PREAPARATION AND STUDY OF FERMENTATION KINETICS, ANTIOXIDANT, ANTIMICROBIAL, α-AMYLASE INHIBITORY ACTIVITY AND QUALITY ANALYSIS OF *BERGENIA CILIATA* ADDED METHEGLIN

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Preparation and Study of Fermentation Kinetics, Antioxidant, Antimicrobial, α -Amylase Inhibitory Activity and Quality Analysis of *Bergenia ciliata* Added Metheglin

A dissertation submitted to the Department of food Technology, Central campus of Technology, Tribhuvan, University, in partial fulfillment of the requirements for the Degree of B. Tech. in Food Technology

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Approval Letter

This ***dissertation*** entitled ***Preparation and Study of Fermentation Kinetics, Antioxidant, Antimicrobial, α -Amylase Inhibitory Activity and Quality Analysis of Bergenia ciliata Added Metheglin*** presented by **Sanjog Kharel** has been accepted as the partial fulfillment of the requirement for the **B. Tech. Degree** in **Food Technology.**

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Abstract

The effect of addition of *Bergenia ciliata* (Pakhanbedh) rhizome at 5 different concentrations (0, 0.25, 0.5, 0.75 and 1% m/v) on fermentation kinetics, physicochemical composition, antioxidant, antimicrobial, and α-amylase inhibitory activity, and organoleptic quality of metheglins produced were examined. The honey used was brassica honey produced by *Apis cerena* from the Chitwan district of Nepal. The aim of this dissertation was to study the effect of *B. ciliata* rhizome concentration on fermentation and yeast growth rate, to find general concentration for best organoleptic property, to assess whether incorporating the *B. ciliata* rhizome (herb) alters the physicochemical properties of the produced metheglins, and to study whether the medicinal properties of the rhizome get extracted to the produced metheglins and study their effect at different concentration.

From studying fermentation kinetics in honey must with the rhizome concentration from 0% to 1% it was found that, at concentration of rhizome ≥ 0.5% overall fermentation rate decreases. The rhizome does not alter the physicochemical properties of the produced metheglins, except for a slight decrease in volatile acidity and a slight increase in higher alcohol content at concentration 0.75% and 1% respectively. Also from sensory analysis of the prepared metheglin, the best concentration for the rhizome incorporation is ≤ 0.5%. Total phenolic content and antioxidant activity increased significantly on incorporation and increment of *B. ciliata* rhizome. The antimicrobial activity on *Staphylococcus aureus* and *Escherichia coli* and α-amylase inhibitory activity increased significantly on incorporation and increment of the rhizome, providing metheglin properties of medicinal wine. Thus, incorporation of *B. ciliata* rhizome to produce metheglin significantly increases the therapeutic properties and the optimum concentration for the best organoleptic, physicochemical and therapeutic properties was found to be 0.5%.

Contents

List of tables

List of figures

List of abbreviations

Abbreviation	Full form
ANOVA	Analysis of Variance
AOAC	Association of Analytical Communities
AWRI	Australian Wine Research Institute
BJCP	Beer Judge Certification Program
DMAB	p-DiMethyl Amino Benzaldehyde
DPPH	2,2-DiPhenyl-1-Picryl-Hydrazyl-hydrate
GAE	Gallic Acid Equivalent
HSD	Honestly Significant Difference
MSA	Mannitol Sugar Agar
OAV	Odor Activity Value
OIV	International organization for vine and wine
PVPP	Poly Vinyl PolyPyrolidone
TPC	Total Phenolic Content
TSS	Total Soluble Solid
YAN	Yeast Assimilable Nitrogen

Part I

Introduction

1.1 General introduction

Metheglin is a mead which contains spices or herbs (Tayleur, 1973). Mead is the alcoholic product generally having 8-18% (v/v) ethanol made by yeast to the honey water must, followed by a period of fermentation time (Iglesias *et al.*, 2014). The amount of alcohol obtained at the end of fermentation depends on the dilution rate of the honey. Mead contains ethanol and many other compounds such as sugars, acids, vitamins, phenolic compounds, mineral (Švecová *et al.*, 2015). The mead is considered as "nectar of gods". There is also some term called "Mead of poetry" in Norse mythology, a mythical beverage that whoever drinks becomes a skald or scholar to recite any information and solve any question. Mead is one of the oldest fermented beverages and was considered drinkable honey. The production of mead has been known since ancient times.

For flavor or for enrichment of wine with chief chemical constituents, different raw materials have been used for preparing wine (Gubhaju, 2006). Mead can also be fermented with various herbs, spice, fruits, grains or hops (Fitch, 2002). Since mead is a fermented diluted honey, it is considered honey wine, but it falls into its own category in alcoholic beverage. Metheglin is as ancient as mead and its recipe is found in a mid-15th century collection of medical recipes, which includes three recipes for mead (honey wine) one of which is metheglin (Digby, 1669). The word metheglin is derived from the Welsh word "Meddyglyn" which comes from the Latin "medicus", a doctor and means "medicinal liquor" (Acton and Duncan, 1965).

Even though honey has antimicrobial properties, ancient people wouldn't have known this. They might have added herbs or spices to preserve the mead as long as possible. Some herbs may have actually had a preservative effect, which allowed for larger batches that stayed drinkable longer. Since there were no "doctors" in those days, the local healers would have added bitter tonics and medicinal herbs into the mead to make them easier to take. Adding medicinal herbs to make metheglins was also a way to preserve the herbs to use in winter months. Whatever the original reasons for adding herbs and spices to meads, metheglins have been with us ever since (Acton and Duncan, 1965)

The spice or herbs are seed, root, bark, leaves, flower or other plant parts which are either ground or used as whole and are mostly used in dry form in must. By definition, metheglins are traditional meads made with herbs and/or spices for flavoring, preserving, medicinal, or other purposes. If medicinal herbs are used, metheglin produced will be a medicinal wine. Various kinds of herbs and spices play an important role in alcoholic beverage production. They are used as enhancer, preservative, and antioxidant sources (Yuwa-Amornpitak *et al.*, 2012).

The alcohol from the fermented sugar in mead, paired with acids, act as an excellent solvent or vehicle for extracting some of the beneficial components of spice/herbs. It may extract components as color and flavor and components with nutritional and medicinal values (Payne, 2016). Metheglin, being a type of mead, not only causes a less impact on the body's systems than distilled spirits, but it also comes with other nutritive benefits and elements that help the body break it down and process it.

Bergenia ciliata, commonly called as Pakhanbedh, is a perennial rhizomatous creeping herb on rocks ledges with stout. The part used is rhizome. In Himalaya region, many rural communities use *B. ciliata* to treat various diseases. For century's rhizome of *B. ciliata* has been used for curing pulmonary infections, leucorrhea, piles and for dissolving bladder and kidney stones (Ahmad *et al.*, 2018; Yadav, 2016). It is popular in the eastern hilly region of Nepal for its many health benefits and there the people make pakhanbedh raksi, a type of alcoholic distillate.

1.2 Statement of problem

Wine culture in Nepal is comparatively a new practice. Since the beginning, wineries of Nepal have been focusing on the very regular types of wines using common raw materials like grapes, locally available seasonal fruits and, to some extent, some Himalayan herbs and berries are found to be used. Studies related to the preparation and quality analysis of herbal wines in Nepalese context are scant. Fermented products from honey are widely consumed around the world. However, compared to wine, the technological and scientific development in this is very low. Mead and metheglin production faces several problems, namely delays and "pouts" fermentations, lack of product uniformity and production of yeast off-flavors. Many factors might be related to these problems, such as honey variety, temperature, medium composition (vitamin and nitrogen content), chemical constituent of herbs,

fermentative yeast, pH and many more. So without adequate technical knowledge and studies it is hard to bloom metheglin production and market. Information on the fermentation kinetics, antioxidant activity, antidiabetic activity, antimicrobial activity and other therapeutic potential of mead and herbal mead are also scanty. People want more diversity in product these days, they want to try new things also, people are becoming more health conscious day by day. With this view, the present study was undertaken to investigate the possibility of preparing metheglin using *Bergenia ciliata* rhizome (pakhanbedh).

1.3 Objectives

1.3.1 General objective

The general aim of this dissertation was to study the kinetics of fermentation and to evaluate therapeutic and quality attributes of *B. ciliata* added metheglins.

1.3.2 Specific objectives

The specific objectives of the study were:

1. To prepare metheglins by incorporating different concentrations of herb (*B. ciliata* rhizome) to honey must.
2. To study the fermentation kinetics of honey must, with different herb concentrations, using pure starter culture of *Saccharomyces cerevisiae*.
3. To analyze and compare some of the therapeutic properties, i.e. antioxidant activity, α-amylase inhibitory activity and antimicrobial activity of mead and metheglins.
4. To carry out physicochemical analysis of mead and metheglins.
5. To assess organoleptic analysis of mead and metheglin and characterize through sensory analysis.

1.4 Significance of the study

Nepal is rich in floral diversity and has a variety of herbs having different therapeutic properties. The mead is also new to Nepalese community. The medicinal properties of herbs can directly be incorporated to mead since people are growing their attention to wine as well as therapeutic foods as of their health concern as they want both luxury and fitness at the same time, which can be provided by such herbal wines. Different local and indigenous herbs can be used and their potential can be extracted and distributed as luxury.

The study of the kinetics of honey must with different proportions of rhizome of *B. ciliata* (pakhanbedh) fermentation can help understand the interaction of yeast with chemical constituent of honey and pakhanbedh, which help to identify the chance of stuck fermentation and eliminate them. Also, with knowledge of fermentation kinetics, optimization of process can be attained.

Pakhanbedh is useful in piles, tumors, urinary discharges, heart diseases, diseases of the bladder and lungs. It is also used as tonic in fever, diarrhea, cough and dysentery (Ahmad *et al.*, 2018) and honey is already considered as a medicinal food in Nepal. So, inclusion of these herbs during fermentation will extract many antimicrobial and health promoting active components from the herbs, resulting in the production of medicinal mead of longer self-life compared to other customary meads and wines. Also, the antioxidant activity, antidiabetic activity and other therapeutic potential of metheglin given by pakhanbedh at different concentration will be assessed. Therefore, this research will help explore the possibility of incorporating *Bergenia ciliata* rhizome in producing metheglin, thus promoting commercial cultivation of these precarious Nepalese medicinal plants and honey production in Nepal.

1.5 Limitations of the study

1. The fermentation was done in ambient room condition because of the unavailability of temperature control instrument in the laboratory.
2. The fermentation for all the samples was carried out at same TSS (23ºBx), room temperature for fermentation of all samples and adjustment of pH for all samples were by addition of same acid (citric acid) i.e. there were no variations for these parameters. Hence, optimization on TSS, temperature and acid used was not done.
3. Prepared metheglin was not aged properly due to time and technical constraints.
4. Only one yeast type was used to carry fermentation.
5. Quantification of active components in the prepared product wasn't assessed.

Part II

Literature review

2.1 Historical background of alcoholic beverage

Alcoholic beverages are believed to have originated in Egypt and Mesopotamia around 6000 years ago. In every part of the world, different civilization had developed some types of alcoholic beverage. The production and consumption of alcoholic beverage is one of the man's oldest activities (Varnam and Sutherland, 2012).

Despite this early application of microbiology, the ability of microorganisms to stimulate the biochemical changes was showed several years later. Alcoholic fermentation was first identified by Gay Lussac in 1810, but at that time yeast was not recognized as a causative organism. Schwan in 1835, demonstrated that yeast could produce alcohol and carbon dioxide when introduced in sugar-containing solution. He termed yeast *Zuckerpilz*, meaning sugar-fungus, from which the name *Saccharomyces* originated. *Saccharomyces* group possesses almost all the credit of producing alcoholic beverages (Samuel and Prescott, 2016).

The yeast cells growing under anaerobic conditions caused the conversion of glucose to alcohol and researchers also showed that fermentation could be carried out using cell-free yeast juice, which led to the discovery of the role of enzymes in fermentation. Such work of pioneers finally revealed the truth that the alcoholic fermentation was in fact anaerobic, because of an enzyme complex known as zymase. Having realized the importance of yeasts in fermentation, people started culturing valuable yeasts and exploiting them for the production of various alcoholic beverages. Today, yeasts are utilized throughout the world for the production of alcoholic beverages in many forms and tastes. The starting materials normally comprise either of sugary materials or starchy materials, which need to be hydrolyzed to simple sugars before fermentation (Buglass *et al.*, 2011).

Over the year a vast range of alcoholic beverage has developed although, in most cases, it is possible to place these in one of three categories- beer, wine or distilled spirit–according to ingredient and method of manufacture (Varnam and Sutherland, 2012).

In Nepal, the history of alcoholic beverage dates back to ancient times. Ethnic groups developed these technologies while celebrating various festivals and settlement of marriage. The knowledge of home brewing has been passed on to generations, but they are quite ignorant about the broad dimensions of microbial biochemistry or their complex mechanisms. In fact, the exact nature of fermentation is still not fully known to them (Gubhaju, 2006).

2.2 History of meads and medicinal wines

Mead is considered the oldest fermented beverage. Pottery vessels discovered in northern China dating from 7000 BCE have shown chemical signatures consistent with the presence of honey, rice, and organic compounds associated with fermentation (McGovern *et al.*, 2004). As long as five thousand years ago, people in the Egyptian, Greek, and Roman empires made honey wine. More "recently," the Vikings also made honey wine, according to the tales. However, we know little about what this old-time mead tasted like or how it was made. Humans have been hunting for honey for over eight thousand years. One can imagine the ancients eating the bulk of the honey and putting the remaining honeycomb and debris into a pouch or vessel and later rinsing out the remaining sweetness with some water. Imagine their surprise if the water was left for a few days, resulting in a beverage with some interesting properties (Steve, 2014).

In 1948 Robert Gayre published Wassail! In Mazers of Mead: An Account of Mead, Metheglin Sack and Other Ancient Liquors, and of the Mazer Cups Out of Which They Were Drunk, with Some Comment upon the Drinking Customs of Our Forebears. His book provides a detailed account of references to mead in writings from ancient mythology to modern works. Gayre wrote that early ale was simply a light mead made from honey but that over time, malted grain was used in production (today, we call this beverage a braggot) and that eventually it became a beverage made entirely from malted grains as a cheap substitute for honey. Gayre also asserted that this early ale differed from modern beer, in that it wasn't bitter, since it predated the use of hops in beer. People used to drink mead from drinking horns, mazers, and mether cups. In Gayre's opinion, mead came first, followed by a lesser beverage made from grapes (wine), and eventually an even lesser beverage made from grains (beer) (Gayre, 1948).

Modern production of mead comprises adding honey to 3-4 volumes of water with whatever addition of fruit, hops, herbs, or spices is required. After boiling, the surface froth is skimmed off and brewer's yeast added. Fermentation is at 15-25°C for 3-6 weeks before aging in oak casks at 10–15°C for up to 10 years (Bamforth, 2014).

Medicinal wine has been used for millennia in Chinese medicine as agents for promoting people's health and corporeity, and enriching people's restorative culture. When the contemporary medicine was not in use, herbal formulations were tried on persons to heal and for body soothing. The earliest evidence of plant additives in fermented beverages was reported in China and Middle East. Chemical analysis data of earthenware gave the proof of herbal incorporation in ancient alcoholic beverages. Also, addition of tree resin in wine was reported to protect the consumer against wine disease. Evidence of tree fragrance additives, along with native species like rice, wheat and millets in the alcoholic formulation, was reported in china (McGovern *et al.*, 2004).

2.3 Some popular mead styles

Mead, also called as honey wine, has its own style and types as wines do. To enhance its character and complexity, a variety of fruits, herbs, or spices may be added to, during, or after fermentation. Traditional mead or show mead is made using either honey from a particular flower source or a multiflora honey. Regarding traditional mead, small amounts of spices, fruits, or herbs are permitted without ever overpowering the honey flavor or aroma (McConnell and Schramm, 1995). Some of the popular styles are:

2.3.1 Plain mead/Show mead

This mead is made with honey after diluting with water without addition of any other substrates (Faubion, 2015).

2.3.2 Metheglin

Mead that also contains spices for flavoring or medicinal herbs is called a metheglin. (Tayleur, 1973).

2.3.3 Cyser

This is mead made with apple juice (cider). Examples include Vander Mill Cyser Van Doom and Green River Ambrosia Bourbon Barrel-Aged Cyser (Faubion, 2015).

2.3.4 Melomel

A mead that contains fruit (such as raspberry, blackberry or strawberry) is called a melomel, which was also used as a means of food preservation, keeping summer produce for the winter (Tayleur, 1973).

2.3.5 Pyment

Mead that is fermented with grape juice is called a pyment. A pyment made with white grape juice is sometimes also called white mead (Tayleur, 1973). Spiced pyment can be classed as a hippocras (McConnell and Schramm, 1995).

2.3.6 Rhodomel

Rhodomel is made from honey, rose hips, rose petals or rose attar, and water. This type of mead could also be considered a metheglin, depending on the intention of the brewer (Adelmann).

2.3.7 Sparkling mead

It is a type of mead that has been carbonated. It is usually created through the use of champagne yeast. It can also be made with artificial carbonation. The meads which are not carbonated are still meads (Faubion, 2015).

2.3.8 Braggot

Mead made with malted grain (usually barley). BeeWolf Braggot, and Kuhnhenn Braggot are examples (Faubion, 2015).

2.3.9 Bochet

Mead made with caramelized honey, which creates such flavors as toffee, chocolate, and marshmallow. It yields a dark, clear mead with a complex flavor (Faubion, 2015).

2.4 Herbal wine

Before the revolution in medicinal area and rise of modern medicines, people were treated using the herbal formulations that were derived from plants (Timothy, 1990). Generally, these herbs are in powder form or dry form. Herbs have many positive effects on health and overall body of the human. These herbs have anti-microbial, anti-cancerous, anti-oxidant

and other medicinal and beneficial properties and benefit both physically and mentally. Also, the herbal infusion in alcoholic drinks and beverage reported having reduced the hypertension and increase overall body performance (Usman and Jawaid, 2012).

Not only did herbs increase the medicinal value of the wine, but increase the organoleptic properties as aroma, color and flavor. Herbs contain tannins, polyphenols and other bioactive compounds. Tannins found in the herbs are astringent, which have aroma enhancing and antioxidant properties. They contain hydroxyl groups and carboxyl groups to form strong complexes with proteins enhancing organoleptic properties of wine (Ashok and Upadhyaya, 2012).

Many studies have shown that red wine can delay ageing and is protective against many diseases (Rodrigo *et al.*, 2011). Herbal wine's constituents are aromatic and helpful in maintaining the health of human beings. Herbal wine has many health benefits like reduction in ovarian cancer, strengthening the bones and overall skeleton, cancer cells deterioration, prevention of heart strokes by keeping the coronary arteries clean, elevating the lung functionality. In a nutshell, these herbs can deliver good anti-microbial, anti-bacterial properties, anti-mutagenic properties (Altenburg and Zouboulis, 2008).

2.5 Honey

According to the Codex Alimentarius in Codex Standard for Honey, honey is "the natural sweet substance produced by honeybees from the nectar of plants (blossom honey or nectar honey) or from secretions of living parts of plants or excretions of plant sucking insects on the living parts of plants (honeydew honey), which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store and leave in the honeycomb to ripen and mature". For a long time in human history, it was an important source of carbohydrates and the only available natural sweetener (Bogdanov *et al.,* 2009). Besides its nutritional properties, because of its therapeutic potential in treating respiratory and gastrointestinal illnesses, in healing wounds and burns, and as an antimicrobial agent, among other biological proprieties, honey is one of the products, referred to in old traditional medicine (Al-Mamary *et al.*, 2002). According to its botanical origin, honey can be classified as mono floral or multiflora, if the bees forage predominantly on one type of plant or several botanical species, respectively (Alvarez-Suarez *et al.*, 2014).

Honey composition is rather variable and dependent on the floral source, climate, environmental and seasonal conditions, as well as the handling and processing practices (Anklam, 1998). Surveys of floral honey composition have established that the three major components are fructose, glucose, and water, averaging 38.2, 31.3 and 17.2%, respectively. Glucose and fructose are the only monosaccharides in honey and it is these sugars, combined in various forms, that comprise the di- and trisaccharide fractions of floral honey (Doner, 1977).

If exposed to moist air, hydrophilic properties of honey pull moisture into the honey, eventually diluting it to the point that fermentation can begin. The fermentation process essentially depends on the initial count of microorganisms in the product, the storage time and temperature, and the moisture content of the honey (Crane, 1975). Gluconic acid, accumulating to a concentration of between 8.6 and 60 mM, is the most abundant acid in honey and the major determinant of its acidity (pH 3.4 - 4.5) (Manyi-Loh *et al.*, 2011a). Masoura *et al.* (2020) published that the antibacterial effect of honey is due to and possibly synergies between, the three main stressors present in honey: sugars, gluconic acid, and hydrogen peroxide (H_2O_2), which result from the enzymatic conversion of glucose on honey dilution and showed that the synergy of H_2O_2 and gluconic acid is essential for the antibacterial activity of honey. This synergy caused membrane depolarization, destruction of the cell wall, and eventually growth inhibition.

