes, volumetric flask, funnel, conical flask, measuring cylinder, micropipette, iodine flask, round bottom flask

3.4 Equipment

The equipment and utensils used in the experiment consisted of a regular laboratory distillation set (glass), electronic balance (±1 mg), hemocytometer (improved Neubauer, China), light microscope (routine), thermometer (±0.5°C), routine laboratory heating mantle, centrifuge (up to 10000 rpm), spectrophotometer (single-beam, Labtronics, India), and plastic buckets (10 L cap).

3.5 Experimental design

For the present study, 2 factors with 3 levels each were used to get a total of 9 runs for the preparation of wine from mahuwa flower. The two treatment factors and levels (in parenthesis) were: (1) Types of yeasts: Fy_1, My_2, and Wy_3; (2) Levels of pH: 3, 3.5, and 4

The meanings of the notation F, M and W are:

Fy_1 = Yeast isolated from mahuwa flower; My_2 = Yeast from *murcha*; Wy_3 = Commercial wine yeast

Table 3.1 Experimental plan for mahuwa wine

Treatment code ↓	Treatments and levels	
	pH	Yeast type
A	3	Fy_1
D	3	My_2
G	3	Wy_3
B	3.5	Fy_1
E	3.5	My_2
H	3.5	Wy_3
C	4	Fy_1
F	4	My_2
I	4	Wy3

Experimental plan (the recipe sheet) for the runs is shown in Table 3.1. The panelists that took part in the sensory analysis has not been shown in the said Table.

3.6 Processing methods

3.6.1 Mahuwa juice

Mahuwa flowers were washed thoroughly with tap water and then converted into pulp by soaking the flowers in water in the ratio of 1:2. The soaked flowers were then crushed (macerated) with hands and lightly pressed for extracting the juice. The extracted juice was filtered through a muslin cloth to remove any pulp traces. The extracted juice was then analyzed for TSS with the help of a hand refractometer. The TSS, which was always > 22°Bx, was adjusted to 22°Bx by adding requisite amounts of water. The adjusted juice was finally heated to 90°C for 10 min for pasteurization.

3.6.2 Inoculum of yeast culture

- The wine yeast strain obtained from the Department of Food Technology, Dharan (*Saccharomyces cerevisiae*) was cultured on Molasses broth at 25°C for 48 h in an incubator. TSS of molasses broth was maintained 15^{o} Bx.

- Isolation of fermentative yeast from mahuwa flower: Mahuwa flowers have a huge number of natural yeasts. According to Karmayogi (2003), the isolation of these local yeasts was done in multiple phases in the laboratory. The initial step consisted of natural fermentation, as practiced by the locals. Following this fermentation step, fermentative yeast was isolated.

- The chosen colony was then subcultured again on MYPG agar medium plates for further isolation, preservation, and maintenance. After incubation of the isolated culture on subcultured medium plates, microscopic examinations were performed once again. MYPG slants were used to preserve the separated subcultures, which were then refrigerated (Bitzilekis and Barnett, 1997). The yeast colonies were examined for features such as shape, size, color, edge, surface, elevation, and consistency of a single isolated colony, as well as microscopic identification using a simple staining approach (Rajopadhyaya *et al.*, 2020).

- Yeast fermentation broth (YFB) containing an inverted Durham's tube (replaced by a test tube) was used to identify *Saccharomyces* species based on the fermentation of various carbohydrate sources (molasses medium), with minor modifications to the technique described by Guimarães *et al.* (2006). The broth was prepared by adding distilled water to the molasses medium to maintain a TSS of 15°Bx, followed by autoclaving. After sterilization, the media were allowed to cool at 35-40°C before being placed into individual tubes with molasses medium solution and a loopful of isolate suspension and incubated at 30°C for 3 days. If the development of CO_2 and the smell of alcohol was be detected after three days, this medium was was selected for the propagation stage.

- Some conventional media, such as MYPG, were changed with molasses media in this study due to their suitability for fermentative yeast (Rai and Subba, 2016).

- The same afore-described approach was used for screening murcha yeast also.

- For all settings, the yeast inoculum contained around 10^5 cells per ml.

3.6.3 Fermentation/Wine making process

For the setting up of the fermentation unit, the must was taken in jars up to 2/4 of its volume, and the yeast culture was then added to the must under aseptic conditions as per the treatment details. The aseptic condition was maintained to minimize the chance of contamination with feral yeasts and other contaminants. The TSS level and yeast cells were kept constant in all the treatments. The pH of juice extract was adjusted at pH 3.5, 4 and 5 (just before pasteurization) with the help of 2% citric acid. The yeast extract was added as a nitrogenous source at the rate of 0.1% (w/v) in the mahuwa juice. The opening of the jar was closed with a plastic wrapper and tightened with a rubber band. Fermentation was carried out at 25-28°C. Samples were analyzed daily until bubbling ceased (indicator to completion of fermentation). Fermentation was terminated after the TSS of the medium remained stabilize (at ~ 7-8°Bx, measured by a hand refractometer) for two successive days. All experiments were conducted in triplicates and a control (un-inoculated) was also taken. Fig. 3.1 shows a recapitulative process flow diagram used in the experiment.

In case the wine was not clear after racking, it was clarified with bentonite to recover wine of crystal-clear quality. Following clarification, the clear wine was siphoned off and

transferred to new sterile bottles, corked, and pasteurized. Finally, pasteurized wine bottles were stored for aging. After maturity, wine samples were organoleptically evaluated for acceptability by sensory panelists.

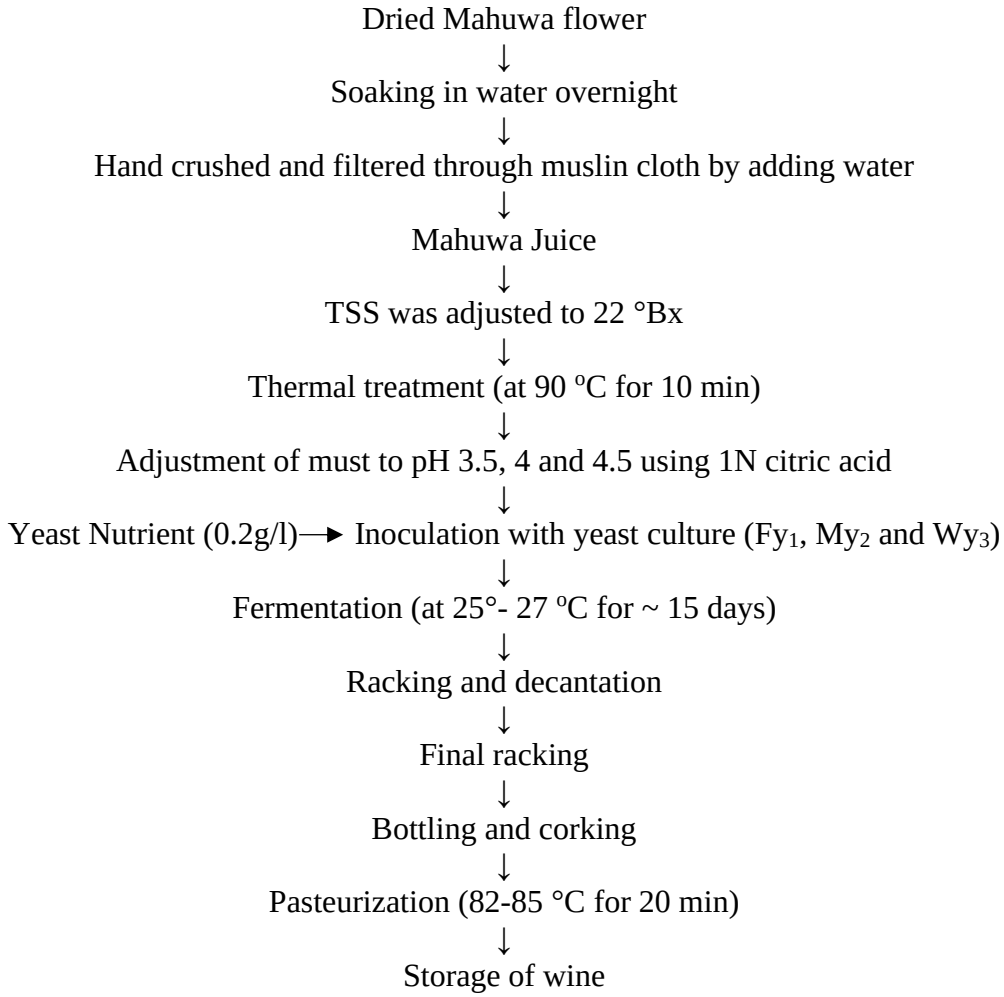

Fig. 3.1 Process preparation chart for mahuwa wine

3.7 Analytical methods

3.7.1 Determination of TSS

TSS was determined by a portable refractometer (Hanna Instrument) at 20°C after degassing of samples and the results were expressed as °Bx.