The physical properties of honey vary depending on water content, the type of flora used to produce it (pasturage), temperature, and the proportion of the specific sugars it contains. Fresh honey is a supersaturated liquid, containing more sugar than the water can typically dissolve at ambient temperatures. At room temperature, honey is a super cooled liquid, in which the glucose will precipitate into solid granules. This forms a semisolid solution of precipitated glucose crystals in a solution of fructose and other ingredients. Temperature also affects the rate of crystallization, with the fastest growth occurring between 13 and 17°C. The tendency of honey to crystallize depends on the ratios involving the composition of honey regarding glucose. A honey with a glucose/water ratio < 1.7 remains liquid for a long time, while one with a ratio > 2.1 usually crystallizes quickly (Doner, 1977). The honey highly supersaturated with glucose like brassica honey crystallize almost immediately after harvesting, while with low concentration of glucose like in chestnut or tupelo honey does not crystallize (Reuber, 2015).

2.5.1 Characterization of honey

Chemical composition of honey (Blossom honey) is given in Table 2.1 (Bogdanov, 2011), values in g/100 g.

Table 2.1 Chemical composition of honey

Parameter	Average	Min-max
Water content	17.2	15-20
Fructose	38.2	30-45
Glucose	31.3	24-40
Sucrose	0.7	0.1-4.0
Other disaccharides	5.0	28
Melezitose	<0.1	-
Erlose	0.8	0.56
Other oligosaccharides	3.6	0.5-1
Total sugar	79.7	-
Minerals	0.2	0.1-0.5
Amino acids and proteins	0.3	0.2-0.4
Acids	0.5	0.2-0.8
pH	3.9	3.5-4.5

Source: Bogdanov (2011)

2.5.1.1 Carbohydrates

Carbohydrates account for about 95% of the dry matter in honey. Fructose (38.2%, mean value) and glucose (mean value of 31.3%) are the major carbohydrates in honey, followed by sucrose (mean value of 0.7%) (Bogdanov *et al.*, 2009). 25 other oligosaccharides have

been detected, including maltose, isomaltose, trehalose, turanose; trisaccharides erlose, raffinose, and melezitose; and trace amounts of tetra- and pentasaccharides, among others (Anklam, 1998). According to the Codex Alimentarius, the minimum concentration of the reducing sugars, glucose and fructose, is 60% (w/w). The ratio of fructose to glucose highly depends on the nectar source (Anklam, 1998) and is usually 1.2:1 (Ojeda de Rodriguez *et al.*, 2004).

2.5.1.2 Water

Water is the second most important component of honey, ranging between 15% and 20%, with an average value of 17.2% (Bogdanov *et al.*, 2009). The water content of honey depends on several factors: climate conditions, maturity of the hive, and treatments applied during nectar and honey collection and storage (Finola *et al.*, 2007). This parameter will influence its physical properties, such as the viscosity. Honey with a high water content usually presents preservation and storage problems because it increases the probability of product fermentation (Olaitan *et al.*, 2007). In fact, low water content contributes to the stability of honey, preventing fermentation (Küçük *et al.*, 2007).

2.5.1.3 Minerals

Minerals come from the soil and plants and are present in small amounts ranging from 0.04% in the clear honeys, to 0.2% in some dark honeys (Anklam, 1998). In addition, other elements may be added during the processes of centrifugation and storage (Freitas *et al.*, 2006). Potassium is the major mineral, with an average of about one-third of the total (Olaitan *et al.*, 2007), followed by calcium, sodium, phosphorus, magnesium, iron, manganese, and copper (Bogdanov *et al.*, 2009). Trace elements like aluminum, iodine, chloride, fluorine, bromine, and barium, among others, are also present in honey (Bogdanov *et al.*, 2009). The mineral composition depends on the environment, geographic location, and botanical species (Anklam, 1998). In fact, honeys from light blossoms commonly have lower mineral content than dark honeys such as honeydew, chestnut, and heather (Bogdanov *et al.*, 2007).

2.5.1.4 Organic acids

Organic acids comprise gluconic acid, resulting from the oxidation of glucose by glucose oxidase, followed in minor concentrations by pyruvic, malic, citric, succinic, and fumaric acids (Olaitan *et al.*, 2007). These acids account for 0.5% of the dry matter, for the acidity,

and for the characteristic taste of honey (Anklam, 1998). Honey acidity also depends on the botanical species (Küçük *et al.*, 2007) and time of harvest (Ojeda de Rodriguez *et al.*, 2004). The presence of osmophilic yeasts adapted to high osmotic pressures, such as high sugar concentrations, may be responsible for the increase in acidity (Ojeda de Rodriguez *et al.*, 2004). So, low acidity, below the maximum limit of 50 mMol/kg, shows the absence of undesirable fermentation (Finola *et al.*, 2007). Most honeys are acidic, with pH ranging from 3.4 to 6.1, and an average value of 3.9 (Iurlina and Fritz, 2006). However, this parameter is not directly related to the free acidity owing to the buffering capacity of honey (Ojeda de Rodriguez *et al.*, 2004), which depends on phosphates, carbonates, and other minerals of honey.

2.5.1.5 Nitrogen compounds

Amino acids, peptides, proteins, and nucleic acid derivatives are the major nitrogenous substances in honey (Alvarez-Suarez *et al.*, 2010). The amino acid composition of honey is highly variable depending on its origin, thus the amino acid profile is a good indicator of the botanical and geographical origin of honey. Proline is the major amino acid in honey, corresponding to values between 50% and 85% of total free amino acids (Anklam, 1998). Proline content should be above 200 mg/kg; values below 180 mg/kg show potential adulteration of the honey by sugar addition. Besides proline, 26 other amino acids have been identified in honey: glutamic acid, aspartic acid, glutamine, histidine, glycine, arginine, tryptophan, and cysteine, among others (Anklam, 1998). The protein content is relatively low, approximately 2 to 4 g/kg. Proteins in honey are mainly enzymes: invertase, diastase, glucose oxidase, catalase, α-glucosidase, β-glucosidase (Won *et al.*, 2008).

Some enzymes come from the bees during the process of honey ripening. The enzymes diastase and invertase are important for assessing honey quality, because they are used as indicators of honey freshness. Diastase catalyzes the hydrolysis of starch into disaccharides and monosaccharides and it is relatively stable to heat and storage, and invertase catalyzes the hydrolysis of sucrose to glucose and fructose. Hydrogen peroxide, H_2O_2, the antibacterial factor found in honey, is regulated by the enzymes glucose oxidase and catalase. Thus, the enzymatic activity may indicate exposure to heat during processing and storage of the honey (Bogdanov *et al.*, 2009).

2.5.1.6 Vitamins

The vitamin content in honey is low and varies with the floral origin. Most are water-soluble vitamins owing to the aqueous nature of honey and a low percentage of lipids (León-Ruiz *et al.*, 2013). Vitamin C (ascorbic acid), B1 (thiamine), B2 (riboflavin), B6 (pyridoxine), B3 (niacin), B5 (pantothenic acid), and K (phyllochinon) have been reported in honey (Alvarez-Suarez *et al.*, 2010; Bogdanov *et al.*, 2009; Olaitan *et al.*, 2007). Ascorbic acid is the main vitamin found in honey, with concentrations ranging from 22 to 25 mg/kg and it is found in almost all honeys (Bogdanov *et al.*, 2009).

2.5.1.7 Phenolic compounds

Honey contains a diversity of phenolic compounds as secondary constituents, such as flavonoids, phenolic acids, and phenolic acid derivatives. The main polyphenols are the flavonoids, in concentrations that can vary between 0.6 and 4.6 g/kg, and are mainly found in honey produced under dry and high-temperature conditions (Bogdanov *et al.*, 2009). The phenolic acids are found in concentrations ranging from 0.01 to 10 mg/kg (Anklam, 1998).

The phenolic content of honey is highly related to its bioactive properties, namely antioxidant and antimicrobial activities. The antioxidant activity of honey has been reported by many authors (Al-Mamary *et al.*, 2002; Al *et al.*, 2009; Gorjanović *et al.*, 2013; Küçük *et al.*, 2007). Others have provided evidence of antibacterial activity of honey against pathogenic bacteria resistant to antibiotics (Amit *et al.*, 2005; Moussa *et al.*, 2012; Sherlock *et al.*, 2010; Taormina *et al.*, 2001) and against food spoilage bacteria (Mundo *et al.*, 2004).

2.5.1.8 Volatile compounds

Volatile compounds of honey are derived from the botanical species or nectar source, from the transformation process carried out by bees, from heating or handling during processing and storage, or from microbial and environmental contamination (Manyi-Loh *et al.*, 2011b). Aroma compounds are present at very low concentrations, mainly as complex mixtures of volatile components with different functionality and relatively low molecular weight (Cuevas-Glory *et al.*, 2007). Indeed, over 300 volatile compounds have been identified in different honeys, including hydrocarbons, aldehydes, alcohols, ketones, acids, esters, benzene derivatives, furans and pyrans, norisoprenoids, terpenes, and sulfur compounds (Cacho *et al.*, 2015). Usually, monofloral honeys possess highly individual aroma profiles

compared to multifloral ones (Kaškonienė and Venskutonis, 2010). The volatile profile represents a chemical fingerprint of monofloral honey because the nature and amount of volatile compounds are related to the floral source. So, the determination of volatile compounds has been used to differentiate honeys according to botanical origin and geographical origin (Jerković *et al.,* 2009).

2.5.1.9 Color

The determination of honey color is a useful classification criterion for monofloral honeys, because it is related to the contents of phenolics, flavonoids, and minerals (Bertoncelj *et al.*, 2007). The mineral content influences the color and the taste; honeys with a higher quantity of minerals have darker color and stronger taste (González-Miret *et al.*, 2005). The color of honey also depends on the processing, temperature, and/or time of storage (Olaitan *et al.*, 2007) and can range from white-water, extra white, white, extra clear amber, light amber, amber, to dark amber (Bertoncelj *et al.*, 2007). However, it is important to find out that the color's intensity increases during storage owing to Maillard reactions, caramelization of fructose, and reactions with polyphenolic compounds (Shafiee *et al.,* 2013).

2.5.2 The natural microbiota of honey

The microbial population of honey includes microorganisms that come from the environment, soil, plants, pollen, and those that usually colonize the digestive tract of bees (primary sources of contamination) (Olaitan *et al.*, 2007). Thus, the microbial population of honey includes fungi (yeasts and molds) and spore-forming bacteria (Kb ániová *et al.*, 2009). The intestine of bees contain high numbers of gram-positive bacteria (*Bacillus, Bacteridium, Streptococcus,* and *Clostridium* spp.) and gram-negative bacteria (*Achromobacter, Citrobacter, Enterobacter, Erwinia, Escherichia coli, Flavobacterium, Klebsiella, Proteus,* and *Pseudomonas*) and lower numbers of yeasts (Al-Waili *et al.*, 2012).

The survival of microorganisms is influenced by honey's chemical composition, particularly by the low water content. Indeed, this parameter hampers microbial growth, especially of bacteria, which are generally less tolerant to high osmotic pressure, compared to fungi (Olaitan *et al.*, 2007). Also, the low pH and high sugar content play key roles in the survival and growth of microorganisms (Iurlina and Fritz, 2006). Even though bacteria can survive in this natural product, they are unlikely to replicate (Snowdon and Cliver, 1996).

As a result, the detection of high numbers of vegetative bacteria might indicate recent contamination by a secondary source (Iurlina and Fritz, 2006).

The consumption of honey contaminated with *Clostridium botulinum* spores is especially dangerous for infants and children, with many reported cases of infant botulism. Although honey itself does not contain the toxin, the spores can theoretically build the toxin after digestion in infants until 1-year-old (Bogdanov *et al.*, 2009).

Molds, or filamentous fungi, normally associated with honey include the genera *Penicillium, Aspergillus, Cladosporium*, *Penicillium,* and *Mucor* (Kb ániová *et al.*, 2009). These microorganisms can survive but do not grow in honey (Snowdon and Cliver, 1996).

Honey naturally contains various osmotolerant/osmophilic yeasts that grow at low pH values and are not inhibited by high osmotic pressure. Most yeasts isolated from this environment include species of the genera Saccharomyces, *Debaryomyces, Hansenula, Lipomyces, Pichia, Schizosaccharomyces, Torula,* and *Zygosaccharomyces* (Snowdon and Cliver, 1996). Honey with moisture content less than 17.1% is safe from fermentation risk regardless of yeast count; however, a value above 20% means that the honey is always in danger of fermentation occurring (Bogdanov *et al.*, 2009).

2.5.3 Health benefits of honey

Some of the therapeutic properties of honey are:

2.5.3.1 Anti-inflammation action

Flavonoids found in honey have showed anti-inflammation properties and ability to inhibit pro-inflammatory enzymes such as of cyclooxygenase-1 and cyclooxygenase-2 and pro-inflammatory mediators, including nitric oxide, cytokines and chemokines (Silva *et al.*, 2021).

2.5.3.2 Antioxidant activity

Honey has exhibited a strong antioxidant potential and its activity is strongly correlated with the content of total phenolic and the color of honey. It was found that dark honey has a higher total phenolic content, and a higher antioxidant capacity (Al-Mamary *et al.*, 2002; Al *et al.*, 2009; Gorjanović *et al.*, 2013; Küçük *et al.*, 2007).

2.5.3.3 Antidiabetic properties

Using honey in Type I and Type II diabetes was associated with significantly lower glycemic index than with glucose or sucrose in normal diabetes. Because of the low glycemic index of the honey, it helps to reduce the absorption of digested food. Honey, compared with dextrose, caused a significantly lower rise in plasma glucose levels in diabetic subjects. Al-Hariri (2018) showed that low glycemic index honey can act as a hypoglycemic agent and delay or prevent the progression of the diabetic outcome.

2.5.3.4 Antimicrobial activity

Honey has been reported to have antibacterial activity against various bacterial species including *Bacillus anthracis, Corynebacterium diphtheriae, Haemophilus influenzae, Klebsiella pneumoniae, Listeria monocytogenes, Mycobacterium tuberculosis, Pasteurella multocida, Yersinia enterocolitica, Proteus species, Pseudomonas aeruginosa, Acinetobacter* spp., *Salmonella diarrhea, Salmonella typhi, Serratia marcescens, Shigella dysentery, Staphylococcus aureus, Streptococcus faecalis, Streptococcus mutans, Strep. pneumoniae, Streptococcus pyogenes,* and *Vibrio cholerae.* An antifungal action has been reported for honey against *Aspergillus, Penicillium,* as well as all the common dermatophytes and *Candida albicans*. Honey has shown antiviral effect as well. Many authors have provided evidence of antibacterial activity of honey against pathogenic bacteria resistant to antibiotics (Amit *et al.*, 2005; Moussa *et al.*, 2012; Sherlock *et al.*, 2010; Taormina *et al.*, 2001) and against food spoilage bacteria (Mundo *et al.*, 2004).

2.6 Pakhanbedh (*Bergenia ciliata*)

Bergenia ciliata in sanskrit: Pashanbheda, in english: Rock-foil (Khan and Kumar, 2016), is a plant species in the genus *Bergenia*. It is a small perennial rhizomatous creeping herb of family saxifragaceae found throughout the temperate Himalayans at an altitude of 900-3000 m (Khan *et al.*, 2017). The Lal meaning of word Pakhanbedh is, one that breaks stones. Rhizome of the plant have been used in Ayurvedic medicine for centuries. There are many plants that are known by this name because of their diuretic and lithotriptic (dissolving or destroying stone in the bladder or kidneys) activities (Prabhakar, 2014).

B. ciliata belong (haw.) Sternb belongs to the family Saxifragaceae, which comprises 30 genera and 580 species. *B. ciliata,* commonly known as hairy *Bergenia,* is a perennial herb

found between the height of 800–3000 m throughout the temperate Himalayas from Afghanistan to Southeast Tibet (Chauhan *et al.*, 2012b). For century's rhizome of *B. ciliata* has been used for curing pulmonary infections, leucorrhea, piles and for dissolving bladder and kidney stones (Ahmad *et al.*, 2018).

Different Ayurvedic treatise mentioned this plant and recommended its use for treatment of urinary stones. Charak Samhita (210 BC-170 AD) mentioned this plant under the name Pashanbhed and recommended it for painful micturition, for curing abdominal tumour and for breaking up calculi. Sushruta Samhita (170 AD- 340 BC) and Ashtang Hridaya (341 AD–434 AD) also mention it for uric acid calculi (Prabhakar, 2014).

2.6.1 Plant description

The *Bergenia* is a genus of 10 species of flowering plants in the family of Saxifragaceae native to Central Asia, from Afghanistan to China and Himalayas. The salient botanical features of the family Saxifragaceae are: leaves, simple or compound, alternate, rarely opposite, usually exstipulate, inflorescence cymose or racemose, rarely flowers solitary flowers; bisexual or occasionally unisexual. Stamens are inserted with the petals, equaling or doubling their number rarely indefinite. Ovary comprised 3-5 united carpels with axial placenta, occasionally celled with partial placentas, ovules many, erect or pendulous. Styles are as many as carpels, free or more or less connate (Prabhakar, 2014).

2.6.2 Scientific classification of pakhanbedh

- **Classification:** *Bergenia* Moench
- **Kingdom:** Plantae-plants
- **Subkingdom:** Tracheobionta-vascular plants
- **Super divison:** Spermatophyta-seed plants
- **Division:** Magnoliphyta-flowering plants
- **Class:** Magnoliopsida-dicotyledons
- **Subclass:** Rosidae
- **Order:** Rosales
- **Family:** Saxifragaceae
- **Genus:** *Bergenia*
- **Species:** *ciliata f. Ciliata*

Source: Khan and Kumar (2016)

2.6.3 Chemical composition (phytochemistry)

The aqueous extract of rhizomes showed the presence of bergenin, phenolic compounds leucocyanidin, gallic acid, methyl gallate, catechin and polymeric tannin. Phytochemical screening of *B. ciliata* showed the presence of terpenoids, tannins, flavonoids, saponins, steroids (Uddin *et al.*, 2012).

Bergenin, also known as cuscutin which is the most abundant and important compound found in family Saxifragaceae. Chemical formula of bergenin is $C_{14}H_{16}O_9 \cdot H_2O$. 346.3 g per mole is the molecular weight of bergenin (Chauhan *et al.*, 2012a). Gurav and Gurav (2014) reported that rhizome of *B. ciliata* contains 0.75% bergenin.

2.6.4 Medicinal uses of pakhanbedh

Pakhanbedh is used in Ayurveda and Yunani's system of medicine for treatment of many diseases, especially for urinary stones. The plant root has cooling, laxative, analgesic, abortifacient (abortion causing) and aphrodisiac properties.

The rhizomes are used in treatment of vesicular calculi, urinary discharges, uterine hemorrhage, diseases of the bladder, dysentery, menorrhagia, splenic enlargement and heart diseases. Ayurveda mentions the roots as bitter, acrid, post digestion pungent and cool in potency. It is tridoshnashak, i.e. balances Vata (energy of movement), Pitta (energy of digestion) and Kapha (energy of lubrication and structure) (Prabhakar, 2014). Some of the medicinal uses of this rhizome are;

a) **Teething troubles:** The roots are rubbed down and given with honey to children when teething.
b) **Ear pain:** The leave juice is extracted in mortar and pestle. This is used as ear drops to cure earache.
c) **Intestinal parasites roundworms:** About 10 g of root paste or juice is taken orally by human adults with the molasses, twice a day for 3-4 days.
d) **Cuts, boils, wounds and burns:** Dried roots paste is applied externally on affected body parts.
e) **Urinary disorders, stomach disorders and urogenital complaints:** Decoction of fresh roots is taken orally for treating these conditions.
f) **Constipation:** Root paste is taken with lukewarm water.

g) **Dysentery:** Approximately 5-10 g root powder is taken with fresh water, two times a day.
h) **Fever:** The root powder tea is given to treat fever (Prabhakar, 2014).

2.6.5 Pharmacological profile of *Bergenia ciliata*

2.6.5.1 Toxicology

The toxicological investigations of *B. ciliata*, with particular reference to acute systematic toxicity and intracutaneous toxicity in experimental animals, displayed that it elicits severe toxicity. The symptoms of toxicity in intracutaneous test showed erythema and edema, whereas assessment of acute systemic toxicity frequently observed breathing problem and initiations of diarrhea with blood in stool of experimental model and caused gastero-intestinal syndrome. *B. ciliata* can produce toxicity, suggesting a role in certain diseases. In higher doses, it is cardio-toxic, shows anti-diuretic action, and has a depressant action on the central nervous system (Islam *et al.*, 2002).

2.6.5.2 Anti-pyretic activity

Sinha *et al.* (2002) found that the methanol extract of *B. ciliata* rhizome exhibited significant antipyretic effects on normal body temperature and yeast-induced pyrexia in rats. *B. ciliata* extract, at 300 mg/kg, significantly reduced the normal body temperature in rats for up to 5 h after its administration. In yeast-induced pyrexia, the extract significantly lowered body temperature for up to 4 h after its administration in a dose-dependent manner and the effect was comparable with that of paracetamol, a standard antipyretic agent.

2.6.5.3 Anti-diabetic activity

The hydroalcoholic extract of the *B. ciliata* exhibited significant anti-diabetic activity in an in vitro model. Extraction and fractionation of the extract lead to the isolation of two active compounds, (-)-3-O-galloylepicatechin and (-)-3-O-galloylcatechin. These isolated compounds showed significant dose dependent enzyme inhibitory activities against rat intestinal α-glucosidase and porcine pancreatic α-amylase. IC50 value for sucrose, maltase and α-amylase were 560, 334 and 739 μM, respectively for [(-)-3-O-galloylepicatechin] and 297, 150 and 401 μM, respectively for [(-)-3-O-galloylcatechin] (Bhandari *et al.*, 2008).

2.6.5.4 Anti-inflammatory activity

Paashanolactone is an important constituent isolated from *Bergenia ligulata* rhizomes that showed significant anti-inflammatory activity and the same activity of an aqueous extract of *B. ciliata* rhizomes was confirmed in a dose dependent manner on carrageenin induced paw oedema in rats (Kumar *et al.,* 2002). The methanol extract of the rhizome of *B. ciliata* exhibited significant anti-inflammatory activity in acute rat models (carrageenan- and serotonin (5-HT)-induced rat paw oedema) and a chronic rat model (cotton pouch-induced granuloma). At 300 mg/kg, the methanol extract exhibited maximum inhibition of 32.4 ± 2.89% in carrageenan-induced rat paw oedema. In the serotonin-induced rat paw oedema model, 300 mg/kg methanol extracts suppressed oedema by 45.33 ± 2.09%. In the cotton pouch granuloma model, the methanol extract inhibited significantly the granuloma weight in a dose-dependent manner (Sinha *et al.*, 2001b).

2.6.5.5 Antimicrobial activity

The methanolic extract of *B. ciliata* rhizome showed a wide spectrum of concentration dependent antibacterial activity of methanolic extract of *B. ciliata* rhizomes at a concentration of 200-1000 µg/disc (Sinha *et al.*, 2001a). The broad spectrum and concentration dependent antibacterial activity was also confirmed in aqueous extract of crude drug. Singh *et al.* (2017) revealed that the extract of *B. ciliata* tested was effective against the bacteria and actinomycetes studied, but showed no activity against fungi. The antimicrobial activity of *B. Ciliata* was also reported by (Khan *et al.*, 2018).

2.6.5.6 Antitussives activity

The methanolic extract of *B. Ciliata* rhizome showed significant and dose dependent antitussive activity in mice using sulphur dioxide gas model. The extract exhibited significant antitussive activity in a dose-dependent manner, as compared to control. The antitussive activity of the extract was comparable to that of codeine phosphate (10 mg/kg body wt.), a standard antitussive agent. The extract at doses of 100, 200 and 300 mg/kg body wt. showed significant inhibition of cough reflex by 28.7, 33.9 and 44.2%, respectively, within 90 min of the experiment (Sinha *et al.,* 2001b).

2.6.5.7 Anti-ulcer activity

B. Ciliata was evaluated for its gastroprotective effects on ethanol/HCl, indomethacin and pylorus ligation induced gastric ulcers in rats. Doses of 15, 30 and 60 mg/(kg body weight) of the aqueous and methanol extracts of the rhizome exhibited anti-ulcer activity. The aqueous extract decreased the ulcer lesion ($p < 0.05$) in all models to a greater extent than the methanol extract, but at the higher doses the effect was reduced. The antiulcer activity appears to be mediated via cytoprotective effects conferred by enhancement of the mucosal barrier, rather than by prevention of gastric acid secretion or the lowering of pH and acidity (Kakub and Gulfraz, 2007).

2.6.5.8 Antioxidant activity

Many authors have listed *B. ciliata* as a strong natural antioxidant (Bagul *et al.*, 2003; Hendrychová *et al.*, 2014; Singh *et al.*, 2017; Venkatadri *et al.*, 2010; Zafar *et al.*, 2019) . The methanolic extract of *B. ciliata* rhizomes was also reported to have free radical scavenging property in superoxide radical and nitric oxide scavenging models. The methanolic extract was found to be a good scavenger of DPPH radical, with an EC of 36.24 μg/ml. The extract scavenged superoxide radical in a dose dependent manner with EC of 106.48 μg/ml (Bagul *et al.*, 2003). In another study, antioxidant activity of methanolic and aqueous extracts of *B. ciliata* revealed that both extracts to be active radical scavengers. Reducing power and lipid peroxidation inhibition efficiency (TBARS assay) of both extracts showed promising activity in preventing lipid peroxidation and might prevent oxidative damages to biomolecules. The ability of the extracts to protect DNA (pBR322) against UV-induced photolysed oxidative damage was analysed. Both the extracts were able to protect DNA from oxidative damage (Venkatadri *et al.*, 2010).

2.6.5.9 Antimalarial activity

The leaf extract of the plant showed good in vitro anti-plasmodial activity, with an IC50 < 0.0005) enhanced the mean survival time of mice compared to infected control, which exhibited a mean survival time of 8.6 ± 1.5 days (Walter *et al.*, 2013)

2.6.5.10 Anti-urolithic activity

The hydro-alcoholic extract of *B. ciliata*/standard drug cystone were administrated simultaneously at a dose of 150 and 300 mg/kg body weight/day along with ethylene glycol

(0.75% v/v) for 28 days. Significant changes were observed in body weight and absolute organ weight of ethylene glycol treated rats. Histopathological results showed disrupted renal parenchyma, degenerative changes in glomeruli and focal calcification in glomerulo-tubular structures in ethylene glycol treated animals. Administration of *Bergenia ciliata* extract/cystone along with ethylene glycol showed a significant protective effect in body weight and organ weight with few stray areas of calcifications in glomeruli. *Bergenia ciliata* extract shows higher renoprotective index than cystone at the same dose level (Saha and Verma, 2011).

2.6.5.11 Anticholinesterase activity

Zafar *et al.* (2019) found the crude extract of *B. ciliata* showed anticholinesterase (acetylcholinesterase = 90.22 ± 1.15% and butyrylcholinesterase = 88.22 ± 0.71%) potential.

2.7 Yeast

Meads can be prepared using either natural yeast flora of the honey (spontaneous fermentation) or pure cultures (culture yeasts). During ancient time, when people probably didn't know about the microorganism or fermentation process, they rely on the spontaneous fermentation of honey must. In spontaneous fermentation, there are various strains of yeasts present. Each yeast type will contribute a unique flavor to the mead. But spontaneous fermentation may sometimes lead to failure and also most strain of yeast do not produce a large amount of alcohol and aren't osmotolerant (i.e. ability to grow in an environment with a high osmotic pressure) to survive in honey must as well few strains produce undesirable organic compounds such as organic acids, H_2S, higher alcohols, etc., that may affect the flavor (Rai, 2012).

Strains of *S. cerevisiae* used for fermenting honey must include C11-3 (Navratil *et al.*, 2001), BRL-7 (Qureshi and Tamhane, 1987), and UCD522 (Mendes-Ferreira *et al.*, 2010) from culture collections, as well as commercial strains, such as Fermol® premier cru (Pereira *et al.*, 2015) and ENSIS-LE5 (Roldán *et al.*, 2011). For honey wine production, wine yeast strains are usually used because the sugar, pH, and nitrogen characteristics in mead are similar to the ones of white grape must (Schramm, 2003) so white wine yeast is also a choice for many mead makers. Some commonly used for mead making yeast Lalvin D-47, Lalvin EC-1118, Lalvin K1-V1116, Lalvin 71B-1122 etc. which are different strain of *Saccaromyces cerevisiae* var, e*llipsoideus* (synonyms: *Sacch. cerevisiae, Sacch.*

ellipsoideus, Sacch, vini.) Nowadays the must is partially 'sterilized' by the use of sulphurdioxide, bisulphate or metabisulphite that eliminates most microorganisms in the must leaving wine yeasts. Yeasts are then inoculated into the must (Okafor and Okeke, 2007).

Honey and wine musts have different compositions regarding sugar content (nearly 3 times higher in the former) and nitrogen concentrations (about 100 times higher in the last). Thus, wine yeast strains are not necessarily optimally suitable for mead production. In order to circumvent this problem, yeasts isolated from honey had been studied in relation to their fermentative abilities (Pereira *et al.*, 2015). Pereira *et al.* (2015) verified that significant differences did not exist between the strains. *S. cerevisiae* strains isolated from honey were similar to commercial and reference strains, all appearing to be suitable for mead production. Other studies have investigated microorganisms inducing alcoholic fermentation of beverages in tropical and subtropical areas. For example, *S. cerevisiae* ET99, isolated from ogol, an indigenous Ethiopian honey wine (Teramoto *et al.*, 2005), has yielded promising results. However, more studies are required to isolate strains ideal for mead production.

Sufficient number of yeast must be used for fermentation, the use of a reduced inoculum of *S. cerevisiae* can be associated with sluggish and stuck fermentations (Carrau *et al.*, 2010). So, to provide evidence for this claim, Pereira *et al.* (2013) studied the effect of the inoculum size on yeast fermentation performance, as well as on mead composition and the volatile compounds production. Increasing the pitching rate resulted in significant fermentation time saving, even though high inocula could lead to lower production of desirable aromatic compounds. In addition, they found out the final aroma composition depended on the yeast strain and inoculum size. Fourteen of the twenty-seven volatile compounds quantified could contribute to mead aroma and flavor because their concentrations rose above their respective thresholds. The formation of these compounds was particularly pronounced at low pitching rates, i.e. 10^5 cfu/ml.

Yeast in the must fermentation can also be used at immobilized state (Pereira *et al.*, 2014). Some studies on continuous mead production have been performed, involving *S. cerevisiae* immobilized in calcium alginate gels (Qureshi and Tamhane, 1987) or calcium pectate (Navratil *et al.*, 2001). In an experiment performed by Navratil *et al.* (2001), using cells immobilized in calcium pectate in a two-column system, fermentation slowed after only 60

h (30°C). Ethanol production fell to about 50% of its maximal rate within 120 h. Much work still needs to be done before it could become commercially viable.

Good mead making yeast is one which will impart a vinous or fruit like flavor, will ferment sugar to 14-16% alcohol, and is characterized by remaining in suspension during fermentation and then agglomerating to yield a coarse granular sediment that settles quickly and is not easily disturbed in racking (Pederson, 1980). Good mead yeast should have the following properties:

a) High alcohol tolerance, i.e. the yeast should continue to ferment despite the increasing concentration of the alcohol, giving stronger, drier wines with up to 16% alcohol (v/v), or even up to 18% (v/v) where the yeast is fed by periodic additions of sugar in small amounts.
b) Good agglutination, i.e. the tendency of the yeast to flocculate into small lumps that give a cohesive sediment as fermentation ceases, so that racking is simple and the wine clears easily.
c) Steady and persistent fermentation capacity, this leads to wines of better quality than when the fermentation falls away after a tempestuous start.
d) Absence of unpleasant flavors generated by dead and dying cells and low production of undesirable aroma and flavors.
e) Growth at the relatively high acidity i.e., low pH of must for fermentation.
f) Osmotolerance, i.e. yeast, should be able to tolerant high osmotic pressure created by high concentration of sugar on must.
g) SO_2 tolerance, i.e. for partial sterilization of must SO_2 as sulfite is used, then the yeast used should not be affected by applied sulfite.

2.8 General cultural condition for alcoholic fermentation of honey must

Cultural condition refers to the environment of yeast, i.e. fermentative media on which the propagation of yeast is conducted and final quality of beverage is largely depended (Samuel and Prescott, 2016). During mead fermentation, several problems are encountered. The likelihood of stuck fermentation is increased as most mead is made empirically, without adjustments. This can lead to subsequent yeast re-fermentation and secondary fermentations by lactic and acetic acid bacteria. These can undesirably increase acidity and the production of volatile esters. The presence of these compounds alters the organoleptic quality of mead,

in particular its aroma and flavor, making its consumption unpleasant (Ramalhosa *et al.*, 2011). Following are the few parameters that determine cultural condition of the fermentative media.

2.8.1 pH

The pH of must is crucial not only to its flavor but also to nearly every aspect of the mead. The pH could affect flavor, aroma, color, carbon dioxide absorption, stability, agility, and fermentation rate. Also, the pH can influence many chemical reactions that take place in wine. The optimum pH for mead production is 3.7 to 4. Low pH increases the efficiency of many preservatives, such as sulfur dioxide and sorbic acid. The pH of must/wine does not remain static during the course of fermentation and maturation. However, as a general rule, the addition of 0.5-1 g/L acid as tartaric drops the pH by about 0.1 units (Rotter, 2008).

The pH of mead fermentation should be taken into account, and preferably earlier than later. Honey is naturally acidic and often attains a pH of 3.5-5.0 once diluted to typical mead, must densities (usually around 21–24° Brix). However, it has very little in the way of natural buffers, and this means that as the fermentation gets underway, with the rise of carbonic acid (CO_2) along with the various organic acids produced by the yeast themselves, the pH of the must can quickly drop to 2.6–2.8 in a 24–36 h period. This is well past the desired, lower-end threshold for a wine yeast fermentation (which is around pH 3.2). This low pH, if left uncorrected, will cause the yeast to become stressed, and the resulting fermentation will often become sluggish or even stuck.

2.8.2 Temperature

Temperature plays an important role in fermentation. Increasing temperature increases the fermentation rate up to a certain level, after which it starts to decrease. Above 38ºC, the fermentative yeast used will have reduced vitality and viability; at too low temperature, it will ferment slowly either way might cause stuck fermentation (Berry, 1996).

For mead fermentation is conducted at temperatures ranging from 22 to 25ºC and is monitored daily to reduce the risk of premature fermentation arrest (Pereira *et al.*, 2017). There is a possibility of stuck fermentation if it is carried at a higher temperature. Aroma composition showed significant differences based on fermentation temperature and lower fermentation temperature was related to greater amounts of esters in mead, including

ethyloctanoate and ethyldecanoate (Tomasino *et al.*, 2018). This can be ascribed to the fact that, lower fermentation temperatures retains more esters, while the warmer ferments drive off and volatilize esters (Saerens *et al.*, 2008). Fermentation temperature affected the amount of phenethyl alcohol and phenethyl acetate in the finished meads. Higher temperatures favor higher alcohols, such as phenethyl alcohol (Albertazzi *et al.*, 1994).

2.8.3 Sugar concentration

The must having very high sugar concentration imparts high osmotic pressure, which has a negative effect on yeast cells, since both growth of yeast and fermentation activity are lowered. Depending on the yeast species, the tolerance of higher sugar concentration varies. The optimum sugar concentration in terms of total soluble solid is 20-24ºBx. The sugar concentration in the honey must determine the alcohol content in mead low concentration gives low alcohol content and vice versa (Samuel and Prescott, 2016).

Fructose utilization by wine yeasts is critically important for the maintenance of a high fermentation rate at the end of alcoholic fermentation. *Saccharomyces cerevisiae* able to ferment grape must sugars to dryness was found to have a high fructose utilization capacity (Guillaume *et al.*, 2007). Therefore, using such yeasts might eliminate such problems.

2.8.4 Nutrient supplementation and Yeast Assimilable Nitrogen (YAN)

Honey is mainly composed of glucose, fructose and water, but it lacks other nutrients, mainly assimilable nitrogen, which is vital for the growth and work of yeast during fermentation. Assimilable nitrogen is essential for yeast metabolism and growth. Nitrogen availability is directly related to biomass production during the yeast exponential growth phase at early stages of alcoholic fermentation (Hernández-Orte *et al.*, 2005). Honey must supplementation is necessary for mead production because of the deficiency in nitrogen materials in this feedstock, despite its high fermentative sugar content. The nitrogen limitation can halt or slow fermentation and lead to the production of unpleasant sensorial compounds, such as sulfur derivatives. According to Luís Menezes de Almeida *et al.* (2020) the addition of supplements resulted in increased cell viability in the first 5 days of fermentation at 20°C, but did not affect the final acidity of the produced meads. Supplementation also leads to increased sugar consumption, and sugar conversion into ethanol increased as nitrogen supplementation increased. This shows that these compounds also regulate yeast metabolic pathways. Supplementary nitrogen acts both in protein anabolism and the gene expression

of glycolytic and fermentative pathway components, favoring, in this case, sugar conversion into ethanol.

The problems of honey must fermentation are due to a deficiency of nitrogen, minerals, and other growth factors (Gupta and Sharma, 2009). The correction of these nutritional deficiencies may reduce stress sensitivity of the yeast, improving fermentation performance (Gibson, 2011).

Vitamins, whose concentration is not usually limiting, are required by yeast cells for many enzymatic reactions (Alfenore *et al.*, 2002). Minerals are required as cofactors for several metabolic pathways influencing the rate of sugar conversion (Pereira *et al.*, 2010). Nitrogen deficiency has been reported as the major cause of stuck or sluggish fermentation of grape juice (Beltran *et al.*, 2005), because nitrogen affects yeast growth, yeast fermentation rate, and fermentation length (Bely *et al.*, 1994). Nitrogen concentration also regulates the formation of byproducts, such as H_2S, fatty acids, higher alcohols, and esters, among others, which affect the chemical and sensorial proprieties of the alcoholic beverage (Torrea *et al.*, 2011). The nitrogen limitation can halt or slow fermentation and lead to the production of unpleasant sensorial compounds, such as sulfur derivatives (Luís Menezes de Almeida *et al.*, 2020). In alcoholic fermentation, *S. cerevisiae* normally requires a minimum of 267 mg/L, expressed as nitrogen, for complete fermentation of a must containing 200 g/L hexoses (glucose and fructose), in an industrially reasonable time (Mendes-Ferreira and Mendes-Faia, 2004). Despite this, there are differences in the nitrogen demand according to the industrial yeast strain or the quality of the nitrogen source or the must sugar concentration (Manginot *et al.*, 1998).

In fact, the supplementation of nitrogen deficiencies with DAP addition is a widespread practice in mead production (Ilha *et al.*, 2000; Morales *et al.*, 2013; Pereira *et al.*, 2013). In other cases, the honey must nutritional deficiencies are supplemented as commercial nutrients (Pereira *et al.*, 2009; Wintersteen *et al.*, 2005). In the fermentation of longan mead, Chen *et al.* (2013) found that the addition of commercial nutrients containing yeast hulls, yeast extract, DAP, vitamin B1, magnesium sulfate, folic acid, niacin, and calcium pantothenate only attained high fermentation rates. Also using commercial nutrients, Gomes *et al.* (2013) detected high sugar consumption and high production of ethanol, acetic acid, and glycerol with a concentration of 0.88 g/L.

There are references in Lature to other natural supplements that can be added to mead to improve yeast growth or yeast fermentative activity: black rice, a natural nutrient for yeast (Koguchi *et al.*, 2009); fruit juices as a source of acids and growth factors (Gupta and Sharma, 2009). The influence of pollen addition to mead elaboration improved fermentation rates, alcohol yield, and final sensory attributes. Pollen addition also reduced mead's total acidity, possibly by supplementing its potassium and calcium content, which could have led to salinization, reducing acidity (Roldán *et al.*, 2011).

Depending on the style desired, the honey is diluted with water or juice, and nutrient mixture/Yeast nutrition added. This may include $(NH_4)_2SO_4$, $CaSO_4$, $(NH_4)_3PO_4$, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$ (DAP), K_3PO_4, $MgCl_2$, $MgSO_4H_2O$, $NaHSO_4$, citric acid, sodium citrate, tartaric acid, potassium tartrate, potassium sodium tartrate 4-hydrate, malic acid, vitamins (biotin, pyridoxine, thiamin), myo-inositol, and peptone which altogether act as yeast energizer or yeast food or commercially available yeast energizers (McConnell and Schramm, 1995; Pereria, 2015).

2.9 Alcohol

The word "alcohol" derives from Arabic *al- kuhul,* which denotes a fine powder of antimony used as an eye makeup. Alcohol originally referred to any fine powder, but medieval alchemists later applied the term to the refined products of distillation, and this led to the current usage (Shakhashiri, 2009)**.**

There are many kinds of alcohol, but when the term is used loosely in alcoholic beverage, it invariably applies to the potable alcohol called ethyl alcohol or ethanol, the common ingredients of alcoholic drinks of all types. Ethanol has been made since ancient times by the fermentation of sugars. All beverage ethanol and more than half of industrial ethanol are still made using this process. Zymase, a set of enzymes from yeast, changes the simple sugars into ethanol and carbon dioxide The ethanol produced by fermentation ranges in concentration from a few percent up to about 14%. Above about 14%, ethanol destroys the zymase enzyme and fermentation stops. Ethanol melts at–114.1°C, boils at 78.5°C, and has a density of 0.789 g/ml at 20°C. It mixes easily with water in any proportion, and where quantities are mixed, there is a contraction in volume. It is clear, colorless, inflammable liquid. It is good solvent for essential oil, ester, tannins, various organic acids and certain other organic compounds. Notably, the production of alcohol during fermentation assists the

physical extraction of numerous compounds (e.g. terpenes) from plant cells (i.e. fruit, herbs, spices etc) which appear in the fermented products (Clarke and Bakker, 2004). It burns easily in air, so that oxidation is possible and then gives a blue smokeless flame, producing water and CO_2 (Shakhashiri, 2009).