3.7.2 Determination of total reducing sugar

Total reducing sugar and total sugar was determined by Lane and Eynon method according to Kirk and Sawyer (1991)

3.7.3 Determination of alcohol content

200 mL of the wine was neutralized with 0.1N NaOH and distilled as described by FSSAI (2019). The alcohol content in the distillate was determined by the hydrometer method.

3.7.4 Determination of total acidity, fixed acidity

The total-, fixed- and volatile acidity were determined as per Kirk and Sawyer (1991). Total and fixed acidities were expressed in % (m/v) as lactic acid.

3.7.5 Volatile acidity determination

The volatile acidity was measured according to FSSAI (2019). 50 ml distillate collected during the determination of ethyl alcohol was taken for volatile acidity determination and was titrated against standard sodium hydroxide using phenolphthalein indicator. Volatile acidity as acetic acid gram per liter of wine was calculated using formula:

$$\text{Volatile acidity (acetic acid, g/L of wine)} = \frac{V \times 0.003 \times 1000}{V_1}$$

Where: V_1 = volume (ml) of wine taken for estimation, V = volume (ml) of standard NaOH used for titration. It may be noted that 1 ml. of 0.05*N* NaOH is equivalent to 0.003 g of acetic acid.

3.7.6 Determination of total ester

Total ester content was determined as per FSSAI (2019). Briefly, to the neutralized distillate from the volatile acidity determination, 10 ml of standard sodium hydroxide was added and refluxed on a steam bath for 1 h. Then it was cooled and back titrated the unspent alkali against standard sulfuric acid. Similarly, blank was carried out simultaneously taking 50 ml of distilled water instead of distillate in the same way. The difference in titer value in milliliters of standard sulfuric acid was calculated and ester content was calculated using formula:

$$\text{Ester (ethy acetate, g/L of absolute alcohol)} = \frac{V \times 0.0088 \times 100 \times 1000 \times 2}{V_1}$$

Where: V = difference of titer value (ml) of standard H_2SO_4 used for blank and sample, V_1 = alcohol % by volume. It may be noted that 1 ml of 0.1*N* NaOH is equivalent to 0.0088g of ethyl acetate.

3.7.7 Total aldehyde content

Total aldehyde as g acetaldehyde/100 L alcohol was determined as per FSSAI (2019). 50 ml of distillate of liquor was taken in a 250-ml iodine flask and 10 ml of sodium bisulfite (0.05*N*) solution was added. The flask was kept for 30 min in dark with occasional shaking. 25 ml of standard iodine solution was added then and was back titrated excess iodine against standard sodium thiosulphate (0.05*N*) solution using starch (1%) indicator to light green end point. Similarly, blank was carried out with 50 ml distilled water. Then the difference in titer value in ml of sodium thiosulphate solution was noted and aldehyde content was calculated using formula:

$$\text{Aldehyde (acetaldehyde, g/100 L of absolute alcohol)} = \frac{V \times 0.0011 \times 100 \times 1000 \times 2}{V_1}$$

Where: V_1 = alcohol % by volume, V = difference in titer of blank and sample, in ml of sodium thiosulphate solution. It may be noted that 1 ml. of 0.05*N* sodium thiosulphate is equivalent to 0.0011 g of acetaldehyde.

3.7.8 Determination of total phenolic content (TPC)

Total phenolic content was determined as per Sadasivam (1996). Briefly, 1 ml of the filtered (Whatman 41 filter paper) wine was diluted to 10 ml with distilled water. One ml of the diluted wine was pipetted into a test tube and 2 ml of distilled water and 0.5 ml of Folin-Ciocalteau reagent was added. After 3 min, 2 ml of sodium carbonate solution (20%) was added, mixed thoroughly, and incubated at room temperature for 1 h after which the absorbance was measured at 650 nm against a reagent blank. Total phenolic content in the wine was calculated from the standard curve prepared using different concentrations of gallic acid and the result was expressed as mg gallic acid equivalent (GAE)/100 ml of wine.

3.7.9 Determination of fusel oil (higher alcohols)

Higher alcohol was determined by spectrophotometric method as per FSSAI (2019). Briefly, 1 g of the fusel oil standard (4 volumes isoamyl alcohol mixed with 1 volume of isobutyl

alcohol) was diluted to 1 L with water. Finally, working standard solutions were prepared by pipetting 0, 5 10, 25 and 35 ml of fusel oil standard solution in to 100 ml volumetric flasks containing 7 ml of 95% neutral ethanol and diluting to volume with distilled water. Distillate (1 ml) was pipetted in a test tube and diluted to 2 ml with distilled. One ml of DMAB (p-dimethyaminobenzaldehyde) solution (1 g DMAB dissolved in a mixture of 5 ml H_2SO_4 and 90 ml distilled water and volume made up to 100 ml with distilled water) was added to the test tube, shaken and placed in ice bath for 3 min. With the tube still in ice bath, 10 ml of chilled H_2SO_4 was added into the tube, shaken and replaced in ice bath for 3 min. Then the tube was placed in a boiling water bath for 20 min and replaced in ice bath for 5 min. The tube was shaken and brought to room temperature. Similar procedure was followed for fusel oil working standard solutions. The transmittance (% T) of both the test sample and working standard solution were read at 540 nm against reagent blank as reference. The concentration of the fusel oil was found out from the fusel oil standard curve prepared by plotting gram fusel oil on linear scale as abscissa against %T as ordinate on log scale of semi log paper. The results were expressed as mg fusel oil/L of wine.

3.7.10 Determination of number of yeast cell using hemocytometer

Yeast cell count of the samples were done according to (Taylor and Francis, 1988). The samples to be counted were well mixed, degassed, and diluted, if required. If the sample to be counted did not require dilution, a 50-ml sample for a minimum of 5 min was stirred using a magnetic stirrer. If dilution was necessary, 0.5% sulfuric acid was used as a diluent to deflocculate cells and serial dilution was carried out if required. The counting chamber and cover slip of hemocytometer was cleaned with clean water and ethanol and dried before use. The cover slip was centered over the counting area such that both counting sections are equally covered and equal amounts of cover slip project over the cover glass slide supports. Then it was filled with the sample for yeast count after which the prepared slide was put on a stand for a few minutes to allow yeast to settle then put on a microscope with 400× magnification.

To eliminate the possibility of counting some yeast cells twice, it was necessary to standardize the counting technique. Cells touching or resting on the top and right boundary lines were not counted. Cells touching or resting on the bottom or left boundary lines were counted. Yeast cells that were budded were counted as one cell if the bud was less than one-

half the size of the mother cell. If the bud was equal to or greater than one half the size of the mother cell, both cells were counted. To obtain an accurate yeast cell count, counting was performed for no fewer than 75 cells on the entire 1-mm^2 ruled area and no more than about 48 cells in one of the 25 squares. The observation was stated correct if counts from both sides of the slide agreed within 10%. The number of cells/ml (*N*) was calculated using the formula:

$$N = \text{Total cells in central 25-square ruled area} \times \text{dilution factor} \times 10^4$$

3.7.11 Sensory evaluation

Mahuwa wine prepared from mahuwa flower were evaluated for sensory qualities viz., appearance, odor, in-mouth sensation, finish (after-taste and lingering), and overall acceptance. Each attribute was given a separate score of 1-7 scale according to the method reported by Jackson (2016). Sensory evaluation was carried out by coded samples kept randomly by a group of semi-trained panelists. The mean values of the score for sensory evaluation were calculated and reported. The scale used for the sensory evaluation was:

Faulty - 1, Poor - 2, Below average - 3, Average - 4, Above average - 5, Very good - 6, Exceptional - 7

3.8 Data analysis

All the data obtained in this work was analyzed by the statistical program known as GenStat (Genstat Discovery Edition 12, 2009). Using this, ANOVA on the data were conducted and the treatment means were compared by Tukey HSD test at 5% level of significance to determine whether the sample were significantly different from each other and to determine which one was superior among them. MS- Excel 2016 was also employed for the general calculations, graphs and diagram construction.