2.9.1 Alcoholic fermentation

Alcoholic fermentation is the anaerobic transformation of sugars, mainly glucose and fructose, into ethanol and carbon dioxide in presence of nitrogen compound. Fruit juices have the highest sugar concentration among the many substrates used for the production of ethanol by fermentation. As a result, the level of ethanol is among the highest seen. This process, which is carried out by yeast and also by some bacteria, can be summarized by this overall reaction:

$$\underset{\text{Hexose}}{C_6H_{12}O_6} \xrightarrow{\text{Yeast (zymase)}} \underset{\text{Ethanol}}{2\,C_2H_5OH} + \underset{\text{Carbon dioxide}}{2CO_2}$$

However, alcoholic fermentation is a much more complex process. At the same time as this overall reaction proceeds, a lot of other biochemical, chemical and physicochemical processes take place, making it possible to turn the sugar into alcohol. Besides ethanol, several other compounds are produced throughout alcoholic fermentation, such as higher alcohols, esters, glycerol, succinic acid, diacetyl, acetoin and 2, 3-butanediol. Simultaneously, some compounds of grape juice are also transformed by yeast metabolism. Without the production of these other substances, wine would have little organoleptic interest (Zamora, 2009). The theoretical conversion of 180 g of sugar into 88 g of carbon dioxide and 92 g of ethanol means that yield of ethanol is 51.1% on a weight basis. But in reality, the yield is not equal to and is always lower than that of this given percentage and the decrease in the percentage varies depending upon inoculum size, fermentation temperature and nutrient availability (Usansa, 2003).

2.9.2 Fermentation kinetics of honey wines

The fermentation kinetics study is the study of the changes in the physicochemical properties of a media over a time, i.e. rate of change of reducing sugar, pH, alcohol content, yeast growth rate, etc. With the help of a study of kinetics, we can optimize our process efficiently and this might help with some fermentation related conundrums, helping us to have new

insights into fermentation. But fermentation kinetics is easily affected by conditions like temperature, media composition, type of inoculum used, size of inoculum etc. so change in any of these parameters changes the outcome of the study (Deindoerfer, 1960).

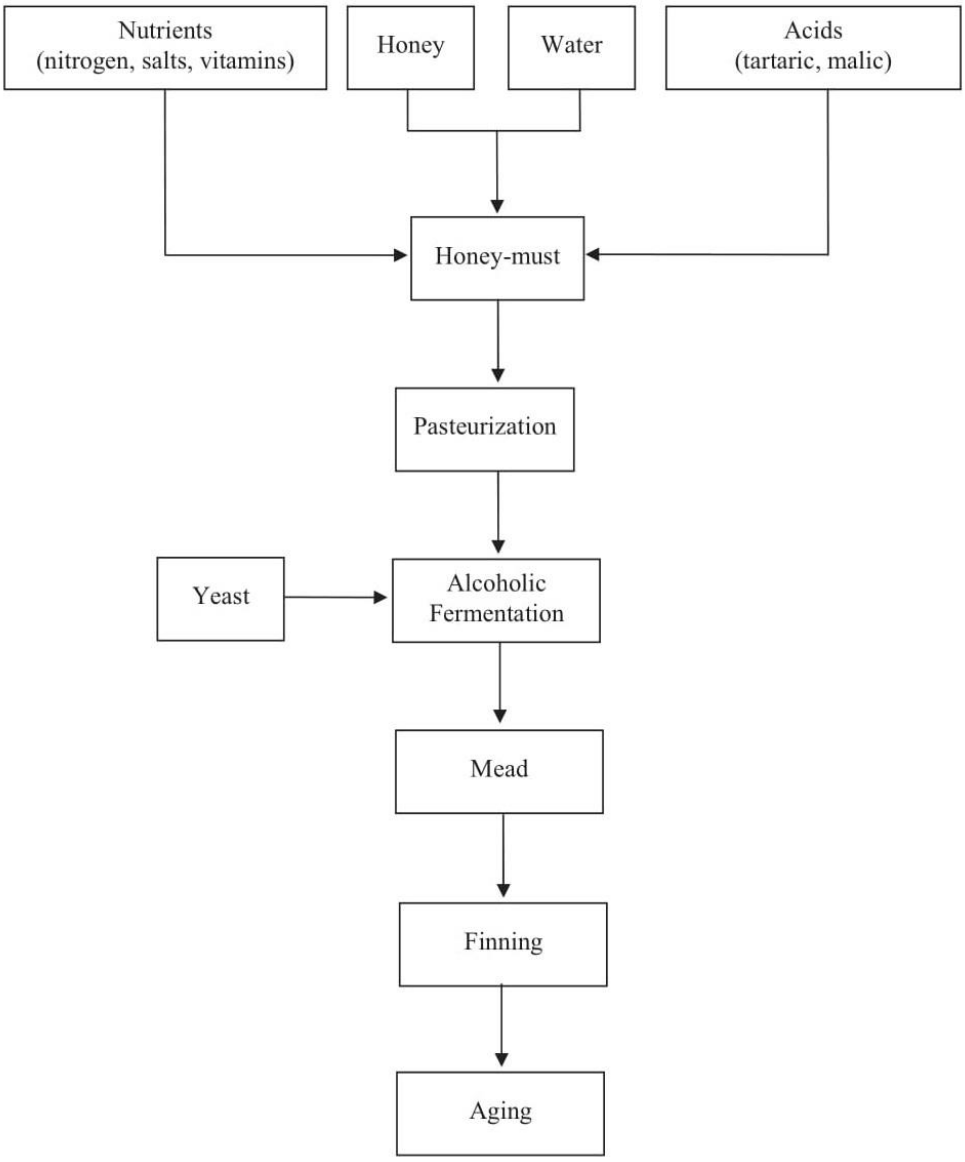
Pereira *et al.* (2013) tested yeast growth with 2 different strains of *S. cerevisiae* i.e. ICV D47 and QA23 at 22°C with five different pitching rates: 1.5×10^5 cfu/ml, 10^6 cfu/ml, 10^7 cfu/ml, 4×10^7 cfu/ml and 10^8 cfu/ml and found that for both, the net growth was highest for the lowest pitching rate and at the highest pitching rates (4×10^7 or 10^8 cfu/ml), no detectable increase in yeast growth was observed, which could be explained by a cell-to-cell contact mechanism at high-cell-density of *S. cerevisiae*. At pitching rate 1.5×10^5, the yeast density in the must reached nearly 10^8 cfu/ml after 48 h after which stationary phase started and yeast number remained nearly constant throughout the fermentation and for this pitching rate there was a 75% reduction in reducing sugars than the initial content of the must after 96 h. For all the pitching rate, yeast goes in stationary phase after the cell density reached near around 10^8 cfu/ml.

2.10 General method of mead preparation

The process of making mead is similar to white wine making. The process of preparation of metheglin is similar to mead production, with just an additional step of herbs incorporation. The desired range for mead during fermentation is between pH 3.7 and 4.0 with citric acid (Sroka and Tuszyński, 2007), mallic acid (Pereira *et al.*, 2013) or tartaric acid (Roldán *et al.*, 2011). A mixture of tartaric and malic acids may be used not only to adjust the acidity but also to increase the buffer capacity of honey must (Mendes-Ferreira *et al.*, 2010).

The must is subsequently sanitized; pasteurization being one of the most commonly used methods (Mendes-Ferreira *et al.*, 2010; Pereira *et al.*, 2014; Wintersteen *et al.*, 2005). Czabaj *et al.* (2017) conducted research showing that the use of heat treatment speeds up the fermentation process, maintaining good product characteristics. Heat treatment had a significant effect on the antioxidant properties of meads, as gently boiled mead having highest antioxidant and total phenolic content than control or pasteurized must. The downside of the heating process is the formation of HMF, which can affect product quality. In contrast, other techniques are used to control or inactivating most wild microorganisms, including the addition of potassium metabisulfite (Roldán *et al.*, 2011) or boiling of the must (Ukpabi, 2006). After the honey must treatment, it is inoculated sufficiently with selected

strains of *Saccharomyces cerevisiae* from culture collections or active dry yeasts (0.5 g/L) (Czabaj *et al.*, 2017). A general flow chart of mead preparation is given in Fig. 2.2.



Source: Pereira *et al.* (2017)

Fig. 2.2 Flow chart of mead (Honey wine) preparation

2.10.1 Fermentation

Fermentation is the soul (heart) of wine making. All the desirable reactions take place during this step, so most of wine makers pay strict attention to this stage. Fermentation is conducted at temperatures ranging from 22 to 25ºC and is monitored daily to reduce the risk of premature fermentation arrest. The duration of fermentation depends on the type of honey,

nutrients added to the honey must, size of the inoculum, etc. The fermentation conditions may last up to 2 weeks to 3 months. In this process, CO_2 is simultaneously released, making fermentation violent at first and then slow. The yeast added is 1-3% of the volume of the must. Generally, 14 days are required for complete alcoholic fermentation. Most of the fermentation takes place in three stages.

1. An initial stage during which the yeast cells are multiplying.
2. A very vigorous stage accompanied by bubbling and marked rise in temperature.
3. Quiet fermentation that can proceed for quite a long time at a lower and lower rate.

High temperature also encourages heat tolerant bacteria to produce acid, mannitol and off flavor (Douglas and Considine, 1982). Srimeena and Gunasekaran (2015) reported, during fermentation of honey, total soluble solids, reducing sugars, pH were decreased and acidity of mead was increased.

Johnson and Peterson (1974) reported that at the usual total sugar content of 19-24%, alcoholic fermentation proceeds rapidly and, with alcohol tolerant strains of yeast, to completion, producing about 10-12.5% alcohol (by volume). If the sugar content is greater than 24%, the high sugar content may inhibit fermentation and the rate of fermentation will be slower and may be incomplete. Under special condition of simulation, 16-18% alcohol can be reached. It is agreed that methanol is not produced by alcoholic fermentation. The amount of higher alcohols produced is less when ammonium phosphate is added prior to fermentation. At very low concentration the higher alcohols may play a desirable role in sensory quality (Amerine *et al.*, 1980).

Guymon *et al.* (1961) Showed that oxidative conditions during fermentation favor higher alcohol production. Most enologists consider that glycerol is of considerable sensory importance because of its sweet taste and its oiliness. Acetaldehyde is a normal by-product of alcoholic fermentation. Acetaldehyde reacts with ethyl alcohol to form acetal, a substance with a strong aldehyde like odor, found very little in wines (Amerine *et al.*, 1980).

The tartaric, malic and citric acids of the must are found in the resulting wines but in decreased amounts. They are important constituents of wine not only for their acid taste but also because they protect the wine from spoilage, maintain the color, and are themselves sometimes attacked by microorganisms (Amerine *et al.*, 1980). The formation of acetic acid,

widely referred to as volatile acidity, is recognized as a normal by-product of alcoholic fermentation. In bacteria-free fermentations, the amount of acetic acid formed does not exceed 0.03 to 0.04 g per 100 ml. By far, the largest increase in acidity during fermentation is brought about by the formation of nonvolatile organic acids, and it varies between 0.1 to 0.4 g per 100 ml but averages about 0.2 g per 100 ml. Succinic acid and lactic acid are nonvolatile acids, which are products of alcoholic fermentation. The predominant nonvolatile organic acid formed during fermentation is succinic acid (90%) and lactic acid appears to be the other acid formed in significant amounts (10%) (Thoukis *et al.*, 1965). Lactic acid has a slight odor and is a weak acid. It is a constant by-product of alcoholic fermentation, 0.04 to 0.75 g/L. Carbonic acid makes up a very special case for both still and sparkling wines. It has no odor and very little taste. But it has a feel and disengagement of the bubbles from the wine probably brings more oxygen away from the surface of wine (Amerine *et al.*, 1980).

Ferreira *et al.* (1996) studied the ability of fermentative CO_2 to blow off the volatile compounds that are synthesized during fermentation. Model solutions simulating a fermenting must were purged at different CO_2 flow rates and temperatures, and the amount of volatile compounds blown off by the stream of CO_2 was recorded by high-resolution gas chromatography. Synthesis takes place during the tumultuous period of fermentation, together with CO_2 production, that blows off the volatile material. Hydrolysis takes place in the last stages of fermentation. In open fermenter, up to 80% of volatile material can be blown off while an average of 10% is retained and residual esterase activity accounts for about 20% of the total amount of ester synthesized.

The end of fermentation is signaled by a clearing of the liquid, by a vinous taste and aroma, and by a drop in temperature, and can be confirmed by checking degrees balling (sugar residual) (Douglas and Considine, 1982).

2.10.2 Post fermentation adjustments

When fermentation is complete, the mead is siphoned from the yeast sediment into barrels (racking) and is left for maturation, in which clarification takes place naturally (Grainger and Tattersall, 2005). Normally, wine should be racked within a month of the end of fermentation. Racking process normally entails a sacrifice of 2-3% wine in lees (Rai, 2012).

To clarify mead, bentonite is often used (McConnell and Schramm, 1995; Pereira *et al.*, 2009; Roldán *et al.*, 2011), as well as gelatin (Roldán *et al.*, 2011). Typically, bentonite can be used at a rate of 1.5 g/L. However, it is essential that the fining agents be tested for dosage optimization before use because over fining can cause a permanently cloudy wine (Rai, 2012). **N**ow days cross flow filtration and membrane filtration are used in meadery. These are more efficient than press and frame filter (Ulrich, 2018).

Aging is important in mead production, particularly in relation to the development of aroma compounds moving from a harsh, acidic, unpleasant taste to a smooth to a mellow beverage with a nice bouquet and fragrance. The length of aging can be from months to years, depending on the type of mead. Aging of wines improves the flavor and bouquet because of oxidation and formation of esters. These esters of higher acids formed during aging give the ultimate pleasing bouquet to the well-aged wine (Clarke and Bakker, 2004). Following filtration and clarification, the wine are bottled and pasteurized. Additions like SO_2 and sorbic acid or potassium sorbate (which does not actually kill yeast cells, but prevents it from reproducing) are used to protect wine from chemical and microbial deterioration (Varnam and Sutherland, 2012).

2.11 Wine analysis

Throughout the history of winemaking, analytical techniques have become increasingly important for the development of technology and increased governmental regulation. Analysis of wine is performed for several reasons, such as quality control, spoilage reduction and process improvement, blending, export certification and global regulatory requirements (Fugelsang and Charles, 2007).

2.11.1 Physical and chemical analysis

At the end of fermentations, oenological parameters such as pH, volatile acidity, sugars, methanol, higher alcohols, ester, aldehyde, ethanol content and other required parameters are determined according to standard methods. All wines should be subjected to appropriate analyzes during their production and storage to meet the requirements of regulatory agencies and to give the winemaker information to monitor the operations properly (Fugelsang and Charles, 2007).

Experimental wines often require additional analyzes to get more complete information and study the specific effects of the experimental conditions. There is no sense in doing the experiments unless analytical methods are available to evaluate the results.

2.11.2 Sensory evaluation of mead

It is almost always necessary to compare wines by sensory analysis, besides chemical and physical methods. This is true of commercial wines, but often especially so with experimental wines (Savits, 2014). Aroma volatile compounds play a key role in determining the quality of beverages because they are the primary contributors to aroma and produce an effect on sensory characteristics (Andreu-Sevilla *et al.*, 2013). Two main types of methodologies are used for evaluation of the quality of food and beverages. The identification and quantification of aroma compounds, as an aim analysis technique, or subjective methods based on human assessment of the quality characteristics of the food (Smyth and Cozzolino, 2013).

Sensory analysis is indispensable for the assessment of food flavor characteristics to identify the significant sensory and quality contributors to food quality and consumer preference (Schmidtke *et al.*, 2010). Overall, the more important sensory characteristics of beverages are the smell, the taste, and, to a lesser extent, the color (Robinson *et al.*, 2011), and their assessment is performed by a panel of experts or consumers. One person's opinion is hardly definitive on any wine's sensory character and quality, not that one tester may not be better than another in natural ability, concentrated effort, amount of experience, and/or comparative memory. In evaluation of the sensory qualities of one or more wines, a panel of testers is necessary. This panel should be sensitive and experienced, but each individual is erratic, biased, or unobservant on some occasions, hence the need for panels and statistical evaluation of the testing results (Lesschaeve, 2007).

No technique is ideal for everyone. Probably the most essential property of a serious taster is the willingness, desire, and ability to focus his or her attention on the wine's characteristics. Where tasters are unfamiliar with the characteristics of the wines to be tasted, it can familiarize the senses to the basic attributes of the wines. However, the introductory sample must be chosen with care to avoid setting an inappropriate standard and distorting expectations. It is safer to encourage tasters to cleanse their palate between each sample. In contrast, olfactory adaptation may have an advantage. For example, it may "unmask" the

presence of other aromatic compounds. Most wines are best sampled in clear, tulip-shaped wine bottle. The primary exception involves sparkling wines. These are normally judged in elongated, flute-shaped glasses. They facilitate observation of the wine's effervescence. All glasses in a tasting should be identical and filled to the same level (about one-quarter to one-third full). This permits each wine to be sampled under equivalent conditions. Between 30 and 50 ml is adequate for most tastings. Not only are small volumes economic, but they facilitate holding the glass at a steep angle (for viewing color and clarity) and permit vigorous swirling (to enhance the release of aromatics) (Jackson, 2002).

Jackson, Jackson (2002) listed the sequence and method of wine sensory evaluation as following

I. **Appearance:** Firstly, view each sample at 30˚ to 45˚against the bright white background. Then record separately the wine's clarity (absence of haze), color (shade or tint) and depth (intensity or amount of pigment), viscosity (resistance to flow) and effervescence (notably sparkling wines).
II. **Odor:** Firstly sniff each at mouth of glass before swirling and then study and record the nature and intensity of fragrance. Now swirl the glass to promote release of the aromatic constituents from wine, then smell the wine initially at the mouth and deeper into the bowl. Now study and record the nature and intensity of fragrance.
III. **In-mouth sensations:** Take a small (6 to 10 ml) sample into mouth. Move wine into mouth to coat all surface of the tongue checks and palate. For various taste sensations (sweet, acid, bitter) note where they perceived, when they first detected, how long they last, and how they change in perception and intensity. Then, concentrate on the tactile (mouth feel) sensation of astringency, prickling, body temperature and heat. Record this perception and how they combine with each other.
IV. **Finish:** concentrate on the olfactory and gustatory sensations that linger in the mouth. Compare these sensations with those previously detected. Note their character and sensations.
V. **Overall quality:** After the sensory aspect has been studied individually, attention shift to integrating their effects the wine's overall quality and finally, make and overall assessment of the pleasure, complexity, subtlety, elegance, power and balance of wine.

2.11.2.1 Color of mead

Honey, which is the raw material to produce mead, shows a lot of variations in color and composition, which are likely to affect the end product (mead) produced (Gupta and Sharma, 2009). Also, the addition materials to the honey must during production also affect the color of wine. The color changes accordingly; from dark purples, bright red, pale pinks, or even blue as use of berries impart the color from berries as in red wine. Mead ranges from almost clear to a molasses amber color, especially if heat is not used in the process. If heat is used; as in a Brochet mead, it can be quite dark in color. It all depends on the material used during preparation of mead.

2.11.2.2 Aroma of mead

The aroma profile is one of the most typical features of a food product, for both its organoleptic quality and its authenticity (Alvarez-Suarez *et al.*, 2010). The aroma of mead has contributions from honey, inoculated yeast, and technological processes (Chen *et al.*, 2013; Gupta and Sharma, 2009; Pereira *et al.*, 2013). Concerning wine, its aroma is composed of the varietal aroma that arises directly from grapes with minor modifications; fermentative aroma compounds, produced by yeasts during the alcoholic fermentation; and the maturation bouquet that results from chemical reactions during storage and ageing (Robinson *et al.*, 2011; Swiegers *et al.*, 2005). Regarding mead aroma, it has contributions from honey, the yeast used for inoculation and fermentation conditions(Chen *et al.*, 2013; Gupta and Sharma, 2009).

2.11.2.2.1 Honey-derived volatiles

Honey aroma is very complex and involves several volatile compounds; however, not all have a significant impact on the aroma. The impact of a compound depends on the extent to which the concentration exceeds its odor threshold. It is important to state that some synergistic and/or antagonistic interactions between various components may occur, and thus, even compounds present in low concentrations may contribute to honey aroma.

To determine the influence of the volatile compounds on overall honey aroma, odor activity values (OAVs) should be assessed by dividing the concentration of each compound by its perception threshold. Only the compounds with OAVs greater than 1 (or near) may have contributed to the honey aroma (Manyi-Loh *et al.*, 2011b). The same volatile

compounds identified in various honey samples can be characterized by a wide range of aroma descriptors, for example, from bitter, rancid, or fishy to sweet and flowery (Manyi-Loh *et al.*, 2011b).

Sensory evaluation, based mainly on attributes of aroma and taste, is one of the most useful tools in honey characterization (Castro-Vázquez *et al.*, 2009). Some of the aroma attributes proposed have been floral, fruity, candy, waxy, resin, wood, citric, acidic, spicy, balsamic, caramel, herbaceous, coffee/chocolate, cheese, chemical, and fermented, among others. The attributes sweet, acid, astringent, ripe fruit, toasty caramel, woody, and spicy have been selected for taste characterization. Honeys from different geographical and botanic origins differ regarding their sensory profile. For instance, the attributes flowery, fruity, waxy, jaggery-like, chemical, and caramel notes were the major variables among honey samples from India (Anupama *et al.*, 2003).