Part IV

Results and discussion

Mahuwa flower was collected from Gadhawa Rural Municipality Ward No. 08 as stated in detail in Section 3.2.

4.1 Survey result

The survey's findings are as follows:

As per the survey, collectors of mahuwa flowers are both male and female, and they have all done so since they were children. Collectors ranged in all age group. All of the interviewees had been instructed by their forefathers. The younger generation used to assist their parents with the collection. Throughout the months of Chaitra and Baisakh (mid-March to mid-May), they pick mahuwa flowers. The flowers were usually conserved and utilized to manufacture the mahuwa spirit.

The majority of the people have been making mahuwa spirit for more than 15 years, while some have been doing so for less than 5 years and have relocated to Gadhawa Rural Municipality. Fig. 4.1 shows is a general flow diagram used by tribes in the manufacturing of mahuwa spirit:

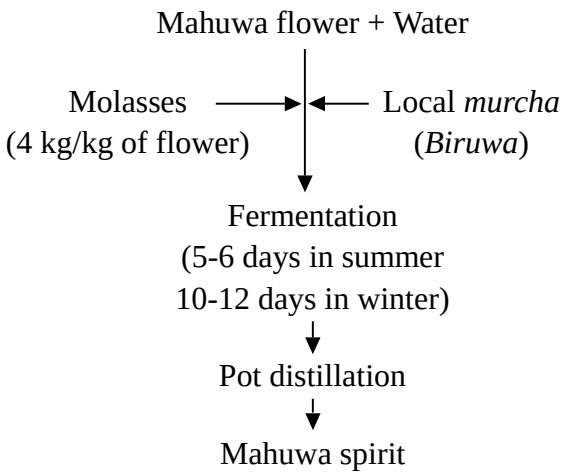

Fig. 4.1 Traditional preparation of mahuwa spirit

Those who sell spirit to others follow the method given in Fig. 4.1. Molasses is not used by individuals who produce spirit for themselves to drink at festivals. They only ferment

mahuwa flowers, the spent wash is re-fermented with molasses. Murcha was employed in fermentation, and some individuals followed spontaneous fermentation. They claimed yeast was present in mahuwa flowers.

The average quantity of flowers collected by the villagers of Gadhawa Gaunpalika (Rural Municipality) was found to range from 80 to 150 kg/tree. The quantity of flower-seeds collected was 75-85 kg/tree. The total quantity of flowers collected in a season by the villagers varied from 24000 to 45000 kg.

Another outcome of this poll indicated that individuals did not attempt to change the traditional mahuwa spirit production technique. They firmly believe that their product is safe, but they are unable to explain why. In the study region, 53% of the 187 homes produce mahuwa spirit. A typical home produces 200 L of alcohol per year, with a yearly production of around 20000 L in Ward No. 08 of the Gaunpalika. People were found to charge ₹ 125 (Indian rupee)/L of spirit. There aren't any distilleries. Their biggest customers are locals from Lamahi (Dang district) and the neighboring places, as well as border security forces from both India and Nepal. Assuming that all the spirit is sold at the price mentioned above, it would amount to an annual income of NRs 4000000.0 for Ward No. 08, which is a decent income for the household (~ NRs 40000.0 per household).

The majority of spirit producers pick flowers in their field by themselves, with just a small minority purchasing flowers from other collectors around their residence called Khabrinaka. Before being stored in containers or bags, the flowers were sun-dried for 4-5 days.

Another finding was that the fresh flower's juice is also consumed, and the dried flowers are consumed later as the baked dish is prepared.

The quality and strength (potency and purity) of the mahuwa spirit are determined by putting it in the fire to see how it burns. Soaring flames are created when a small amount of spirit is flung into a flame. Similarly, the color of flames represents their strength; when flames burn with a low flame, they are weak; yet, when flames burn with a high blaze and a blue tint, they are powerful. Furthermore, the quality of the mahuwa spirit may be determined by tasting one teaspoon and assessing its flavor. The high-quality mahuwa spirit, according to them, is aromatic, extraordinarily floral, and leaves a burning path down the throat with a sweet and smoky aftertaste. Another important finding was that females had a liking for the mahuwa spirit, while children did not.

According to the survey results, the natives use uncontrolled fermentation processes. Process improvements and standardizations in spirit manufacturing will enhance output while also increasing annual revenue. Although the local government is prepared to assist them, there is no legislation modification in Nepal permitting home scale distilled alcohol.

4.2 Chemical analysis of mahuwa must

Parameters such as TSS, acidity, pH, moisture content, reducing sugar and total phenolic content were measured. Chemical composition of must obtained is given in Table 4.1.

Table 4.1 Chemical composition of mahuwa must

Parameter	Value*
TSS (ºBx)	22 (0)
Acidity (% as lactic acid)	0.21 (0.02)
Total ash (%)	0.27 (0.02)
Reducing sugar (% as dextrose)	17.2 (0.40)

*: Values are the means of three determinations. Figures in the parentheses are the standard deviations.

4.3 Fermentation kinetics of mahuwa must

Physiochemical properties as Total Soluble Solid (TSS), reducing sugar, total titratable acidity and growth kinetics of different strain of *S. cerevisiae* mahuwa must were studied.

4.3.1 Kinetics of Total Soluble Solid (TSS)

Total soluble solid of the different musts recorded every 2 days up to the 20 days are shown in Table E. 1 (Appendix E). Fig. 4.2 shows the graphical representations of TSS depletion with fermentation time (days). The TSS changed significantly along with the fermentation days up to day 12 after with the rate of depletion in TSS dropped for all the samples. In the fermentation process, low pH slows down the depletion of TSS.

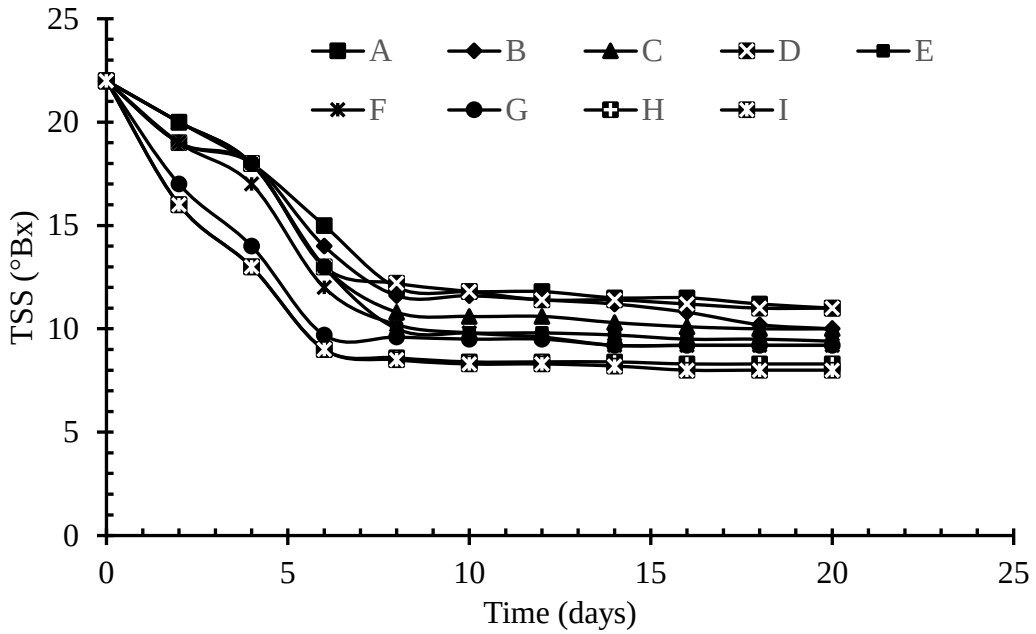

Fig. 4.2 TSS decrease during fermentation

The initial TSS of wines was 22°Bx. The sugar depletion rates were high in samples G, H, and I during fermentation. Similarly, for samples A, B, C, D, E, and F, depletion of TSS was slower. Then after 12 days of fermentation system sugar was depleted at a slow and steady rate. When the TSS was depleted up to 11-8°Bx it was observed that there was no significant depletion of sugar for the following days.