Castro-Vázquez *et al.* (2009) identified the volatile compounds and the sensory descriptors that are more representative of different monofloral honeys, namely citrus, rosemary, eucalyptus, lavender, thyme, and heather. These authors verified, citrus honeys were characterized by higher amounts of linalool derivatives and by fresh fruit and citric aromas; eucalyptus honeys had hydroxyketones and p-cymene derivatives together with cheese and hay aromas; lavender honeys had mainly hexanal, nerolidol oxide, and coumarin and the sensorial attributes balsamic and aromatic herb aromas; finally, heather honeys were characterized by high contents of benzene and phenolic compounds and ripe fruit and spicy aromas. Regarding chestnut honeys from Spain it was verified that the volatile composition and sensory profile are greatly influenced by the geographic origin, i.e., honeys from the Spanish northeast presented significantly higher concentrations of aldehydes, alcohols, lactones, and volatile phenols, which are associated with herbaceous, woody, and spicy notes; honeys from the northwest area showed superior levels of terpenes, esters, and some benzene derivatives, closely related to honey-like, floral, and fruity notes (Castro-Vázquez *et al.*, 2010).

2.11.2.2.2 Fermentation yeast-derived volatiles

During alcoholic fermentation, yeasts produce a range of compounds with strong sensorial importance to the quality of the final product. Fermentative compounds, resulting from the metabolic activity of yeasts, represent quantitatively most volatile compounds in wines

(Vilanova and Oliveira, 2012); therefore, these microorganisms play an important role in the development of wine aroma. Since 2005, some research has been conducted on volatile compounds formation during mead fermentation. The production of volatile compounds is affected by several factors, including the yeast strain (Chen *et al.*, 2013; Teramoto *et al.*, 2005), cell condition (free or immobilized) (Pereira *et al.*, 2014), and inoculum size (Pereira *et al.*, 2013), as well as by the fermentation conditions(Wintersteen *et al.*, 2005). In addition, the type of honey (Vidrih and Hribar, 2007), and the honey must composition/formulation (Mendes-Ferreira *et al.*, 2010; Roldán *et al.*, 2011) can also modulate the formation of volatile compounds. The volatile compounds produced by yeasts are alcohols, organic acids, esters, volatile fatty acids, carbonyl compounds, and volatile phenols, among others.

2.12 Mead faults

Like beer and wine, mead has its defects from non-microbial causes and spoilage caused by microorganisms like yeasts, acetic acid bacteria and lactic acid bacteria. Some defects of honey beverage are acetic, acidic, alcoholic, waxy, yeasty, chemical, cloudy, cork taint, cloying, fruity, metallic, moldy oxidation, sherry, tannic etc. which can be prevented using right fermentation methods (BJCP, 2008).

Part III

Material and methods

3.1 Materials

3.1.1 Raw materials

3.1.1.1 Honey

Brassica honey from *Apis cerena* fed on *Brassica napus* was obtained from a honey farm named "API enterprises" located at Cancer gate, Bharatpur - 7, Chitwan, Nepal (Google plus code 7MV6MC79+42) which was farmed there locally.

3.1.1.2 *Bergenia ciliata* (pakhanbedh)

The herb (rhizome of *Bergenia ciliata*) was brought from the local market of Dharan (Google plus code 7MR9R76P+GF), Nepal, which was grown in Namche (Google plus code 7MV8WJ42 + J6) and was cleaned and air-dried.

3.1.2 Yeast

The Active wine yeast used for pitching and fermentation was *Saccharomyces cerevisiae*, from Fermentis SAFŒNO™ SC 22, which is a rapid fermentation starting yeast with alcohol tolerance of 15% (v/v) and working fermentation temperature for this yeast 12 to 35°C. Medium nitrogen requirement is required for the yeast, which is between 150 and 180 mg/L of available nitrogen. It is necessary to supply 20 g/HL of Springferm® and 20 g/HL of DAP at yeast inoculation (Fermentis, 2021).

3.1.3 Yeast nutrient

The yeast nutrient used was Fermentis SpringFerm™, which is a multi-purpose fermentation activator, made of partially autolyzed yeast that has organic nitrogen, sterols, minerals, and vitamins.

3.1.4 Glassware and equipment

All standardized glassware and equipment used were obtained from the campus, Central Campus of Technology, Dharan. The list of equipments used is given in Table 3.1.

Table 3.1 List of equipment used

Equipment	Equipment
Stainless steel vessels	Weighing arrangement
Hand refractometer (0-30ºBx) (Hanna Instrument, Portugal)	Heating arrangement
pH meter (Deluxe pH meter)	Distillation set
Thermometer	Titration apparatus
Pycnometer	Other routine glasswares
Food grade silicon tube rubber pipe	Plastic jars
Microprocessor UV-vis spectrophotometer (Labtronics Model LT–291, India)	Wine bottles with cap
Hemocytometer (improved neubauer China)	Microscope (NIKE)

3.1.5 Chemicals

All chemicals used were of analytical grade and were obtained from laboratory of Central Campus of Technology.

3.2 Methodology

The total work was based on preparation of metheglin with varying proportion of rhizome of *B. ciliata* and analysis of final mead as shown in detailed flow diagram in Fig 3.1.

3.2.1 Experimental procedure

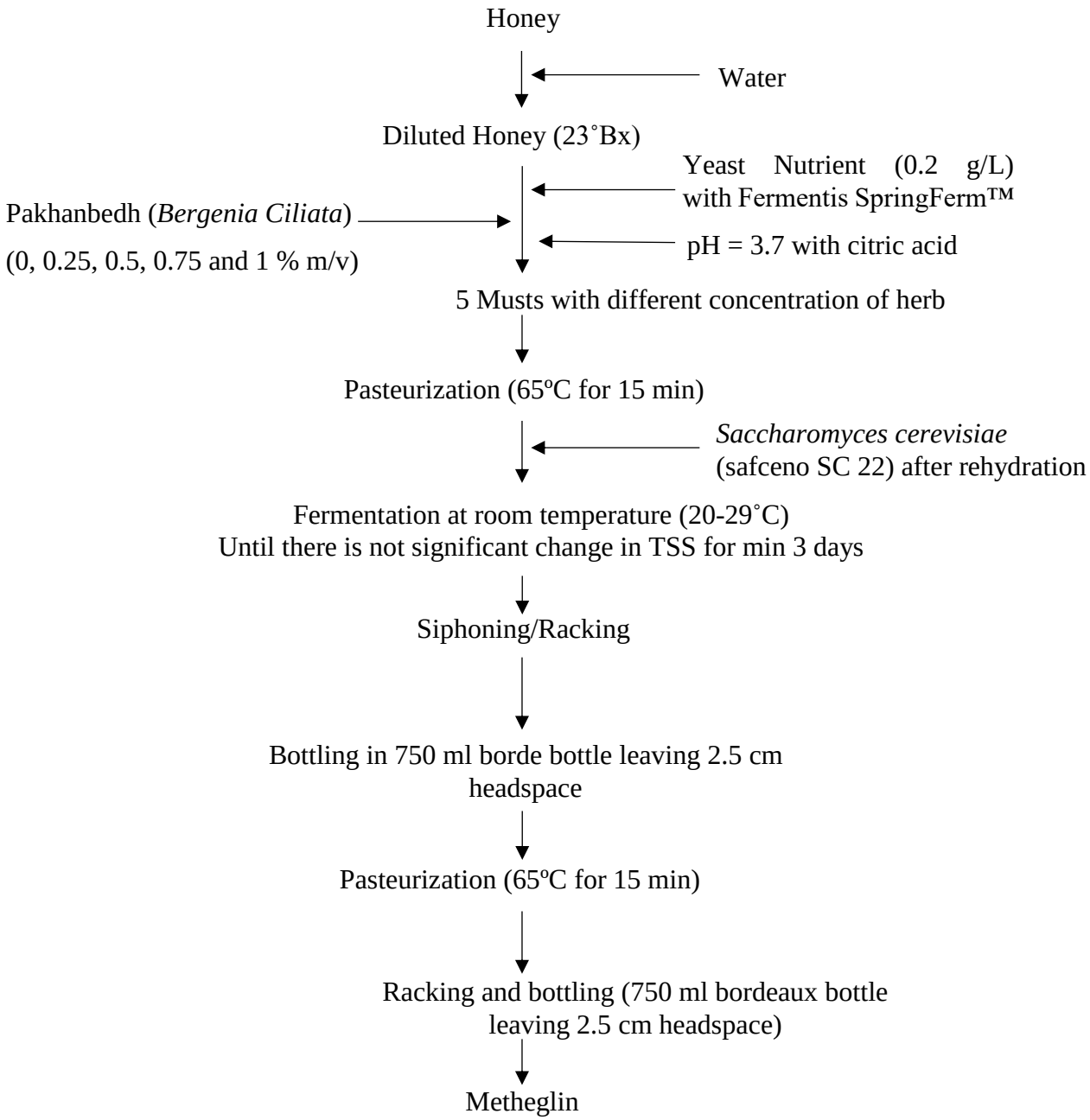


Fig. 3.1 General flow diagram for the experimentation

3.2.1.1 Preparation of must composition

After analysis of honey, 5 different samples with 5 different proportion of *B. ciliata* (pakhanbedh) were prepared i.e. (0, 0.25, 0.5, 0.75 and 1% m/v). The TSS of 23˚Bx was

maintained by addition of water to honey and was measured with a hand refractometer (Hanna Instrument, Portugal). The pH was maintained at 3.7 by use of citric acid and was measured with a digital pH meter. The yeast nutrient was added at the rate of 0.2 g/L to the must. Then varying amount of herb to must was added to make different samples with different herb concentration (0, 0.25, 0.5, 0.75 and 1% m/v) with granules of size (500-2500 μm) diameter got after hammer milling of *B. ciliata* rhizome.

3.2.1.2 Pasteurization

The must was pasteurized with gradual heating at 65ºC for 15 min on an open pan heating system and cooled to room temperature.

3.2.1.3 Pitching

Saccharomyces cerevisiae, from Fermentis SAFŒNO™ SC 22, was used for pitching. It was activated/rehydrated with mildly heated water and pitching was done at the rate of 10^5 cfu/ml for all musts. The general flow sheet for procedure is given in Fig. 3.1.

3.2.1.4 Fermentation

Must after pitching were kept in plastic jars for fermentation after vigorous shaking of must for aeration of must. The exact process followed in this study is given in Fig. 3.1. It was necessary to create an anaerobic condition inside the jars during fermentation for improving the quality of product so the jars were sealed with airlock system. The process of fermentation was followed by measuring the drop in degree brix. The Fermentation temperature was not consistent because it was carried out at room temperature, which fluctuates and was 20-29˚C (min-max). The kinetics of the fermentation were studied at 2 days interval. And fermentation was stopped after gassing cease, no significant change in TSS and yeast flocculation and clearing of metheglin.

3.2.1.5 Racking, fining, pasteurization and bottling

After fermentation, the clear metheglin was drawn off from the sediment known as 'lees' by siphoning, which was done using sterilized food grade silicon tube rubber pipe. Then the metheglin was tested with bentonite test to find the concentration of bentonite required to clarify it. Then, after fining it with bentonite, it was bottled and was pasteurized by heating bottles by boiling water in order to maintain the temperature of wine 65°C for 15 min and

cooled to room temperature and were kept for further analysis. A bottle with water was filled to and kept with other bottles during pasteurization for to keep a record of the temperature.

3.2.2 Analytical procedure

Although different authors have described different methods and parameters to analyze honey, must and metheglin only those parameters and related methods, which were feasible in the laboratory, were determined in this study. The determinations were conducted in triplicates.

For honey TSS, pH, mineral content, moisture content, total titratable acidity, total phenolic content and reducing sugar were analyzed. The fermentation kinetics study of TSS, reducing sugar content, ethanol, total titratable acidity and yeast count were studied. The final metheglins were analyzed for chemical composition and properties like TSS, pH, total acidity, volatile acidity, specific gravity, alcohol content (ethanol, methanol and higher alcohols), ester, and aldehyde and for prepared metheglin sensory analysis based on following parameters appearance, odor, in mouth sensation, finish and overall acceptance was done to select best product.

3.2.2.1 Determination of total soluble solid (TSS)

The TSS of the honey, must mead and metheglins were determined using a hand refractometer (Hanna Instrument, Portugal) at 20ºC after degassing of samples and the results were expressed as °Bx.

3.2.2.2 Determination of reducing sugar

The percentage of reducing sugar in samples was determined by the Lane and Eynon method according to (Kirk and Sawyor, 1991).

3.2.2.3 Determination of pH

pH of honey, must, mead and metheglins were determined by the digital pH meter of Labtronic ™ (Deluxe pH meter) of model LT-10 provided by Central Campus of Technology, Nepal and standardized with standard buffers at 25ºC after degassing.

3.2.2.4 Determination of total acidity, fixed acidity and volatile acidity

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

3.2.2.5 Preparation of distillate from mead and metheglins

By transferring exactly 200 ml of neutralized fermented honey musts into a 500 ml distillation flask containing about 25 ml of distilled water and a few pieces of pumice stone. Distilled the contents in about 35 min and collected the distillate in a 200 ml volumetric flask until the volume almost reaches the mark. Brought the distillate to room temperature and made up to volume (200 ml) with distilled water and mixed thoroughly.

3.2.2.6 Determination of ethanol content from specific gravity method

The percentage of alcohol by volume from specific gravity was determined according to AOAC (2005). The relative specific gravity of the distillates were determined by dividing the weight of 25 ml of the distillate by the weight of an equal volume of water using a 25 ml specific gravity bottle (pycnometer) and ethanol (%v/v) was found by referring to the reference table.

3.2.2.7 Determination of ester content

Total esters content was determined by the titrimetric method as per Kirk and Sawyor (1991). Total esters content was expressed as gram of ethyl acetate in 100 L of alcohol.

3.2.2.8 Determination of aldehyde content

Total aldehyde content was determined by the titrimetric method as per FSSAI (2015). Total aldehyde content was expressed as gram of acetaldehyde in 100 L of alcohol.

3.2.2.9 Determination of fusel oil (higher alcohol)

Higher alcohol was determined by DMAB spectrophotometric method as per AOAC (2005). Distillate (1 ml) was pipetted in a test tube and diluted to 5 ml with distilled water prior to analyzing. The higher alcohol was expressed as milligram per L of wine (mg/L).

3.2.2.10 Determination of methanol content

Methanol content was determined by chromotropic acid colorimetric method as per OIV (2020) and was expressed in mg/L of metheglin.

3.2.2.11 Determination of total phenolic content (TPC)

Total phenolic content was determined as per Prior *et al.* (2005) using Folin-Ciocalteau method. Briefly, 1 ml of the filtered (Whatman 42 filter paper) wine was diluted to 10 ml with distilled water prior to assessment and was expressed as milligram of gallic acid equivalent (GAE) per 100 ml wine.

3.2.2.12 Determination of antioxidant activity

The antioxidant activity of wine was determined by DPPH method as per Sing *et al.* (2008). Briefly, wine sample was filtered through Whatman No. 42 filter paper. 1 ml of the filtered wine was diluted to 10 ml with methanol. One ml of the diluted wine was taken in a test tube and 4 ml of 0.004% methanolic solution of DPPH was added. Then the test tube was left for 30 min in the dark and absorbance was measured at 517 nm using a UV-vis spectrophotometer. Similarly, blank was also run using methanol instead of the sample. The DPPH scavenging activity was calculated:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{Blank absorbance} - \text{Sample absorbance}) \times 100}{\text{Blank absorbance}}$$

3.2.2.13 Determination of antimicrobial activity

Antibacterial activity of prepared wines against a Gram-positive bacteria, *Staphylococcus aureus* and a Gram-negative bacteria *Escherichia coli* was performed by well diffusion technique (Deans and Ritchie, 1987). The *E. coli* in MacConkey agar and *S. aureus* in mannitol salt agar (MSA) were isolated from raw cow milk from the local farm of Dharan. The *S. aureus* was identified based on Gram staining (+ve), colony morphology, oxidase (+ve) and catalase test (+ve). The *E. coli* was identified based on gram staining (-ve), colony morphology, catalase (+ve) and oxidase (-ve) test. Media was prepared by dissolving 5.6 g of nutrient agar and 2.6 g of nutrient broth in 200 ml of distilled water in the flask. The nutrient broth was taken approximately 7-8 ml per test tube. All the apparatus

and media viz. Petri plates, tips and normal saline etc. used in the activity were autoclaved for 20 min at 121°C. After sterilization, nutrient agar was poured into the petri plates and allowed to solidify. The culture was actively grown in nutrient broth for 16 h at 37°C. 100 µL liquid culture of each microbe was spread on each nutrient agar plate to create a bacterial lawn of the respective microbe. 6 wells with a diameter of 6 mm were punched in nutrient agar plate and 100 µL mead and metheglin samples were added to the bored wells in each plate under aseptic condition as well as 10% (v/v) pure ethanol was also loaded in one of the well. The plates were left for 30 min at room temperature for the diffusion of the test samples before being incubated at 37°C for 24 h, after which the diameter of zones of inhibition (in mm) were measured using vernier caliper. Analyses were carried out in triplicate.

3.2.2.14 Determination of number of yeast cell using hemocytometer

Yeast cell count of the samples was 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 min to allow yeast to settle, then put on a microscope with 400x 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 are 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 get an accurate yeast cell count, it was advisable to count no fewer than 75 cells on the entire (1-mm^2×9) 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%.

Number of cells/ml = Total cells in central 25-square ruled area × dilution factor × 10^4

3.2.2.15 Determination of pancreatic α-amylase inhibitory activity

α-amylase inhibitory activity of the prepared metheglins was carried out according to Ademiluyi and Oboh (2013) with minor modification. Reaction mixture containing 500 µL sodium phosphate buffer (100 mM, pH = 6.9), 100 µl α–amylase (2 Unit/ml i.e. 1 unit = 1 µmol/min) and 200 µl of sample was pre-incubated at 37°C for 20 min. Then, the 200 µL of 1% soluble starch (100 mM phosphate buffer pH 6.9) was added as a substrate and incubated further at 37°C for 30 min; 1000 µL of the DNS color reagent was then added and boiled for 10 min. The reaction mixture was then diluted after adding 10 ml of distilled water, and absorbance was measured at 540 nm. In parallel, control was created with the addition of water as a sample and each experiment was performed in triplicates. The results were expressed as percentage inhibition, which was calculated using the formula,

$$\text{Inhibitory activity (\%)} = \frac{\text{Ac- As}}{\text{Ac}} \times 100$$

Where,

As is the absorbance in the presence of test substance, and Ac is the absorbance of control.

3.2.2.16 Bentonite fining trial

Bentonite fining trial was done as per AWRI (2021). A 10% (w/v) calcium bentonite slurry was made in water at 60°C by stirring slowly and sprinkling the bentonite into the water to disperse it thoroughly and kept overnight at sterile condition to hydrate the slurry during which the bentonite swells up and efficiency of it increases. 20 ml of each sample was taken in a clean and clear test tube and bentonite slurry was added at rates of 0.1 ml to 0.5 ml and was left overnight and after which the best result was observed visually.

3.2.2.17 Sensory evaluation

The prepared samples were subjected to sensory evaluation for consumer's acceptability. The samples were served in clean wine glass at silent environment. Sensory attributes (such as appearance, odor, in mouth sensation, finish and overall quality) were evaluated using 7 points hedonic rating test ranging from faulty (1) to exceptional (7) as described by Jackson, Jackson (2002) with the help of 15 semi- trained panelist whom were teachers and students

of food technology who were familiar with alcoholic beverage. The format of used specimen card is given in Appendices A.

The sequence and method of wine sensory evaluation can be listed as following

I. **Appearance:** Firstly, view each sample at 30º to 45º against the bright white background. Then record separately the wine's clarity (absence of haze), color (shade or tint) and depth (intensity or amount of pigment) and viscosity (resistance to flow).
II. **Odor:** Firstly sniff each at mouth of glass before swirling and then study and record the nature and intensity of fragrance. Now swirl the glass to promote release of the aromatic constituents from wine, then smell the wine initially at the mouth and deeper into the bowl. Now study and record the nature and intensity of fragrance.
III. **In-mouth sensations:** Take a small (6 to 10 ml) sample into mouth. Move wine into mouth to coat all surface of the tongue checks and palate. For various taste sensations (sweet, acid, bitter) note where they perceived, when they first detected, how long they last, and how they change in perception and intensity. Then, concentrate on the tactile (mouth feel) sensation of astringency, prickling, body temperature and heat. Record these perceptions and how they combine with each other.
IV. **Finish:** concentrate on the olfactory and gustatory sensations that linger in the mouth compare these sensations with those previously detected. Note their character and sensations.
V. **Overall quality:** After the sensory aspect has been studied individually, attention shift to integrating their effects the wine's overall quality and finally, make and overall assessment of the pleasurableness, complexity, subtlety, elegance, power, balance and memorableness of wine.

3.2.3 Statistical 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 the treatment means were compared by Tukey HSD test at 5% level of significance to determine whether the sample differed significantly from each other and to determine which one is superior among them and superscript was assigned to each of them in descending order with mean value. MS- Excel 2016 was also employed for the general calculations, graph and diagram construction.