Singh *et al*. (2013) reported similar pattern for reduction of TSS during fermentation of mahua juice and linked the reduction TSS to consumption of sugar present in juice extract of mahuwa.

4.3.2 Kinetics of reducing sugar

Reducing sugar (as % dextrose) of the different musts were recorded at day 0, 4, 8, 12, 16, 20. The recorded data for different samples are as follows in Table E. 2. Graphical representation of kinetics of reducing sugar shows in Fig. 4.3.

Reducing sugar depleted at a higher rate during the first 8 days of fermentation in samples G, H, and I. In samples A, B, C, D, E, and F depletion rate was lower for the first 12 days of fermentation. After 12 days, the depletion rate remained constant. Afterward, there was no significant change in reducing sugar in samples A, B, C, D, E and, F from day 16. Similarly, from day 12 there was no significant change in RS in samples G, H, and I.

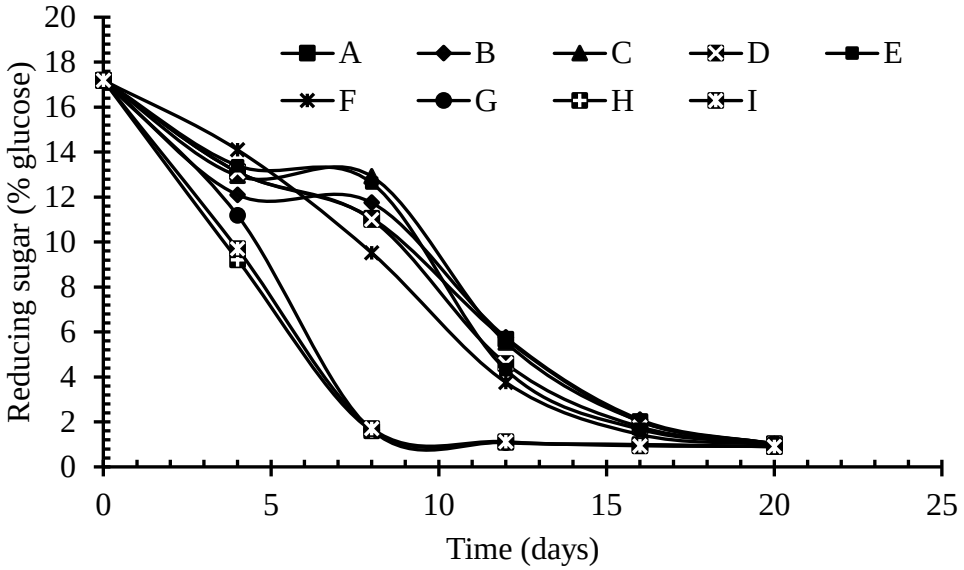

Fig. 4.3 Kinetics of reducing sugar during the course of fermentation

The increased rate of sugar intake displayed by G, H, and I when compared to samples A–F can be attributable to the reduced lag phase of commercial wine yeast when compared to isolated yeast. Singh *et al.* (2013) found a similar lowering sugar drop pattern during mahuwa juice fermentation and linked the source of depletion to sugar to alcohol and carbon dioxide.

4.3.3 Kinetics of total titratable acidity

Total titratable acidity (expressed as % lactic acid) of the different musts were recorded at day 0, 4, 8, 12, 16, 20. The recorded data for different samples are as follows in Table E. 3. The graphical representation is given in Fig. 4.4.

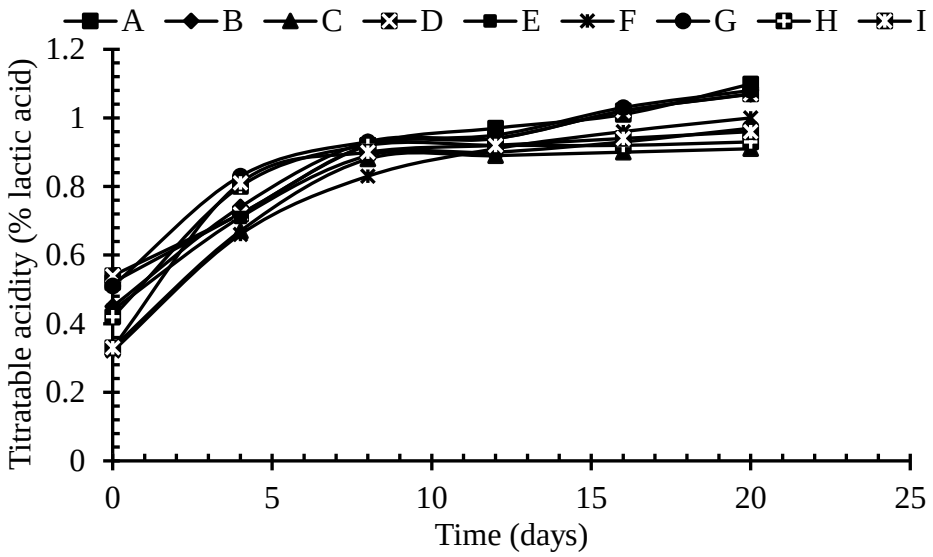

Fig. 4.4 Kinetics of total titratable acidity during the course of fermentation

In this study, early phases of fermentation show the greatest rise in total titratable acidity in all samples afterward. the rate of increase leveled off gradually. Peng *et al.* (2016) reported that titratable acidity increases in early stage of fermentation and the rate of titratable acidity pattern was similar to this study Thoukis *et al.* (1965) reported that yeast strain and the starting pH of the fermenting medium had no effect on the net rise in nonvolatile organic acidity. Similar result was obtained in this study.

4.3.4 Growth kinetics of *S. cerevisiae*

The yeast cells of *Saccharomyces cerevisiae* were counted at day 0, 4, 8, 12, 16 and 20. The recorded data for different samples are as follows in Table E. 4, (Appendix E). Growth curve of yeast cells are shows in Fig. 4.5.

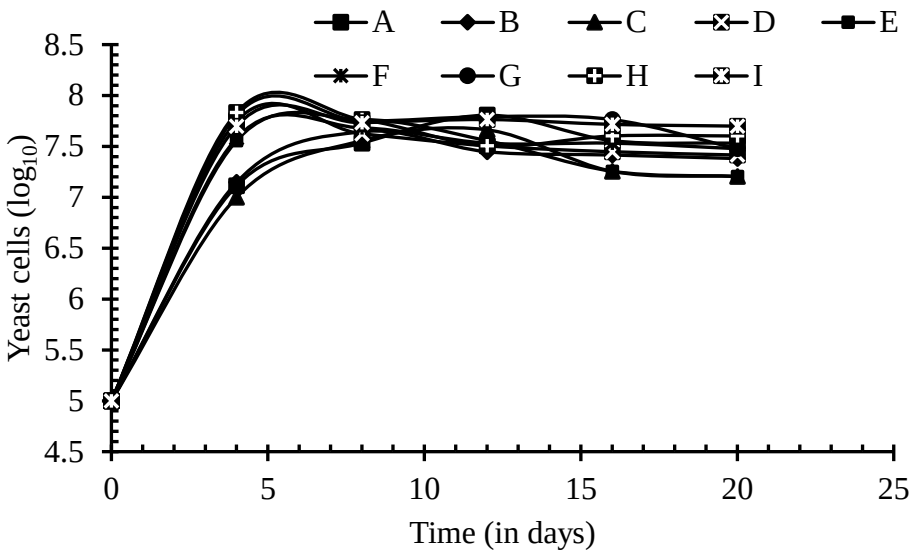

Fig. 4.5 Growth kinetics of *S. cerevisiae*

The yeast growth increased exponentially during the first 8 days of fermentation after which the growth rate was slowed down.

The initial pH of the must have a significant effect on the yeast growth kinetics affecting the metabolism of sugar, acidity, and ethanol production during alcoholic fermentation of the mahuwa must. Decreasing the pH decreased the cell number and growth rate, as well as substrate utilization and product formation for isolated yeast strain, which may be due to the optimal pH of wild yeast, which is 4-6 pH. Yang (1973) reported that *Sch. pombe* and *Saccharomyces cerevisiae*, the fermentation rate increased as the pH increased. Flower yeast and Murcha yeast encounter low pH stress at the beginning of fermentation, so initially their growth is slow down as compared to wine yeast. Liu *et al.* (2015) says that the initial pH of must was a vital factor influencing yeast growth and alcoholic fermentation. Except in a few cases, low beginning pH had the properties of lengthening the yeast lag phase, altering cumulative mass loss, modifying the consumption rate of total sugar, raising the final content of acetic acid and glycerol, and lowering the final amount of ethanol and l- succinic acid. Charoenchai *et al.* (1998) found that the yeast strains examined in his study exhibited similar growth behavior at either pH 3, 3.5, or 4. In this study the growth pattern did not significantly differ in each pH, but the isolated yeast strain exhibited more lag phase due to the low initial pH.

4.4 Effect of different pH and yeast strains on physicochemical composition of produced mahuwa wine.

The physicochemical parameter assessed were Total Soluble Solid, Reducing sugar, Total titratable acidity, fixed acidity, volatile acidity, and Total Phenolic Content (TPC).

4.4.1 Effect on Total Soluble Solid (TSS) and reducing sugar

The effects of pH and yeast strains on TSS and reducing sugars is summarized in Table 4.2.

Table 4.2 Final TSS and reducing sugar content of produced mahuwa wine

Samples	TSS (°Bx)	Reducing sugar (as % dextrose)
A	11.2^{e} (0.2)	1.07^{b} (0.03)
B	10^{cd} (0.2)	1.05^{b} (0.04)
C	10.06^{d} (0.3)	1.02^{b} (0.02)
D	11^{e} (0.2)	1.04^{b} (0.03)
E	9.4^{bc} (0.36)	1.04^{b} (0.03)
F	9.2^{b} (0.3)	1.01^{b} (0.02)
G	9.3^{bc} (0.15)	0.89^{a} (0.02)
H	8.2^{a} (0.1)	0.91^{a} (0.03)
I	8^{a} (0)	0.87^{a} (0.01)

Values are the means of three determinations. Figures in the parentheses are standard deviations. Means having similar superscripts in a column are not significantly different ($p>0.05$).

No significant difference in TSS among the samples grouped as HI, EFG, BC, and AD were found. The TSS of the samples with the highest value being for sample A with 11.2±0.2°Bx and lowest being for sample G with 8±0°Bx. There was no significant difference on reducing sugar found in sample A-F, fermented by isolated flower and murcha yeast. Similarly, no significant difference in reducing sugar in sample G-I, fermented by commercial wine yeast. Reducing sugar 1.07±0.03% (as dextrose) was maximum in sample A wine and minimum for sample G wine 0.87±0.01% (as dextrose).

4.4.2 Effect on total titratable, fixed-, and volatile acidity

Effect of different pH level and yeast strains on acidities of the final produced mahuwa wine were studied. Total titratable acidity expressed as lactic acid, volatile acidity expressed as acetic acid and fixed or nonvolatile acidity as lactic acid were evaluated. The data for acidities are given on Table 4.3.

Table 4.3 Final acidities of mahuwa wine

Samples	Total titratable acidity (% as lactic acid)	Volatile acidity (% as acetic acid)	Fixed acidity (% as lactic acid)
A	1.1^{c} (0.01)	0.13^{abc} (0.01)	0.91^{c} (0.01)
B	1.07^{bc} (0.01)	0.096^{ab} (0.005)	0.93^{c} (0.008)
C	0.92^{a}(0.05)	0.08^{a} (0.003)	0.80^{abc} (0.05)
D	1.07^{bc} (0.04)	0.2^{c}(0.02)	0.76^{abc} (0.04)
E	0.97^{abc} (0.07)	0.083^{a} (0.006)	0.84^{bc} (0.08)
F	1.01^{abc} (0.01)	0.13^{abc} (0.02)	0.82^{abc} (0.03)
G	1.09^{c} (0.05)	0.19^{bc} (0.01)	0.80^{abc} (0.04)
H	0.94^{ab} (0.08)	0.19^{c} (0.01)	0.64^{a}(0.1)
I	0.96^{abc} (0.03)	0.20^{c} (0.01)	0.66^{ab} (0.02)

Values are the means of three determinations. Figures in the parentheses are standard deviations. Means having similar superscripts in a column are not significantly different ($p>0.05$).

Wines that lack acid are described as "flat." The acids impact titratable acidity, which influences flavor chemically. According to numerous studies, the titratable acidity of mahuwa wine ranged between 0.39 and 0.65%. When different yeast concentrations were utilized in different TSS, the titratable acidity ranged from 0.39 to 0.58% , according to Belkhede (2019). Yadav *et al.* (2009) discovered 0.63% acidity in mahuwa wine as well. The greatest acidity found in this experiment was 1.1±0.01%, while the minimum acidity was 0.92±0.05%. which does not correspond to the maximum prior result. Nevertheless, Singh *et al.* (2013) reported that titratable acidity was 1.1% in mahuwa wine. The greater

amount of acidity in the samples might be attributed to the media's low initial total acidity. The percentage increase in non-volatile acidity was largest in medium with low beginning total acidity, according to the study of Thoukis *et al.* (1965). From the reports high titratable acidity was the result of high fermenting temperature (Singh *et al.*, 2013).

Yadav *et al.* (2009) reported that the volatile acidity in wine prepared from different treatments to the juice was in the range of 0.115-0.137%.The maximum volatile acidity (0.134%) was observed at 30°C (Singh *et al.*, 2013). In this study, the volatile was in the range. Nevertheless, in samples G, H, and I the volatile acidity was slightly high.

4.4.3 Effect on Total Phenolic Content (TPC)

The final Total Phenolic Content (TPC) was assessed in prepared mahuwa wine using Folin-Ciocalteau reagent and UV-vis spectrophotometer at 765 nm using standard curve given in Appendix C. The data for TPC of produced fermented products are given in Table 4.4.

The total phenolic content of mahuwa wine was 273.3±3.2 mg GAE/L for sample A was maximum and 234.9±7.6 mg GAE/L for sample B was minimum. Samples B, C, D, F, G, and I didn't show significant different nevertheless samples A, and E, H was different. The variation in phenolic concentrations seen after fermentation might be attributable to a variety of processes, including polyphenol adsorption on yeast cell walls, condensation and polymerization events, and enzyme activity (Czyzowska and Pogorzelski, 2002; Ginjom *et al.*, 2011; Pérez-Gregorio *et al.*, 2011; Soni and Dey, 2013). Soni and Dey (2013) reported that the TPC of mahuwa wine fermented at 18°C, 25ºC, and 35°C was 366.8± 9.5, 265±8.4, and 366±7.9 respectively. According to Manoj (2018) phenol % of the mahuwa-pomegranate wine fermented at different temperatures and pH using two different strains of yeast was in the range of 0.25-0.40%, which agrees with the results of this study.

Table 4.4 Total phenolic content of mahuwa wine

Samples	TPC (mg GAE/L)
A	273.3^{c} (3.2)
B	234.9^{a} (7.6)
C	237.7^{a} (5.04)
D	246.3^{a} (4)
E	250.7^{ab} (5.3)
F	247.7^{a} (6.8)
G	238.3^{a} (8)
H	266.3^{bc} (5.6)
I	248.1^{a} (2.5)

Values are the means of three determinations. Figures in the parentheses are standard deviations. Means having similar superscripts in a column are not significantly different ($p>0.05$).