Part IV

Results and discussion

Honey must with different concentrations of *B. Ciliata* i.e. 0, 0.25, 0.5, 0.75 and 1% (m/v) was fermented for 21 days, producing metheglin, as stated in material and methods. Effects of different concentrations of *B. ciliata* on fermentation kinetics of honey must, physicochemical, sensory and therapeutic activity of produced metheglin were studied in the laboratory of Central Campus of Technology, Dharan.

4.1 Chemical analysis of honey

Parameters as TSS, acidity, pH, moisture content, reducing sugar and total phenolic content were measured. And the values obtained from this analysis show the honey is of good quality. Chemical composition of honey after analysis is given in Table 4.1.

Table 4.1 chemical composition of honey

Parameter	Value
TSS (ºBx)	81 (0)
Acidity (% as lactic acid)	0.21 (0.02)
pH	4.6 (0)
Moisture content (%)	17.61 (0.28)
Total ash (%)	0.17 (0.02)
Reducing sugar (% as dextrose)	77 (0.40)
Total phenolic content(mg GAE/100 g)	68 (0.23)

Values in the above table are the means of three determinations. Figures in the parentheses are the standard deviations.

4.2 Fermentation kinetics of honey must with different concentrations of *B. Ciliata*

Changes in physio-chemical properties as Total Soluble Solid (TSS), reducing sugar, total titratable acidity and ethanol content and growth kinetics of *S. Cerevisiae* in honey must with different concentration of *B. Ciliata* was studied.

4.2.1 Kinetics of Total Soluble Solid (TSS)

Total soluble solid of the different musts were recorded for 21 days at day 0, 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 17, 19, 21. The recorded data for different samples varying with *B. ciliata* concentration are as follows in Table B.3 in Appendix B.

The TSS changed significantly along with the fermentation days up to day 12, after which the rate of depletion of TSS dropped for all the samples. The slope is highest up to day 4, similar to results of Pereira *et al.* (2013). The depletion of TSS for samples increases with low *B. Ciliata* concentration in honey must, increasing the *B. Ciliata* concentration slows down the depletion of TSS. The TSS generally represents the sugar content of the honey must. As yeast consumes the sugar during fermentation, the sugar content drops, which ultimately drops the TSS of the must. The graphical representation of the tabulated data for comprehending TSS change is given in Fig. 4.1. From the presented graph, it can be seen that for all the samples the slope is highest for up to day 4 meaning the rate in change of TSS was highest during that course of fermentation which is highest being 43% decrease in sample 0% and lowest being 34% decrease in sample 1%.

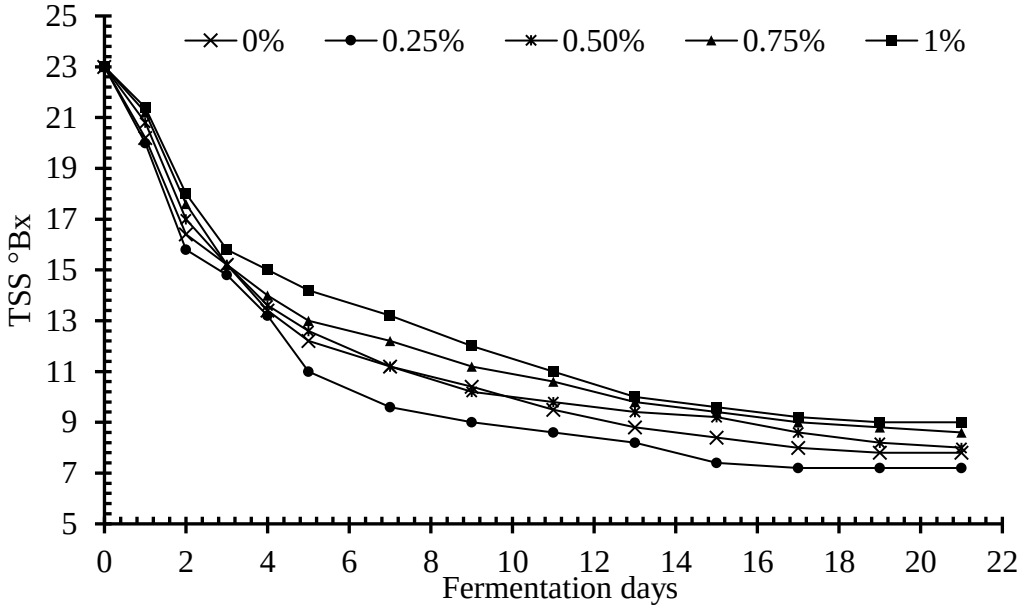


Fig 4.1 TSS depletion during the course of fermentation

4.2.2 Kinetics of reducing sugar

Reducing sugar (as %dextrose) of the different musts was recorded for 21 days on day 0, 2, 4, 6, 10, 14, 21. The recorded data for different samples varying with *B. Ciliata* concentration are tabulated in Table B.2 and presented graphically in Fig. 4.2.

Since the values for TSS and sugar content in honey must are close and similar and the sugar in honey must is almost completely reducing sugars, the rate of change in the reducing sugar content is very similar to the rate of change in TSS content. The sugar decreases due to consumption by yeast during fermentation. The sugar is used for the cell growth by yeast, which converts the sugar to ethanol. The reducing sugar depleted quickly during the initial days of fermentation and the rate of consumption of reducing sugar decreased later after 14 days, after which the rate of depletion occurred slowly. Increasing the *B. Ciliata* concentration decreased the sugar consumption by yeast. On the last day of fermentation, there was 77% and 70% decrease in reducing sugar content for herb concentration 0% and 1% (m/v) respectively.

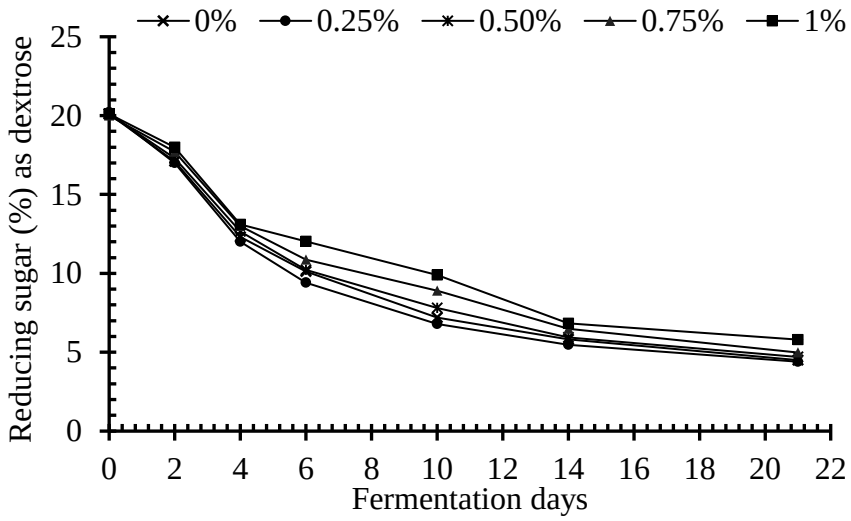


Fig. 4.2 Kinetics of reducing sugar during the course of fermentation

4.2.3 Kinetics of total titratable acidity

Total titratable acidity of the different musts were recorded for 21 days at day 0, 1, 2, 3, 4, 5, 7, 9, 11, 13, 15, 17, 19, 21. The recorded data for different samples varying with *B. Ciliata* concentration are as shown in Table B.5.

The acidity increased during the initial fermentation days for up to 9 days, which increased approx. by 37-43% (min-max %) of the initial value, after which it slowed down and the total increase was approx. by 51-60% (min-max %) of the initial value. The increase in acidity is due to the increase in volatile (acetic acid) and non-volatile acid (succinic acid and lactic acid) production by yeast metabolism during alcoholic fermentation as suggested by (Thoukis *et al.*, 1965). The graphical presentation of the changes in acidity for all 5 samples is shown in Fig. 4.3.

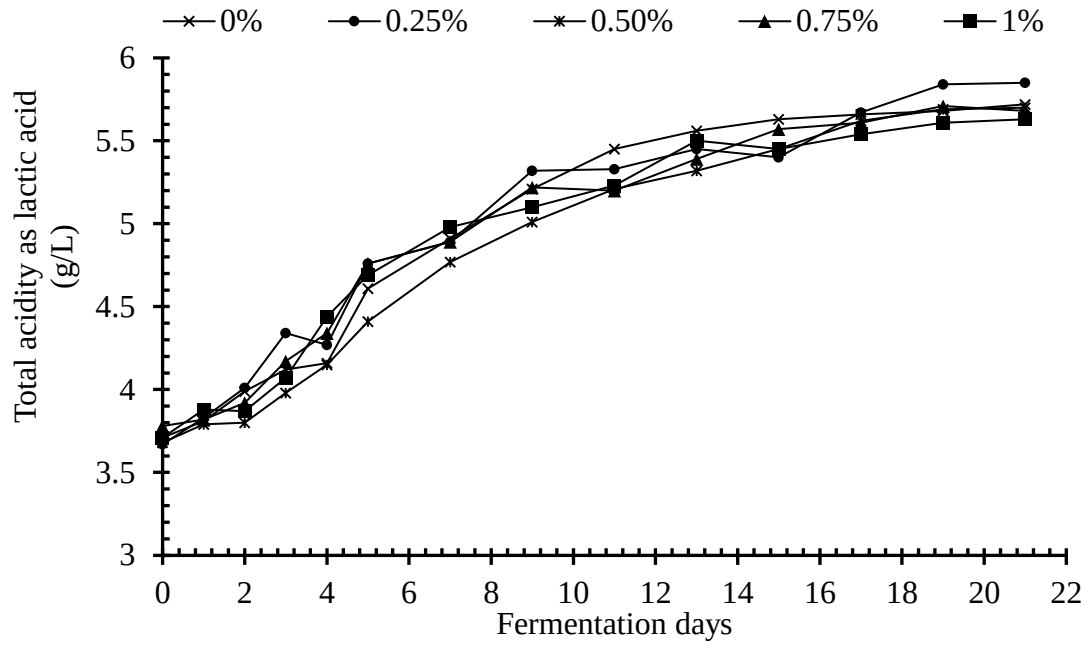


Fig. 4.3 Kinetics of total titratable acidity during the course of fermentation

4.2.4 Kinetics of ethanol content

Ethanol content (% v/v) of the different musts were recorded for 21 days on day 0, 2, 4, 6, 8, 12, 16, 21. The recorded data for different samples varying with *B. ciliata* concentration are as follows in Table B.4.

The ethanol is formed by conversion of sugar in must to ethanol through metabolism of sugar by yeast. The ethanol production rate was high at the initial phase of fermentation for up to 12 days where most of the ethanol (>85%) was produced in the samples, after which rate decreased during the later phase of fermentation. The ethanol yield of the 5 samples 0, 0.25, 0.5, 0.75, and 1% m/v for this fermentation were found to be 47.7, 48.1, 47.1, 48 and 49.8% respectively. Increasing herb concentration to must decrease the ethanol production

due to slow down of yeast metabolism for sugar intake and conversion. Since the ethanol is converted from the reducing sugar in the honey must, the pattern for the rate of ethanol production is somewhat inverse to the rate of reducing sugar depletion, as shown in Fig. 4.4.

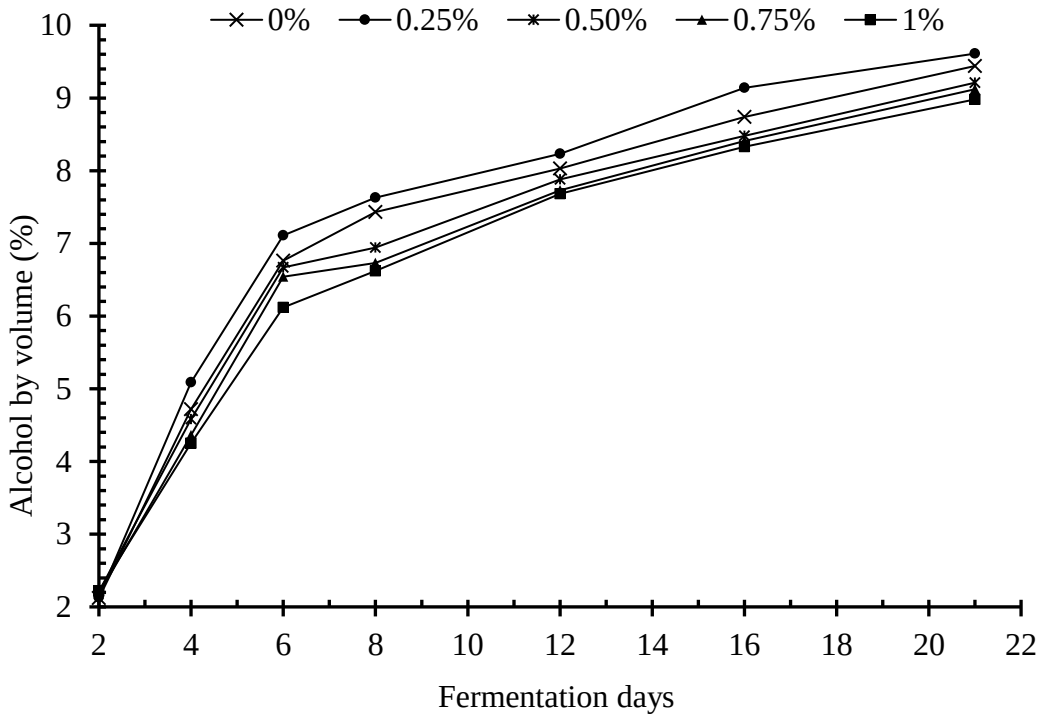


Fig. 4.4 Kinetics of ethanol content during the course of fermentation

4.2.5 Growth kinetics of *S. cerevisiae*

The yeast cells of *Saccharomyces cerevisiae* were counted at fermentation days 0, 1, 3, 5, 9, 13, 17 and 21. The recorded data for different samples varying with *B. ciliata* concentration are as follows in Table B.6.

The yeast growth (i.e. increase in the number of yeast cells) increased exponentially during the first 5 days of fermentation, which was over 500 fold increase in number of cells in all the samples, after which the growth was slowed down and yeast entered the stationary phase which is like what Pereira *et al.* (2013) found in their study. After 21 days, the largest growth was increased by 1100 times for sample 0.25% and smallest growth was by 700 times for sample 1%. From the presented data, we can conclude that the *B. Ciliata* concentration had a significant effect on the yeast growth kinetics affecting the metabolism of sugar,

acidity and ethanol production during alcoholic fermentation of the honey must. Increasing the concentration decreased the cell number and growth rate as well as substrate utilization and product formation except for concentration 0.25 (% m/v), which might be due to low antimicrobial activity at the given concentration and supplement of yeast growth factors or there might not be a significant difference between control and sample 0.25% so, more study may help to get on a rigid conclusion. This can be described as *B. Ciliata* being a powerful antimicrobial herb (Khan *et al.*, 2018; Singh *et al.*, 2017). The graphical presentation of the changes in yeast number regarding fermentation days is presented in Fig. 4.5.

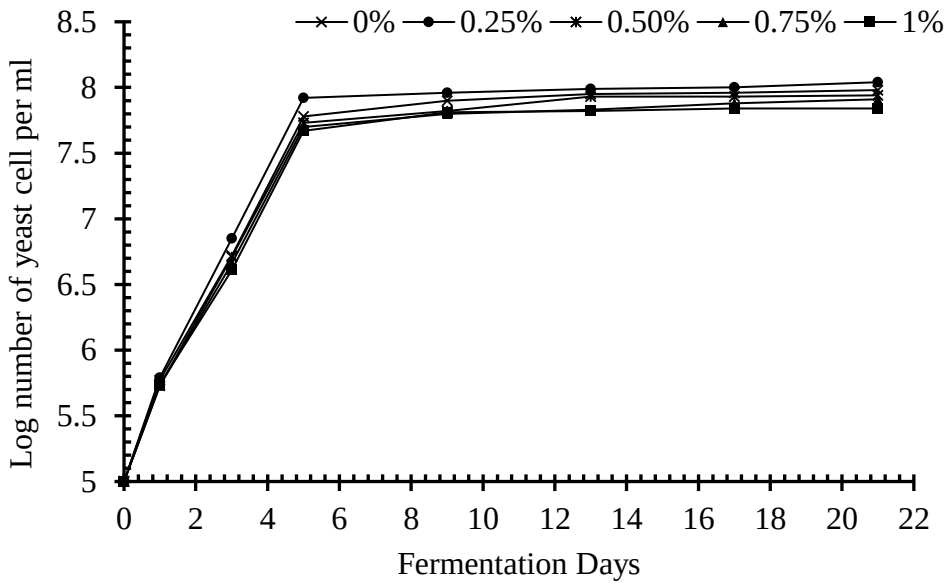


Fig. 4.5 Growth kinetics of *S. cerevisiae*

4.3 Effect of different concentrations of *B. Ciliata* on physicochemical composition of produced metheglins.

The physicochemical parameter assessed were Total Soluble Solid, Reducing sugar, Total titratable acidity, fixed acidity, volatile acidity and pH.

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

The reducing sugars are the primary food source for yeast growth and ethanol precursor in fermentation and the honey is rich in reducing sugar (>90% of total sugar) so no amylase source or hydrolysis treatment is needed for honey must fermentation as in starchy commodities. Since the reducing sugar is the major soluble solid in honey, TSS and reducing

sugar are presented simultaneously and these seem to correlate highly as shown in data. The data for the final TSS and reducing sugar of analyzed metheglins are given in Table 4.2.

Table 4.2 Final TSS and reducing sugar content of *B. Ciliata* added metheglins

B. ciliata concentration (% m/v)	TSS (°Bx)	Reducing sugar (as dextrose g/L)
0	7.53^{c} (0.23)	45.21^{bc} (2.49)
0.25	7.27^{c} (0.12)	44.58^{c} (3.55)
0.50	8.13^{b} (0.12)	47.03^{b} (1.78)
0.75	8.60^{a} (0.20)	51.78^{a} (1.51)
1	8.86^{a} (0.16)	58.29^{a} (2.25)

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 ($p>0.05$).

Increasing the *B. Ciliata* concentration $\geq 0.5\%$ increased the final TSS and reducing sugar content of produced metheglin. 0.25% of *B. Ciliata* didn't show a significant difference in TSS and reducing sugar with control. The high TSS in metheglin at high herb concentration is due to the low fermentation rate and low yeast growth rate because of the effect of *B. Ciliata* components in the product that hinder the growth of yeast and decrease the efficiency of its metabolism. From this we might establish, increasing the concentration of *B. Ciliata* severely decreases the sugar consumption potential of yeast.

4.3.2 Effect on pH, total titratable, fixed and volatile acidity

Effect of *B. ciliata* on the pH and acidities of the final produced metheglins 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 pH and acidities are presented in Table B.7.

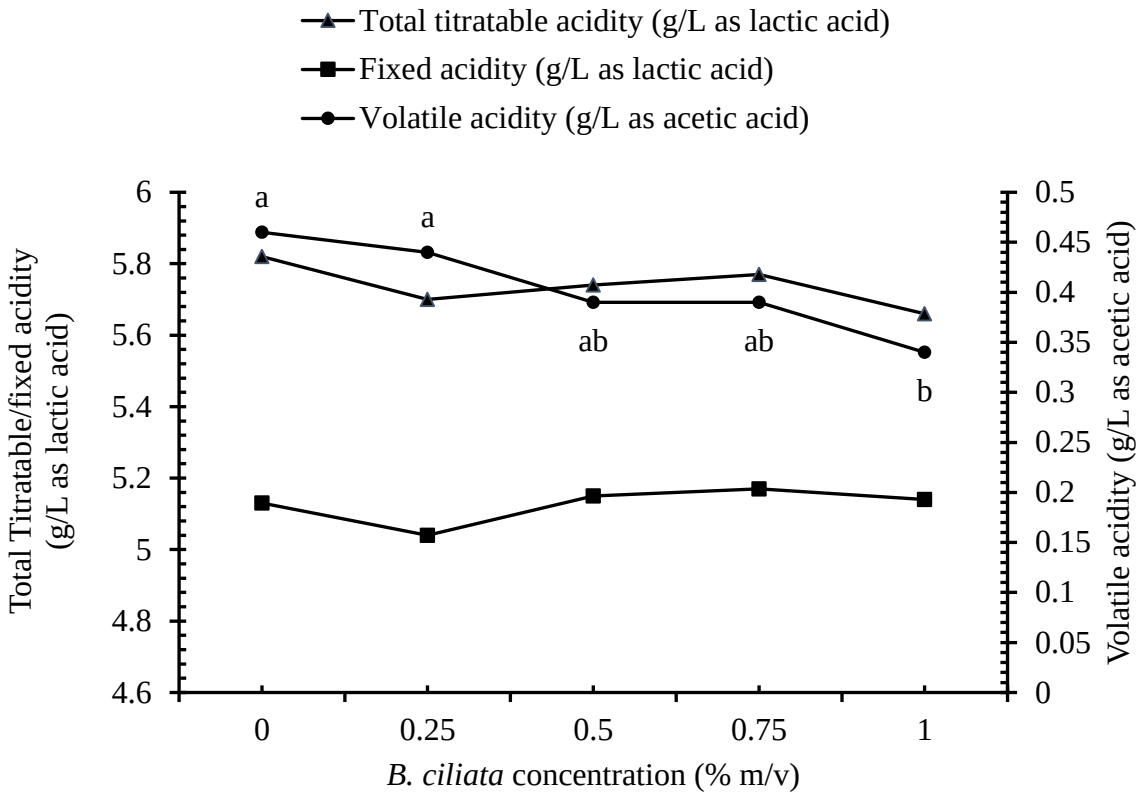


Fig. 4.6 Different acidities of produced metheglins

There was no significant difference of herb addition on the pH, titratable and fixed acidity of the produced fermented products. Volatile acidity decreased on adding herb at higher concentration ≥ 1%. This might be due to the lower metabolism of yeast at higher herb concentration. Since yeast produces volatile acetic acid during the fermentation. Steinkraus and Morse (1973) reported acetic acid in honey wines to be 0·014–0·0779 g/100 ml, which corresponds with our data 0.04 ± 0.004 g/100 ml. So we can conclude that the herb doesn't have any acidic or basic properties to itself and does not affect the acidities profoundly.