4.5 Effect of different pH and yeast strains on volatile composition of produced mahuwa wines

Effect of different pH and yeast strains on volatile composition of produced mahuwa wines has been summarized in Table 4.5. In this analysis, the ethanol concentration of samples B, E, F, H, and I was almost identical. However, the ethanol concentration in samples A, C, D, and G was minimal. This is related to yeast's low sugar intake and growth rate at low pH. Another cause of low alcohol production might be a yeast strain. The maximum ethanol concentration found in this investigation was 12.37±0.32% abv for sample F (*murcha* yeast) and 10.4±0.1% abv for sample A (flower yeast). The greatest ethanol %age in mahuwa wine fermented under multiple yeast strain concentrations and varying TSS level, according to Belkhede (2019), was 12.30% abv. According to the findings of the different research on mahuwa wine the ethanol content reported was 9.10% abv fermented at 25°C and 7.5% abv fermented at 18°C as mentioned by Soni and Dey (2013) similarly, Preeti *et al.* (2009) reported 9.9 %abv in mahuwa wine. The ethanol %age of the mahuwa-pomegranate wine was even lower at 7.62 % abv, according to Manoj (2018).

Table 4.5 Volatile constituents of mahuwa wine

Samples	Ethanol content (% v/v)	Ester content (g ethyl acetate /100 L alc.)	Aldehyde (g acetaldehyde /100 L alc.)	Higher alcohol (mg/L)
A	10.4^{a} (0.1)	204.3^{a} (5.5)	99.8^{a} (0.8)	98.7^{cd} (2)
B	11.83^{cd} (0.15)	146^{b} (7.6)	113.5^{ab} (4.4)	110.2^{d}(2.2)
C	11.53^{bc} (0.3)	157.4^{b} (2.7)	112.1^{ab} (5.4)	74.7^{ab} (4.5)
D	11^{ab} (0.2)	145.4^{bc} (7.5)	140.8^{cd} (3)	63.3^{a} (6)
E	12.13^{cd} (0.23)	236^{cd} (6.7)	148.8^{d} (4.3)	181.7^{f} (6.6)
F	12.37^{d} (0.32)	202^{d} (1.8)	167.5^{e} (10)	92.1^{c} (4.5)
G	11.53^{bc} (0.05)	177.3^{e} (6.8)	141.5^{cd} (6)	86.7^{bc} (3.5)
H	12.1^{cd} (0.17)	100.3^{e} (1.7)	126.4^{bc} (5.6)	157.3^{e} (3)
I	12.33^{d} (0.3)	163.7^{f} (3.5)	112^{ab} (10)	93.3^{c} (4.1)

Values are the means of three determinations. Figures in the parentheses are standard deviations. Means having similar superscripts in a column are not significantly different ($p>0.05$).

In this study, we determined the maximum level of ethyl acetate in sample E (236±6.7 g/100 L) and the minimum level of ethyl acetate in sample H (100.3±1.7 g/100 L). Katarína *et al.* (2017); Plata *et al.* (2003) stated that many variables influence the synthesis of esters, including aeration, the content of fatty acids, higher alcohols, and their precursors. similarly, Vilanova *et al.* (2007) mentioned that the average ester production and relative proportions of each ester are strongly dependent on yeast strain, and the effect of other factors such as temperature, oxygen, and nitrogen may also be strain-dependent. Differences across strains may also be caused by variations in the expression of genes involved in ester production (Sumby *et al.*, 2010). In article reviewed by Cliff and Pickering (2006), the thresholds for ethyl acetate to be 170 ppm for white wine and 160 ppm for red wine. In the mahuwa wine literature, there were no results for correlation.

Acetaldehyde levels in this research vary from 99.8±0.8 to 167.5±10 g/100L. There were no results for correlation in the mahuwa wine literature. The highest acetaldehyde levels in white wine and sherry, according to Liu *et al.* (2015) analysis of the literature, were 493 mg/L and 500 mg/L, respectively. Other wines made without the mahuwa flower do not have the same value as ours. According to Henschke (1993), metabolism of amino acids contributed to the production of acetaldehyde. Because of the amino acid present in the mahuwa flower, acetaldehyde levels in the wine high. Oxygen availability, and higher temperatures also influence acetaldehyde levels in wine (Romano *et al.*, 1994). Acetaldehyde influences the color, astringency, and aroma of wine (Sheridan and Elias, 2015). The presence of large amounts of these volatile compounds is thought to be a defining feature of sherry-style wines (Cortes *et al.*, 1998; Sponholz, 1993).

In this study, the maximum alcohol concentration was 181.7±6.6 mg/L and the lowest was 63.3±6 mg/L. Wine has a total of 80-540 mg/L of higher alcohols. Concentrations up to 300 mg/L contribute to pleasant flavor, however concentrations above 400 mg/L cause unpleasant flavor and harsh flavor (Rapp and Mandery, 1987; Vidrih and Hribar, 1999). Suomalainen and Lehtonen (1979) stated that the yeast strain and the fermentation environment can have an impact on the development of aroma components. Other aroma molecules, such as sulfur compounds and phenols, are also greatly influenced by yeast. Which directly influence sensory characteristics of the wine. Rankine (1967) found that, the pH of the juice was the same, but the amount of higher alcohols varied significantly. Temperature has a broad impact, as well as a yeast/temperature connection (Rankine, 1967). In our study, higher alcohol variations within samples were driven by a combination of yeast strain, aerobic environment, pH, and fermentation temperature.

4.6 Effect of different pH and yeast strains on organoleptic properties of produced mahuwa wine.

The sensory analysis was conducted with 10 semi-trained panelists who are familiar with fermented beverages. The panelists were teaching staffs and students of M. tech (Food) and B. Tech (Food) programs. A 7-points hedonic rating test was done for the evaluation of wine where, 1: faulty, 2: poor, 3: below average, 4: average, 5: above average, 6: very good and 7: exceptional. There were five sensory parameters, viz., (i) appearance, (ii) odor, (iii)

mouthfeel, (iv) aftertaste and (v) overall acceptance. The table for the mean scores for the sensory attributes is given in Table 4.6.

Table 4.6 Mean sensory scores for different attributes

Samples	Quality attributes				
	Appearance	Odor	Mouthfeel	Finish	Overall Acceptance
A	4.1^{a}	4.7^{a}	3.8^{a}	4.6^{ab}	4.3^{a}
B	4.1^{a}	5.2^{a}	3.8^{a}	4.5^{ab}	4.2^{a}
C	5.3^{bc}	5.2^{a}	4.2^{ab}	4.1^{a}	4.9^{a}
D	4.9^{b}	5^{a}	4.3^{ab}	4.4^{a}	4.6^{a}
E	5.7^{cd}	5.5^{a}	5.4^{b}	5.7^{b}	5.8^{b}
F	5.9^{cd}	5.3^{a}	4.3^{ab}	4.3^{a}	4.9^{a}
G	6^{d}	5.1^{a}	4.7^{ab}	4.5^{ab}	5^{ab}
H	5.9^{cd}	5.3^{a}	4.6^{ab}	4.6^{ab}	4.6^{a}
I	4.9^{b}	4.4^{a}	4.4^{ab}	4.2^{a}	4.2^{a}

Using Table 4.6, the prepared wines can be ranked into groups for different attributes as follows:

Appearance : G > E/F/H > C > D/I > A/B

Odor : No significant difference

Mouthfeel : E > C/D/F/G/H/I > A/B

Finish : E > G > A/B/C/D/F/H/I

For all samples, the score for wine appearance was above average. The mouthfeel score in samples A and B indicates that the wine was below average. C, D, and F-I samples were statistically identical. Sample A and I were commented to be distinctly smokey. The mean scores for the majority of the sensory quality descriptors were higher for sample E (*murcha* yeast fermented at pH 3.5) wine than for the other wines assessed.

PART V

Conclusions and recommendations

5.1 Conclusion

The experiment was set up in a Randomized Design using three varieties of yeast (commercial wine yeast and yeasts isolated from mahuwa flower and *murcha*) and three levels of pH (3, 3.5, 4) at 22°Bx TSS, and it was carried out in triplicate. The following are the findings of the chemical analysis and sensory score of mahuwa wine with various types of yeast and pH:

1. Around 53% of the households in Gadhawa Gaunpalika Ward No. 08 are engaged in making mahuwa spirit, with an annual production and income of 20000 L and NRs 4000000.0, respectively. This represents a decent income of about NRs 40000.0 per household.
2. The growth and kinetics) exhibited by indigenous yeast isolated from mahuwa flowers and *murcha* samples are similar to those of commercial wine yeast.
3. Although the effects of pH and yeast variations on physicochemical properties of the mahuwa wines are significant, the correlation between sensory quality and physicochemical qualities is still too complex to establish.
4. The sensory properties of mahuwa wines prepared using indigenous yeasts isolated from mahuwa flowers and *murcha* samples can be as good as (or even superior to) those from commercial wine yeast.

5.2 Recommendations

The findings are based on the outcomes of a single-season experiment; thus they are just illustrative rather than definitive. As a result, for final recommendations, the current study's findings require additional confirmation. Nonetheless, the following recommendations are suggested:

- Treatment levels and fermentation conditions can be varied when fermenting the mahuwa must.

- A study on the influence of sensory evaluations of aged mahuwa wine can be conducted.
- It is possible to investigate the genotype and phenotype of isolated yeast.
- Research on the various products of the mahuwa flower can be conducted.

PART VI

Summary

Mahuwa wine is made from the flowers of *M. indica* (syn. *M. latifolia*) and *M. longifolia*, which are grown locally. These flowers are picked, dried, and preserved before utilizing them in winemaking. These flowers are an excellent source of fermentation substrate. Prior to the collection of mahuwa flowers for the research, a brief survey was carried out at Gadhawa Gaunpalika (Dand district) to generate data on preparation of mahuwa spirit by the natives. Fermentative native yeasts from mahuwa flower and *murcha* (a starter cake) were screened in the laboratory for use in the present research. For juice preparation, the dried mahuwa flowers were soaked in water overnight, squeezed with hand, and extraction carried out by filtering through muslin cloth. The juice was analyzed for acidity, total ash, and reducing sugar content. Then the total soluble solids (TSS) of the juice was adjusted to 22°Bx and fermentation carried out at three pH levels (3.0, 3.5 and 4.0) with three yeast types, viz., commercial wine yeast and fermentative yeast isolates from *murcha* and mahuwa flowers. Fermentation kinetics were studied in terms of TSS, reducing sugar, titratable acidity, and yeast cells for a fermentation duration of 20 days. Wine samples were examined for sensory attributes (hedonic rating) and physicochemical properties (alcohol, methanol, higher alcohols, aldehydes, and acidity, total phenolic content) after 21 days of fermentation.

The survey showed that 53% of the total of 187 households of Gadhawa Gaunpalika, Ward No. 08 are involved in the preparation of mahuwa spirit. The annual production and sale are 20000 L and NRs 4000000.0, respectively. This is a decent income for the natives of this Ward.

On analysis, the extracted mahuwa juice (prepared for fermentation) contained an acidity of 0.21±0.02% (as lactic acid), a total ash content of 0.27±0.02%, and a reducing sugar content of 17.2±0.40% as dextrose. The highest values of ethanol, esters, aldehydes, higher alcohol and total phenolic content were observed, respectively, in sample F (12.37±0.32% abv, wine yeast, pH 3.5), sample A (204.3±5.5 g/100 L alcohol, flower yeast, pH 3), sample F (167.5±10 mg/100 L alcohol, wine yeast, pH 3.5), sample H (157±3 mg/L, mahuwa yeast, pH 3.5), and sample A (273.3±3.2 mg GAE/L, mahuwa yeast, pH 3). Sample E (*murcha* yeast, pH 3.5) had the highest overall sensory score (5.8 out of 7), implying it is too complex to correlate the physicochemical properties to sensory qualities.

Statistical analysis of the sensory data also show that the overall acceptance is positively related to properties like odor, mouthfeel and finish of the mahuwa wine. The kinetic study shows that an initial must pH of 4 leads to rapid sugar assimilation by all the yeast types, which is also reflected corresponding increase in the number of yeast cells. It can thus be concluded that mahuwa wine can be prepared using any fermentative yeast types, including the natural flora of mahuwa flower. However, if a good quality *murcha* cake is readily available, it can be also used to produce a good quality mahuwa wine.

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Appendices

Appendix A

Date: .....

Survey for mahua spirit production

1. Informant detail

Name: ***Sex:*** M | F ***Age:*** yrs.

Municipality: ***Ward:***

Profession: ***Education:*** Illiterate / Literate / School level / College level

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2. Questionnaire

1. How long have you been producing mahua spirit?

☐ 1-5 years ☐ 6-10 years ☐ 10-15 years ☐ more than 15 years

2. What is the process that you follow for mahua spirit production?

3. Have you tried to modify the traditional process of mahua spirit production? If yes, give detail.

4. What type of culture do you use for fermentation?

5. How do you get mahua flowers?

☐ collect and store ☐ purchase from other collectors ☐ both

6. If you collect yourself, how do you preserve/process mahua flowers?

7. What are the other uses of mahua flower?

8. How much mahua spirit do you produce annually?

9. What is your annual income from mahua spirit business?

10. What do you generally do with the money earned from mahua spirit?

11. How many mahua spirit distillers are there in this region (municipality)?

12. Who are the general costumers?

13. Have you heard your spirit sometimes making people sick? If yes, what could be the reason?

14. Where/how do you sell your product?

15. Don't you think that mahua spirit production should be banned? Yes | No
(please give reason for the answer)

16. Is mahua spirit production allowed by the local government bodies? Yes | No
(please give reason for the answer)

17. What is the role of women/children in mahua spirit making?

18. How do you ensure quality of mahua spirit?

19. How do you find the strength of the spirit?

20. Do women and children in your family also taste mahua spirit?

Enumerator: .....

Appendix B

Specimen card of sensory evaluation by 7-point hedonic rating test

Sensory evaluation of mahuwa wine

Name of panelist: ……. Date: ……

Name of Product: Mahuwa wine

Please evaluate the product by your sense organ to show your perception by checking at the point that best describes your feelings about the product and also write to any of the defect as described below. An honest expression of personal feeling will help me.

Parameters	Samples								
	A	B	C	D	E	F	G	H	I
Appearance									
Odor									
Mouthfeel									
Finish									
Overall acceptance									

Quality description:

1: Faulty 2: poor 3: Below average 4: Average 5: Above average
6: very good 7: Exceptional

Comments: ……

Signature

Appendix C

ANOVA result for analysis of mahuwa wine

Table B. 1 Two-way ANOVA (no blocking) for appearance

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	44.8000	5.6000	31.61	<.001
Panelist	9	2.8444	0.3160	1.78	0.086
Residual	72	12.7556	0.1772		
Total	89	60.4000			

Since, F pr. < 0.05, there is significant difference between the sample at 5% level of significance so LSD testing is necessary.

Table B. 2 Two-way ANOVA (no blocking) for odor

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	9.1556	1.1444	1.73	0.105
Panelist	9	15.7889	1.7543	2.66	0.010
Residual	72	47.5111	0.6599		
Total	89	72.4556			

Since, F pr. > 0.05, there is no significant difference between the sample at 5% level of significance so LSD testing is not necessary.

Table B. 3 Two-way ANOVA (no blocking) for mouthfeel

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	19.0889	2.3861	3.22	0.003
Panelist	9	12.9444	1.4383	1.94	0.059
Residual	72	53.3556	0.7410		
Total	89	85.3889			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 4 Two-way ANOVA (no blocking) for finish

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	17.4222	2.1778	2.99	0.006
Panelist	9	26.5444	2.9494	4.06	<.001
Residual	72	52.3556	0.7272		
Total	89	96.3222			

Since, F pr. < 0.05, there is significant difference between the sample at 5% level of significance so LSD testing is necessary.

Table B. 5 Two-way ANOVA (no blocking) for overall acceptance

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	20.5556	2.5694	7.60	<.001
Panelist	9	3.1667	0.3519	1.04	0.417
Residual	72	24.3333	0.3380		
Total	89	48.0556			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 6 One way ANOVA (no blocking) for final TSS in produced mahuwa wine

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Samples	8	28.86667	3.60833	69.10	<.001
Residual	18	0.94000	0.05222		
Total	26	29.80667			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 7 One way ANOVA (no blocking) for final reducing sugar in produced mahuwa wine

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	0.1301630	0.0162704	17.50	<.001
Residual	18	0.0167333	0.0009296		
Total	26	0.1468963			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 8 One way ANOVA (no blocking) for total titratable acidity

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	0.117945	0.014743	6.14	<.001
Residual	18	0.043231	0.002402		
Total	26	0.161176			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 9 One way ANOVA (no blocking) for volatile acidity

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	0.070540	0.008818	7.91	<.001
Residual	18	0.020072	0.001115		
Total	26	0.090612			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 10 One way ANOVA (no blocking) for fixed acidity

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	0.229679	0.028710	6.09	<.001
Residual	18	0.084885	0.004716		
Total	26	0.314564			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 11 One way ANOVA (no blocking) for ethanol content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	10.33852	1.29231	25.85	<.001
Residual	18	0.90000	0.05000		
Total	26	11.23852			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, LSD testing is necessary.