4.4 Effect of different concentrations of *B. Ciliata* on volatile constituents of produced metheglins.

Volatile constituents, viz., alcohol, total esters, total aldehydes, higher alcohols, and methanol contents of samples were analyzed. Esters, aldehydes, volatile acids and higher alcohols are the ones mainly responsible for giving the nose to the fermented beverage. The data for volatile constituents of analyzed metheglins are given in Table 4.3.

Table 4.3 Volatile constituents of *B. Ciliata* added metheglin

B. ciliata concentration (% m/v)	Ethanol content (% v/v)	Ester content (g ethyl acetate /100 L alc.)	Aldehyde (g acetaldehyde /100 L alc.)	Methanol (mg/L)	Higher alcohol (mg/L)
0	9.44^{a} (0.10)	48.32^{a} (3.31)	201.43^{a} (10.55)	28.90^{a} (8.87)	311.42^{b} (9.40)
0.25	9.61^{a} (0.11)	45.45^{a} (5.34)	198.72^{a} (6.89)	31.16^{a} (2.41)	337.3^{ab} (13.14)
0.50	9.21^{b} (0.14)	50.26^{a} (6.21)	206.16^{a} (5.46)	33.42^{a} (7.12)	333.52^{ab} (9.82)
0.75	9.12^{bc} (0.11)	43.76^{a} (7.67)	201.12^{a} (17.86)	31.56^{a} (3.46)	354.76^{a} (18.98)
1	8.98^{c} (0.08)	44.2^{a} (5.32)	205.53^{a} (3.46)	30.19^{a} (5.56)	352.89^{a} (5.73)

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 ($p>0.05$).

The ethanol content decreased significantly after herb concentration increased from 0.5% than that from control. This is due to low sugar consumption rate and growth rate of yeast in high *B. Ciliata* concentration.

The ester content of the fermented products doesn't differ from each other and with control significantly. Pereira *et al.* (2019) reported ester content in mead to be 30 -65 mg/L and concentration is affected by strain of yeast used, to which our data corresponds which is 46.28 ± 6.21 mg/L. So addition of B. ciliata doesn't affect the ester content of metheglin.

The aldehyde content of *B. ciliata* added metheglins showed no significant difference between the control and with each other. So *B. Ciliata* doesn't affect the aldehyde content of fermented honey must. Some authors stated carbonyl content in meads produced by *S. cerevisiae* in concentrations between 5 and 30 mg/L (Pereira *et al.*, 2013; Roldán *et al.*, 2011). Steinkraus and Morse (1973) stated acetaldehyde concentration in meads usually ranges between 18.2 and 125.5 mg/L. But aldehyde content varies with the type of honey and yeast strain. Since our yeast strain and honey type differ from those stated in their study, the data might not correspond with them. Also, the difference is not myriad.

The methanol content of *B. ciliata* added metheglins showed no significant difference between the control and with each other and methanol content of mead is very low since there almost no pectin compound in honey and pasteurization of must deactivated any pectinase present from honey or herb and it has been found *S. cerevisiae* doesn't produce pectinase enzyme.

The higher alcohol content increased by little on adding *B. ciliata* to the honey must. This might be due to the stress given by *B. ciliata* to yeast. The higher alcohol in mead ranges from 90 to 350 mg/L (Mendes-Ferreira *et al.*, 2010; Pereira *et al.*, 2013; Roldán *et al.*, 2011) which corresponds with our data. The higher level may be due to the rising of temperature during day time leading to higher alcohol formation since high temperature favors higher alcohol production during fermentation (Albertazzi *et al.*, 1994). Since *B. ciliata* is a non-aromatic herb, it is very low on aromatic or volatile, odor giving compounds and these data support this statement.

4.5 Effect of different concentrations of *B. Ciliata* on therapeutic properties of produced metheglins.

B. ciliata having many reported therapeutic activities is incorporated in our honey must and some therapeutic activities feasible in our lab were assessed i.e. TPC, antioxidant activity, antimicrobial activity and α-amylase inhibitory activity to see how much therapeutic potential of the herb retains to the final fermented product.

4.5.1 Effect on Total Phenolic Content (TPC)

The final Total Phenolic Content (TPC) was assessed in prepared mead and metheglins using Folin-Ciocalteu reagent and UV-vis spectrophotometer at 765 nm using standard curve given in Appendices C. The data for TPC of produced fermented products are given in Table B.8.

The TPC content of metheglins was significantly different with each other and increasing the *B. ciliata* concentration increased the TPC content in produced metheglin as shown in Fig. 4.6. Water and ethanol present in the fermenting must act as solvent to extract the polyphenols from herb. Mead (0%) the control in the study, also had TPC content that got along from the honey used to make mead and metheglins since honey also contains phenolic compounds. From the data, it is shown that adding just 0.25% to 1% m/v of herb to must increase the musts TPC content by > 140% to > 630% respectively. So we can conclude that inducing *B. ciliata* to a fermenting must can significantly increase the TPC content of the final resulting wine.

4.5.2 Effect on antioxidant activity

The final antioxidant activity was assessed in prepared mead and metheglins using DPPH radical scavenging activity and UV-vis spectrophotometer at 765 nm. The data for %DPPH RSA of produced fermented products are given in Table B.8.

The % DPPH Radical Scavenging Activity (RSA) of metheglins were significantly different with each other and increasing the *B. ciliata* concentration increased the value in produced metheglin. % RSA is increasing similarly with TPC content of respective metheglins as shown in Fig. 4.6 so we can say the phenolic compounds from the herb *B. ciliata* are primarily responsible for the antioxidant activity in the metheglin and looking at the pattern of the graph for TPC and %RSA in Fig. 4.6 we can establish they are highly correlated as stated by Agnihotri *et al.* (2014). Mead (0%), the control in the study, also had % RSA activity that got along with the honey used to make mead and metheglins. So, fermented beverages made from incorporation of herb *B. ciliata* to the must can have a significant increase in the antioxidant activity in the final fermented product and increasing the concentration of the herb increases the antioxidant activity.

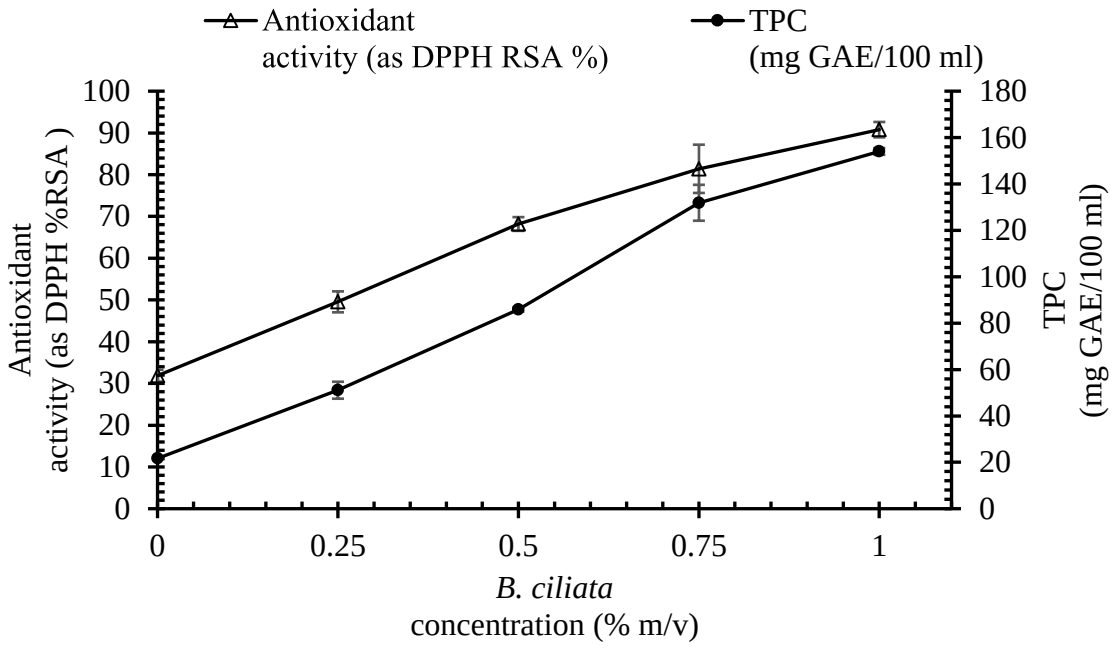


Fig. 4.7 TPC content and % RSA of different *B. ciliata* concentration of metheglins

4.5.3 Effect on antimicrobial activity

The antimicrobial activity of the produced metheglins was assessed with disc diffusion method and two food pathogens, i.e. *Staphylococcus aureus* and *Escherichia coli*, were selected for studying the inhibitory effect. Since ethanol also exhibits an inhibitory effect on microorganisms, 10% (v/v) ethanol was used as a control. The data for antimicrobial activity of produced metheglins are presented in Table B.9.

The zone of inhibition produced from metheglins were significantly different with each other and increasing the *B. ciliata* concentration increased the value in produced metheglin. Mead also had an inhibitory effect on *S. aureus* and *E. coli* that is because of the honey, which contains antibiotics properties as stated by many authors (Amit *et al.*, 2005; Moussa *et al.*, 2012; Sherlock *et al.*, 2010; Taormina *et al.*, 2001). Also, from the data, it can be seen that ethanol at 10% v/v has some antimicrobial properties. So *B. ciliata* incorporated wines can suppress pathogens in gut. Also, it might preserve fermented products from spoilage causing microbes and might act as a preserving fluid for herbs or fruits. So we can conclude that the inhibitory effect of metheglins is due to ethanol, herb components and honey antimicrobial activity.

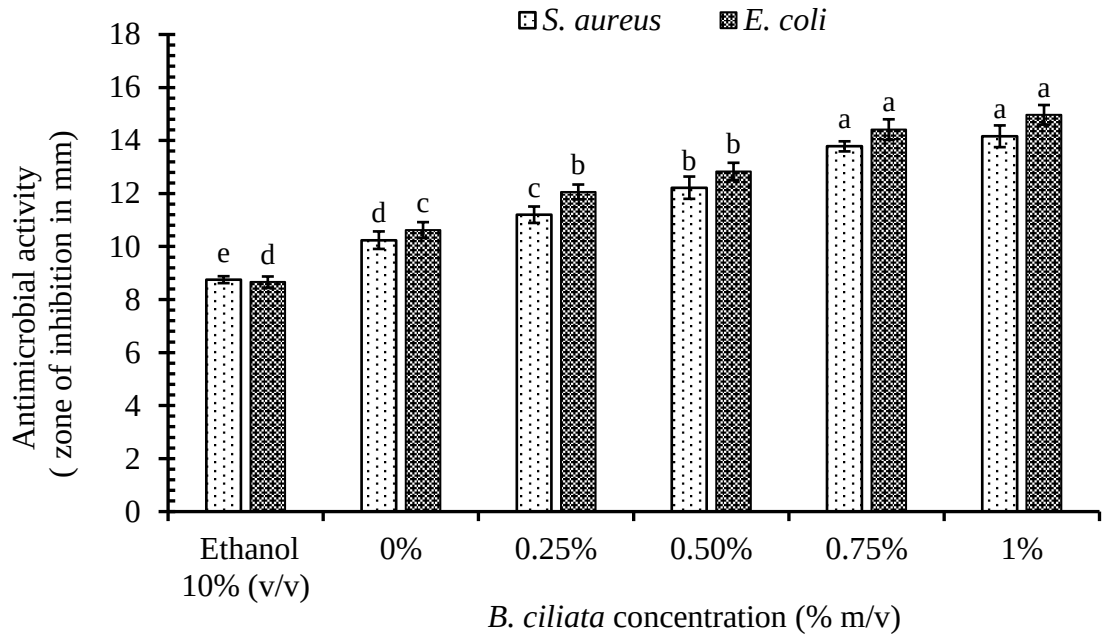


Fig. 4.8 Inhibitory effect of different *B. ciliata* concentration of metheglins on *E. coli* and *S. aureus*

4.5.4 α-amylase inhibitory activity

The α-amylase inhibitory activity was assessed using porcine pancreatic α-amylase. The data for the α-amylase inhibitory activity of all the samples are given in Table B.10.

Incorporation of the herb *B. ciliata* significantly boosted the inhibitory effect on porcine pancreatic α-amylase. So, this might slow down the breakdown of starch in human intestine too, leading to slow formation of glucosides for glucosidase to work on, resulting in low glucose level thus having an antidiabetic effect. Increasing the herb concentration increased the inhibitory effect on amylase with maximum inhibition at herb concentration 1% at which the inhibitory effect increased over 100% than at 0.25% herb concentration, as shown in Fig. 4.8. Using *B. ciliata* in a starchy material for fermentation might cause slow fermentation due to inhibition of amylase enzyme and slow growth rate of microbes in higher concentration of herb. Bhandari *et al.* (2008) reported two active compounds (-)-3-O-galloylepicatechin and (-)-3-O-galloylcatechin to have dose dependent enzyme inhibitory activities against porcine pancreatic α-amylase which probably got extracted by water and ethanol during fermentation in the metheglins and are responsible in our case also for the inhibitory action.

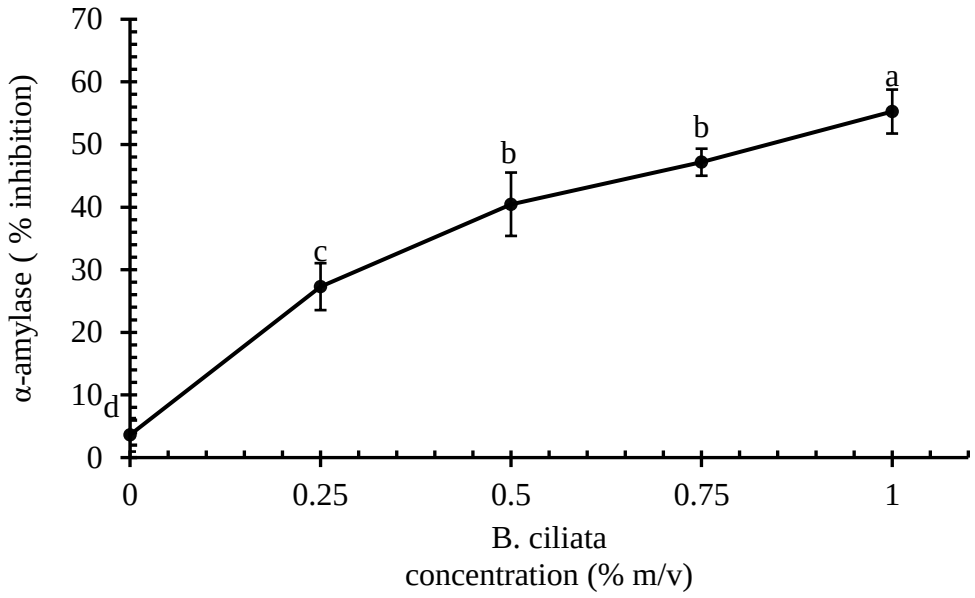


Fig. 4.9 α-amylase inhibitory activity of metheglins with different proportion of *B. ciliata*

4.6 Bentonite fining trial

0.2 ml of the 10 (% m/v) slurry gave a very clear product in 20 ml of all samples showing 1 g/L of calcium bentonite is required to clarify the products and such similar products but a trail must always be performed for better result and to avert errors.

4.7 Effect of different concentrations of *B. Ciliata* on organoleptic properties of produced metheglins.

The sensory analysis was conducted with 15 semi-trained panelists who are familiar with fermented beverages. The panelists were teaching staffs and students of M. Tech (Food) and B. Tech (Food). 7 points hedonic rating test was conducted for the evaluation of the products where, 1: faulty, 2: poor, 3: below average, 4: average, 5: above average, 6: very good and 7: exceptional. There were five sensory parameters, namely appearance, odor, mouthfeel, aftertaste and overall acceptance. And a comment section for leaving comments (if any) was provided on the scorecard. The table for the mean scores for the sensory attributes is given in Table B.1. Fig. 4.9 shows the mean sensory scores. Values on the top of the bars bearing similar superscript are not significantly different at 5% level of significance. Vertical error bars represent ± standard deviation of scores given by panelists.

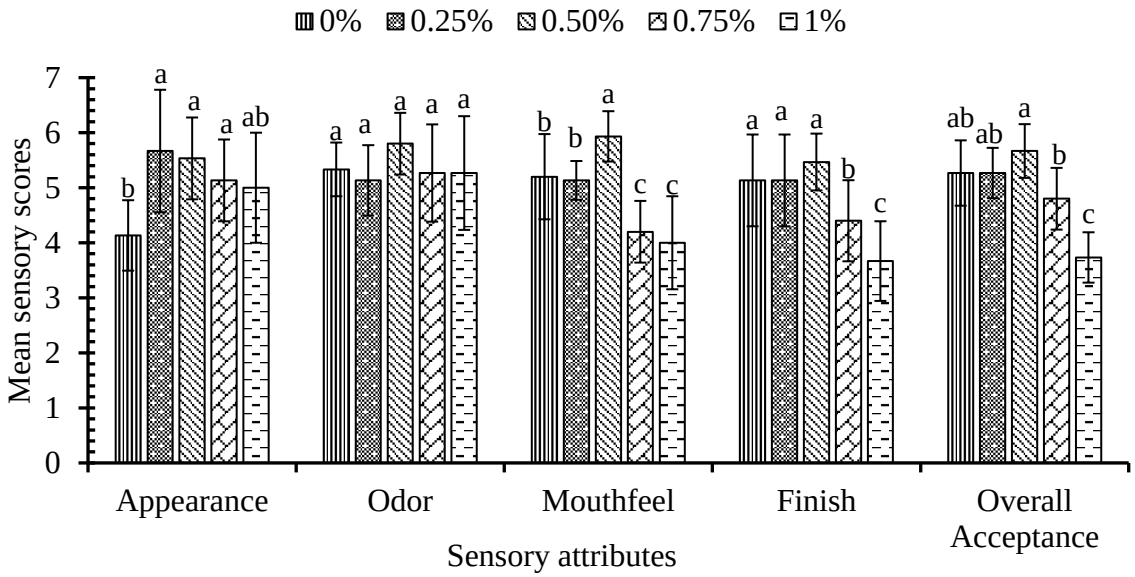


Fig. 4.10 Effects of different concentration of *B. Ciliata* on the organoleptic quality of metheglins

The mean score for the appearance of metheglins with concentration of *B. Ciliata* 0, 0.25, 0.5, 0.75 and 1% are 5.667, 5.533, 5.133, 5 and 4.133 respectively. The score for appearance of the product increased on the addition of *B. Ciliata* to that of control. This is due to the herb giving color, a golden yellowish hue to the product increasing its aesthetic value, increasing herb concentration increased the color intensity. High intensity of color was less appreciated by panelists, i.e. 1%. So concentration of 0.25% to 0.75% is optimum range for best appearance of metheglin.

The mean score for the odor of metheglins with concentration of *B. Ciliata* 0, 0.25, 0.5, 0.75 and 1% are 5.33, 5.13, 5.8, 5.26 and 5.26 respectively. There is no significant difference in odor by adding *B. Ciliata* at given different concentrations. The dried rhizome of *B. Ciliata* is not an aromatic herb, therefore, it doesn't impart any odor to the product.

The mean score for the mouthfeel of metheglins with concentration of *B. Ciliata* 0, 0.25, 0.5, 0.75 and 1% are 5.20, 5.13, 5.93, 4.20, and 4.00 respectively. The score for mouthfeel increased on increasing *B. Ciliata* concentration up to 0.5% after which it decreased. This is mainly because of the polyphenols extracted on the wine from herb giving slight astringency, which is desirable at low concentration and is undesirable at high concentration.

The mean score for the aftertaste/finish of metheglins with concentration of *B. Ciliata* 0, 0.25, 0.5, 0.75 and 1% are 5.13, 5.13, 5.46, 4.40 and 3.66 respectively. The score for finish increased on increasing *B. Ciliata* concentration up to 0.5% after which it decreased. This is because of the phenolic compounds extracted on the metheglin from herb. Too much phenolic compounds immensely gave bitter note to the metheglin, which was found to be undesirable.

The mean score for the overall acceptance of metheglins with concentration of *B. Ciliata* 0, 0.25, 0.5, 0.75 and 1% are 5.26, 5.26, 5.66, 4.80 and 3.73, respectively. Metheglin with concentration 0.5% was found to be best among all the samples. Increasing *B. Ciliata* concentration after 0.5% increased the bitterness in the final product, which was undesirable.