Table B. 12 One way ANOVA (no blocking) for higher alcohol content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	35878.34	4484.79	236.92	<.001
Residual	18	340.74	18.93		
Total	26	36219.08			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, LSD testing is necessary.

Table B. 13 One way ANOVA (no blocking) for ester content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	38527.23	4815.90	163.38	<.001
Residual	18	530.59	29.48		
Total	26	39057.82			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 14 One way ANOVA (no blocking) for aldehyde content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Sample	8	11544.88	1443.11	37.53	<.001
Residual	18	692.10	38.45		
Total	26	12236.98			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Table B. 15 One way ANOVA (no blocking) for total phenolic content in produced mahuwa wine

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F pr.
Samples	8	4013.67	501.71	15.82	<.001
Residual	18	570.92	31.72		
Total	26	4584.59			

Since F pr. < 0.05, there is significant difference between the sample at 5% level of significance, so LSD testing is necessary.

Appendix D

Standard curves

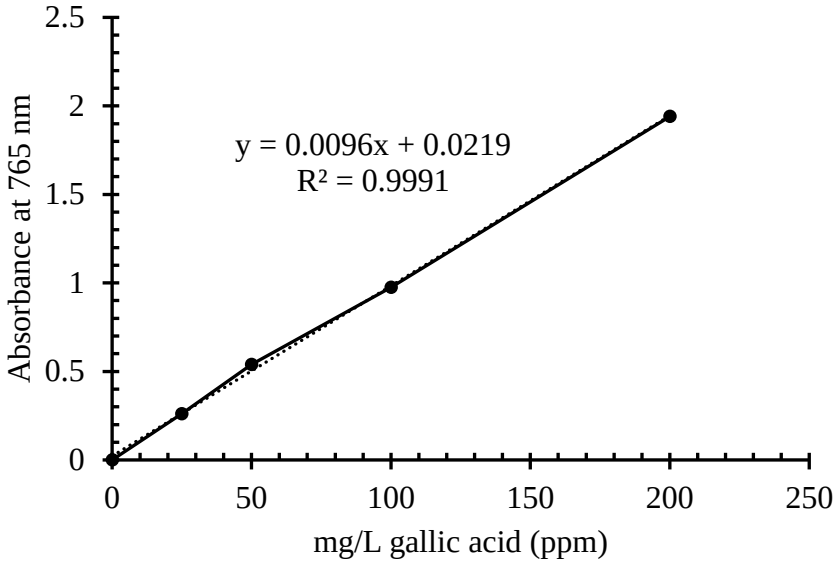

Fig. C.1 Standard curve for total phenolic content determination

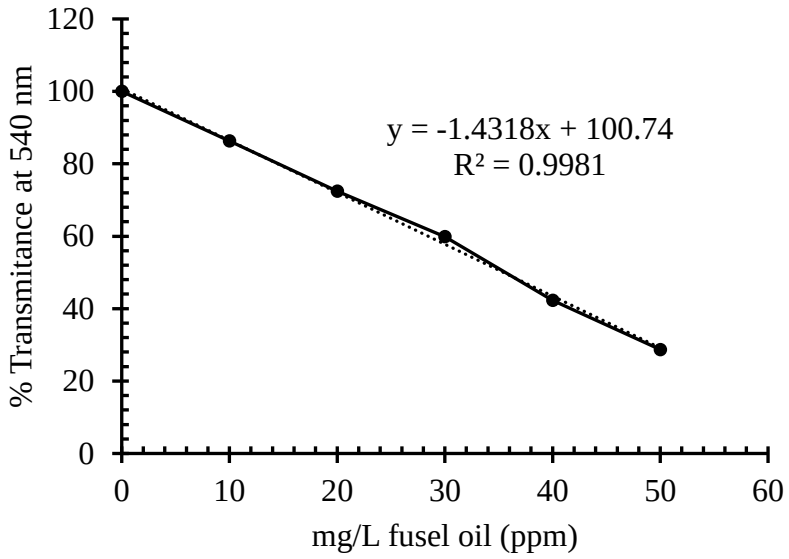

Fig. C.2 Standard curve for fusel oil content determination

Appendix E

Raw data tables

Table E. 1 Change in TSS (ºBx) during the course of fermentation

Day	A	B	C	D	E	F	G	H	I
0	22	22	22	22	22	22	22	22	22
2	20	19	20	19	20	19	17	16	16
4	18	18	18	18	18	17	14	13	13
6	15	14	13	13	13	12	9.7	9	9
8	12	11.6	10.8	12.2	10	10.2	9.6	8.6	8.5
10	11.8	11.6	10.6	11.8	9.8	9.8	9.5	8.4	8.3
12	11.8	11.4	10.6	11.4	9.8	9.6	9.5	8.4	8.3
14	11.5	11.2	10.3	11.4	9.7	9.2	9.2	8.4	8.2
16	11.5	10.8	10.1	11.2	9.5	9.2	9.2	8.3	8
18	11.2	10.2	10	11	9.5	9.2	9.2	8.3	8
20	11	10	10	11	9.4	9.2	9.2	8.3	8

Table E. 2 Change in reducing sugar (% dextrose) content during fermentation

Day	A	B	C	D	E	F	G	H	I
0	17.2	17.2	17.2	17.2	17.2	17.2	17.2	17.2	17.2
4	13.12	12.1	12.95	13.12	13.4	14.1	11.19	9.21	9.7
8	11.06	11.75	12.92	11	12.63	9.52	1.64	1.61	1.71
12	5.67	5.76	5.54	4.6	4.32	3.76	1.08	1.09	1.12
16	2.02	2.1	2.03	1.78	1.67	1.44	0.99	0.99	0.94
20	1.03	1.01	1.02	1.01	1.03	0.99	0.9	0.9	0.91

Table E. 3 Change in acidity (% lactic acid) during the course of fermentation

Day	A	B	C	D	E	F	G	H	I
0	0.52	0.45	0.33	0.54	0.44	0.32	0.51	0.42	0.33
4	0.72	0.74	0.67	0.72	0.71	0.66	0.83	0.8	0.81
8	0.92	0.93	0.88	0.92	0.89	0.83	0.93	0.92	0.9
12	0.97	0.94	0.89	0.94	0.9	0.91	0.95	0.92	0.92
16	1.01	1.02	0.9	1.02	0.93	0.96	1.03	0.92	0.94
20	1.099	1.069	0.91	1.07	0.97	1.00	1.08	0.93	0.96

Table E. 4 Growth of yeast cell during fermentation

Day	A	B	C	D	E	F	G	H	I
0	5	5	5	5	5	5	5	5	5
4	7.1139	7.1461	7	7.7481	7.5563	7.5797	7.8061	7.8325	7.6989
8	7.5314	7.6434	7.5563	7.6232	7.7634	7.6812	7.7481	7.763	7.7323
12	7.8061	7.4471	7.6627	7.5051	7.5563	7.5314	7.7923	7.505	7.7634
16	7.5563	7.4149	7.2552	7.4471	7.2552	7.5314	7.7634	7.602	7.716
20	7.531	7.380	7.204	7.414	7.204	7.477	7.477	7.602	7.698

Color plates

P 1 Survey site (red circle=Koilabas), Google imagery from mobile

P 2 Researcher (right) against a mahuwa tree backdrop

P 3 Researcher (left) taking survey on mahuwa at Gadhawa Gaunpalika

Image source: Chaudhary (2021)

P 4 Mahuwa flower collection in Nepal (mid-March)

(a) (b) (c)

Image source: Kumar *et al.* (2017)

P 5 Mahuwa flowers (a) blooming, (b) picked flowers, (c) sun-dried flowers

P 6 Screening of yeasts from mahuwa flower and *murcha* in the laboratory

P 7 Inoculum build-up for fermentation

P 7 Panelist performing sensory analysis