From this we can conclude that, *B. ciliata* enhance the appearance factor of the produced metheglin at concentration < 0.75% and is found optimum at concentration 0.25%-0.5%, the herb doesn't affect the odor of the produced metheglins up to ≤ 1%, both mouthfeel and finish is enhanced up to ≤ 0.5% and best product for mouthfeel among 5 samples is fount at concentration 0.5% after which bitter note increased which was detrimental for the results. So for sample 0.5%, the overall acceptance among the 5 samples had highest positive rating. So, from an organoleptic point of view, increasing the concentration of herb above 0.5% would be considered unpalatable.

Part V

Conclusions and recommendations

5.1 Conclusions

The honey musts with different proportion of *B. ciliata* were fermented with a pure culture of *Saccharomyces cerevisiae*. Based on the results and discussion, the following conclusions were drawn:

1. Increasing *B. ciliata* concentration in the honey must decrease the yeast growth and sugar consumption rate leading to slower fermentation, so using the herb after fermentation is complete might be a better suited option for faster fermentation.
2. 0.5% of the herb enhanced the appearance and mouthfeel of the final metheglins, increasing the herb above concentration give negative impact to appearance, mouthfeel and aftertaste, especially increasing bitterness in the resulting fermented honey beverage.
3. Incorporating *B. ciliata* enhances the therapeutic properties, i.e. antioxidant antimicrobial and α-amylase inhibiting activity to the metheglin and increasing its concentration in the honey must increases this activity in the produced metheglin. Since *B. ciliata* considered a medicinal herb thus, the product extracted its properties. Thus, the product can be considered as a medicinal wine.
4. Addition of *B. ciliata* at the rate of 0.5% (m/v) of the honey must significantly enhances the therapeutic properties without scarifying its chemical and sensory attributes.

5.2 Recommendations

Based on the present study, the following recommendations have been made:

1. Carry out fermentation using temperature control at different temperatures.
2. Use *B. ciliata* at concentration 0.5% m/v of must during fermentation while producing metheglin using Brassica honey.
3. Different yeast strain can be used to study the fermentation kinetics.
4. The antimicrobial activity can be studied with more food pathogens and food spoiling microbes.

5. Quantification of active components in the prepared product can be assessed.
6. Anti-inflammatory, anti-urolithic, antipyretic and anticholinesterase activity of metheglins with *B. Ciliata* can be studied. In-vivo study of antioxidant and antidiabetics effect can be studied. B-glucosidase inhibitory activity of such metheglins can be studied.
7. Locally available herbs with high medicinal values can be used for preparing metheglins and studying their effects and properties.
8. Different potent herbs locally available can be used to make metheglins having high medicinal values.
9. Effects of ageing of metheglins and effects of incorporating *B. Ciliata* during ageing of mead can be studied.

Part VI

Summary

Metheglin, a mead style prepared from incorporating herbs in the honey, must before fermentation or to mead after fermentation. *Bergenia ciliata* rhizome has high medicinal value that's recommended to treat urinary stones, painful micturition and for curing abdominal tumor. The aim of this study was to study the fermentation kinetics of honey must prepared by incorporating this herb at different concentration, to study the therapeutic properties induced or promoted by this herb and its effect on the physicochemical and organoleptic properties of the resulting metheglin.

In this study, brassica honey and *B. ciliata* (pakhanbedh) were used for preparing metheglins. The honey must with TSS of 23°Bx and pH 3.7 was prepared with adding 0.2 g/L of yeast nutrient and different must were prepared from it with different concentrations of herb (0, 0.25, 0.5, 0.75 and 1% m/v) and pasteurization was done at 65°C for 15 min.

The musts were pitched with a pure strain of *Saccharomyces cerevisiae* at 10^5 cfu/ml. Then the fermentation was carried out for 21 days studying kinetics of yeast growth, change in reducing sugar, acidity, alcohol production and change in TSS. It was found that higher concentration i.e. 0.75% and 1% of *B. ciliata* slowed down yeast growth, sugar consumption and ethanol production rate. The physicochemical analysis of metheglins, i.e. aldehyde, ester, total titratable acidity, methanol and higher alcohol varied little with the control expect for the volatile acidity which was found to be decreasing slightly and higher alcohol increasing slightly at higher concentration of herb. Increasing the *B. ciliata* concentration increased the therapeutic activity of the metheglins but decreased the organoleptic properties, mainly being inferior to mouthfeel and aftertaste because of high bitterness imparted by the herb. *B. ciliata* considered a medicinal herb, and its therapeutic properties got along in our final product (metheglin), so our product can be considered as a medicinal wine. So from this study, we can conclude *B. ciliata* at concentration 0.5% was found to be best for the organoleptic properties and also increase the therapeutic properties of produced metheglins significantly.

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Appendices

Appendix A

Specimen card of sensory evaluation by 7 point hedonic rating test

Sensory evaluation of metheglin

Name of panelist: ……. Date: ……

Name of Product: Honey wine with different proportion of *B. ciliata*

Please evaluate the given organoleptic properties of my product according to 7 point hedonic rating 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
Appearance					
Odor					
Mouthfeel					
Finish/Aftertaste					
Overall acceptance					

Quality description:

1: Faulty 2: poor 3: Below average 4: Average 5: Above average

6: very good 7: Exceptional

Comments (if any):

……

(Signature)

Appendix B

Tabulated data form analysis of metheglins

Table B.1 Mean sensory scores for different attributes

B. ciliata concentration (% m/v)	Quality attributes				
	Appearance	Odor	Mouthfeel	Finish	Overall Acceptance
0	4.13^{b}	5.33^{a}	5.20^{b}	5.13^{a}	5.27^{ab}
	(0.64)	(0.49)	(0.77)	(0.83)	(0.59)
0.25	5.67^{a}	5.13^{a}	5.13^{b}	5.13^{a}	5.27^{ab}
	(1.11)	(0.64)	(0.35)	(0.83)	(0.46)
0.50	5.53^{a}	5.80^{a}	5.93^{a}	5.47^{a}	5.67^{a}
	(0.74)	(0.56)	(0.45)	(0.52)	(0.49)
0.75	5.13^{a}	5.26^{a}	4.20^{c}	4.40^{b}	4.80^{b}
	(0.74)	(0.88)	(0.56)	(0.74)	(0.56)
1	5.00^{ab}	5.26^{a}	4.00^{c}	3.67^{c}	3.73^{c}
	(1.00)	(1.03)	(0.84)	(0.72)	(0.45)

Values in Table C.1 are the means of 15 panelists. Figures in the parenthesis are the standard deviation. Values in the column bearing similar superscript are not significantly different at 5% level of significance.

Table B.2 Change in reducing sugar (% dextrose) as during the course of fermentation

Day	*B. Ciliata* concentration (% m/v)				
	0%	0.25%	0.50%	0.75%	1%
0	20.1	20.2	20.1	20	20.1
2	17.1	17	17.3	17.7	18
4	12.3	12	12.65	13	13.1
6	10.11	9.4	10.22	10.86	12.02
10	7.2	6.8	7.8	8.9	9.9
14	5.82	5.48	5.94	6.48	6.84
21	4.5	4.4	4.7	5.08	5.8

Table B.3 Change in TSS during the course of fermentation

Day	*B. Ciliata* concentration (% m/v)				
	0% (°Bx)	0.25% (°Bx)	0.5% (°Bx)	0.75% (°Bx)	1% (°Bx)
0	23	23	23	23	23
1	20.2	20	20.8	21.2	21.4
2	16.4	15.8	17	17.6	18
3	15.2	14.8	15.2	15.2	15.8
4	13.4	13.2	13.6	14	15
5	12.2	11	12.6	13	14.2
7	11.2	9.6	11.2	12.2	13.2
9	10.4	9	10.2	11.2	12
11	9.5	8.6	9.8	10.6	11
13	8.8	8.2	9.4	9.8	10
15	8.4	7.4	9.2	9.4	9.6
17	8	7.2	8.6	9	9.2
19	7.8	7.2	8.2	8.8	9
21	7.8	7.2	8	8.6	9

Table B.4 Ethanol content during the course of fermentation

Day	*B. Ciliata* concentration (% m/v)				
	0% (% v/v)	0.25% (% v/v)	0.50% (% v/v)	0.75% (% v/v)	1% (% v/v)
2	2.12	2.13	2.21	2.18	2.22
4	4.72	5.09	4.58	4.36	4.25
6	6.76	7.11	6.67	6.54	6.12
8	7.43	7.63	6.94	6.73	6.62
12	8.07	8.23	7.88	7.79	7.68
16	8.74	9.14	8.48	8.41	8.33
21	9.44	9.61	9.21	9.12	8.98

Table B.5 Change in acidity during the course of fermentation

Day	*B. Ciliata* concentration (% m/v)				
	0% (g/L)	0.25% (g/L)	0.50% (g/L)	0.75% (g/L)	1% (g/L)
0	3.71	3.67	3.68	3.74	3.71
1	3.81	3.83	3.79	3.82	3.88
2	3.99	4.01	3.88	3.92	3.87
3	4.12	4.34	3.98	4.17	4.07
4	4.16	4.27	4.15	4.34	4.44
5	4.61	4.76	4.41	4.76	4.69
7	4.91	4.89	4.77	4.89	4.98
9	5.21	5.32	5.01	5.22	5.1
11	5.45	5.33	5.21	5.2	5.23
13	5.56	5.45	5.32	5.39	5.5
15	5.63	5.4	5.45	5.57	5.45
17	5.66	5.67	5.62	5.61	5.54
19	5.73	5.84	5.69	5.71	5.61
21	5.77	5.85	5.70	5.68	5.63

Table B.6 Yeast growth during the course of fermentation

Day	*B. Ciliata* concentration (% m/v)				
	0%	0.25%	0.50%	0.75%	1%
	Log number of yeast cell per ml				
0	5.00	5.00	5.00	5.00	5.00
1	5.77	5.79	5.73	5.73	5.74
3	6.72	6.85	6.71	6.66	6.61
5	7.78	7.92	7.73	7.70	7.67
9	7.90	7.96	7.82	7.80	7.81
13	7.95	7.99	7.93	7.83	7.82
17	7.96	8.00	7.93	7.88	7.84
21	7.98	8.04	7.94	7.91	7.84

Table B.7 Final pH and acidities of *B. Ciliata* added metheglin

B. ciliata concentration (% m/v)	Total titratable acidity (g/L as lactic acid)	Volatile acidity (g/L as acetic acid)	Fixed acidity (g/L as lactic acid)	pH
0	5.82^{a} (0.10)	0.46^{a} (0.03)	5.13^{a} (0.14)	3.63^{a} (0.05)
0.25	5.70^{a} (0.10)	0.44^{a} (0.04)	5.04^{a} (0.11)	3.56^{a} (0.05)
0.50	5.74^{a} (0.08)	0.39^{ab} (0.02)	5.15^{a} (0.08)	3.56^{a} (0.05)
0.75	5.77^{a} (0.05)	0.39^{ab} (0.02)	5.17^{a} (0.07)	3.63^{a} (0.05)
1	5.66^{a} (0.09)	0.34^{b} (0.02)	5.14^{a} (0.07)	3.60^{a} (0.10)

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 ($p>0.05$).

Table B.8 Total phenolic content of *B. ciliata* added metheglins

B. ciliata concentration (% m/v)	TPC (mg GAE/100 ml)	Antioxidant activity (as %DPPH RSA)
0	21.68^{e} (0.52)	31.87^{e} (1.43)
0.25	51.08^{d} (3.65)	49.54^{d} (2.51)
0.50	85.88^{c} (0.95)	68.19^{c} (1.64)
0.75	131.88^{b} (7.74)	81.40^{b} (5.78)
1	154.01^{a} (1.45)	90.78^{a} (1.84)

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 ($p>0.05$).

Table B.9 Inhibitory effect of *B. Ciliata* added metheglins on *S. aureus* and *E. coli*

B. ciliata concentration (% m/v)	Antimicrobial activity (zone of inhibition in mm)	
	Staphylococcus aureus	*Escherichia coli*
Ethanol 10% (v/v)	8.76^{e} (0.13)	8.66^{d} (0.21)
0	10.24^{d} (0.33)	10.62^{c} (0.30)
0.25	11.20^{c} (0.31)	12.06^{b} (0.28)
0.50	12.23^{b} (0.42)	12.83^{b} (0.33)
0.75	13.78^{a} (0.19)	14.41^{a} (0.39)
1	14.16^{a} (0.41)	14.97^{a} (0.37)

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 ($p>0.05$).

Table B.10 Inhibitory effect of *B. Ciliata* added metheglins on porcine pancreatic α-amylase

B. ciliata concentration (% m/v)	α-amylase (% inhibition)
0	3.63^{d} (2.67)
0.25	27.30^{c} (3.75)
0.50	40.46^{b} (5.06)
0.75	47.17^{b} (2.16)
1	55.26^{a} (3.31)

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 ($p>0.05$).

Appendix C

ANOVA results

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

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
Sample	4	21.8133	5.4533	7.04	<.001
Panelist	14	9.1467	0.6533	0.84	0.621
Residual	56	43.3867	0.7748		
Total	74	74.3467			

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

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

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
Sample	4	3.9467	0.9867	2.30	0.070
Panelist	14	15.2800	1.0914	2.54	0.007
Residual	56	24.0533	0.4295		
Total	74	43.2800			

Since F pr. > 0.05, there is no significant difference between the sample at 5% level of significance, so LSD testing is unnecessary.

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

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
Sample	4	37.6800	9.4200	25.96	<.001
Panelist	14	7.1467	0.5105	1.41	0.181
Residual	56	20.3200	0.3629		
Total	74	65.1467			

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

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

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
Sample	4	31.5467	7.8867	18.06	<.001
Panelist	14	13.6800	0.9771	2.24	0.017
Residual	56	24.4533	0.4367		
Total	74	69.6800			

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

Table C.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	4	33.2533	8.3133	32.45	<.001
Panelist	14	4.1867	0.2990	1.17	0.325
Residual	56	14.3467	0.2562		
Total	74	51.7867			

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

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Table C.6 One way ANOVA (no blocking) for final TSS in produced metheglins

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	6.41067	1.60267	55.91	<.001
Residual	10	0.28667	0.02867		
Total	14	6.69733			

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

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Table C.7 One way ANOVA (no blocking) for final reducing sugar in produced metheglins

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	835.911	208.978	36.74	<.001
Residual	10	56.887	5.689		
Total	14	892.798			

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

Table C.8 One way ANOVA (no blocking) for final pH

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	0.013333	0.003333	0.71	0.601
Residual	10	0.046667	0.004667		
Total	14	0.060000			

Since F pr. > 0.05, there is no significant difference between the sample at 5% level of significance, so LSD testing is unnecessary.

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Table C.9 One way ANOVA (no blocking) for total titratable acidity

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	0.044493	0.011123	1.48	0.281
Residual	10	0.075400	0.007540		
Total	14	0.119893			

Since F pr. > 0.05, there is no significant difference between the sample at 5% level of significance, so LSD testing is unnecessary.

Table C.10 One way ANOVA (no blocking) for volatile acidity

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	0.0238000	0.0059500	6.11	0.009
Residual	10	0.0097333	0.0009733		
Total	14	0.0335333			

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

Table C.11 One way ANOVA (no blocking) for fixed acidity

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	0.03364	0.00841	0.83	0.537
Residual	10	0.10160	0.01016		
Total	14	0.13524			

Since F pr. > 0.05, there is no significant difference between the sample at 5% level of significance, so LSD testing is unnecessary.

Table C.12 One way ANOVA (no blocking) for ethanol content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	1.20897	0.30224	23.97	<.001
Residual	10	0.12607	0.01261		
Total	14	1.33504			

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

Table C.13 One way ANOVA (no blocking) for methanol content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	110.90	27.72	0.78	0.566
Residual	10	357.58	35.76		
Total	14	468.48			

Since F pr. > 0.05, there is no significant difference between the sample at 5% level of significance, so LSD testing is unnecessary.

Table C.14 One way ANOVA (no blocking) for higher alcohol content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	3590.9	897.7	5.97	0.010
Residual	10	1504.4	150.4		
Total	14	5095.2			

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

Table C.15 One way ANOVA (no blocking) for ester content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	92.74	23.19	0.51	0.733
Residual	10	458.01	45.80		
Total	14	550.75			

Since F pr. > 0.05, there is no significant difference between the sample at 5% level of significance, so LSD testing is unnecessary.

Table C.16 One way ANOVA (no blocking) for aldehyde content

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	154.7	38.7	0.37	0.823
Residual	10	1039.6	104.0		
Total	14	1194.3			

Since F pr. > 0.05, there is no significant difference between the sample at 5% level of significance, so LSD testing is unnecessary.

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Table C.17 One way ANOVA (no blocking) for total phenolic content in produced metheglins

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	29224.09	7306.02	485.96	<.001
Residual	10	150.34	15.03		
Total	14	29374.43			

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

Table C.18 One way ANOVA (no blocking) for antioxidant activity in produced metheglins

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	6836.048	1709.012	694.63	<.001
Residual	10	24.603	2.460		
Total	14	6860.652			

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

Table C.19 One way ANOVA (no blocking) for antimicrobial activity on *S. aureus* by metheglins

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	5	68.2094	13.6419	134.76	<.001
Residual	12	1.2148	0.1012		
Total	17	69.4242			

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

Table C.20 One way ANOVA (no blocking) for antimicrobial activity on *E. coli* by produced metheglins

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	5	65.86018	13.17204	133.27	<.001
Residual	12	1.18607	0.09884		
Total	17	67.04625			

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

Table C.21 One way ANOVA (no blocking) for α-amylase inhibitory activity in produced metheglins

Source of variation	Degree of freedom	Sum of squares	Mean squares	Variance ratio	F Pr.
B. ciliata concentration (% m/v)	4	5132.71	1283.18	68.25	<.001
Residual	10	188.01	18.80		
Total	14	5320.72			

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

Appendix D

1. Standard curve for total phenolic determination.

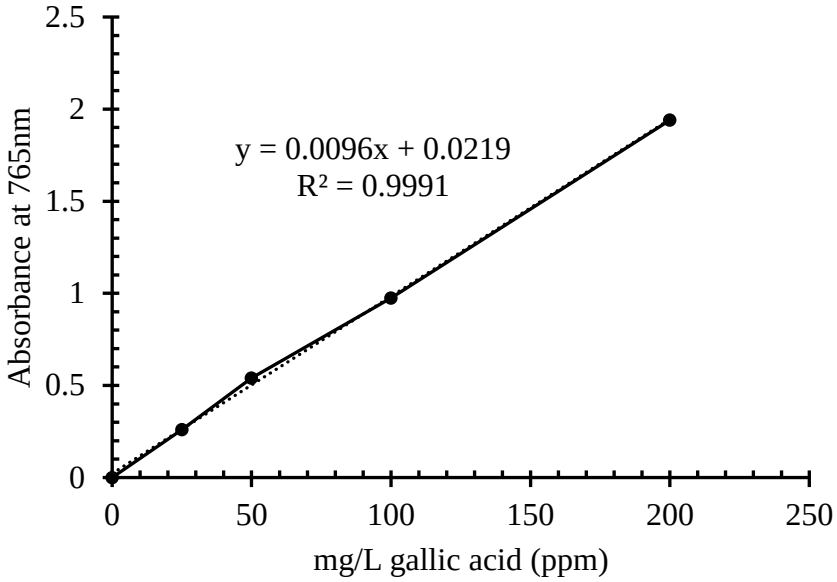


Fig. D.1 Standard curve for total phenolic content determination

2. Standard curve for methanol content determination.

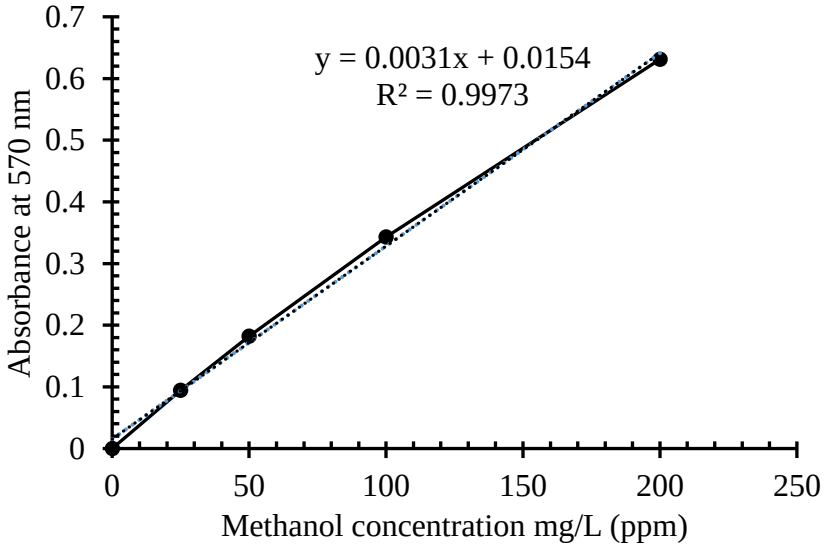


Fig. D.2 Standard curve for methanol content determination

3. Standard curve for fusel oil content determination.

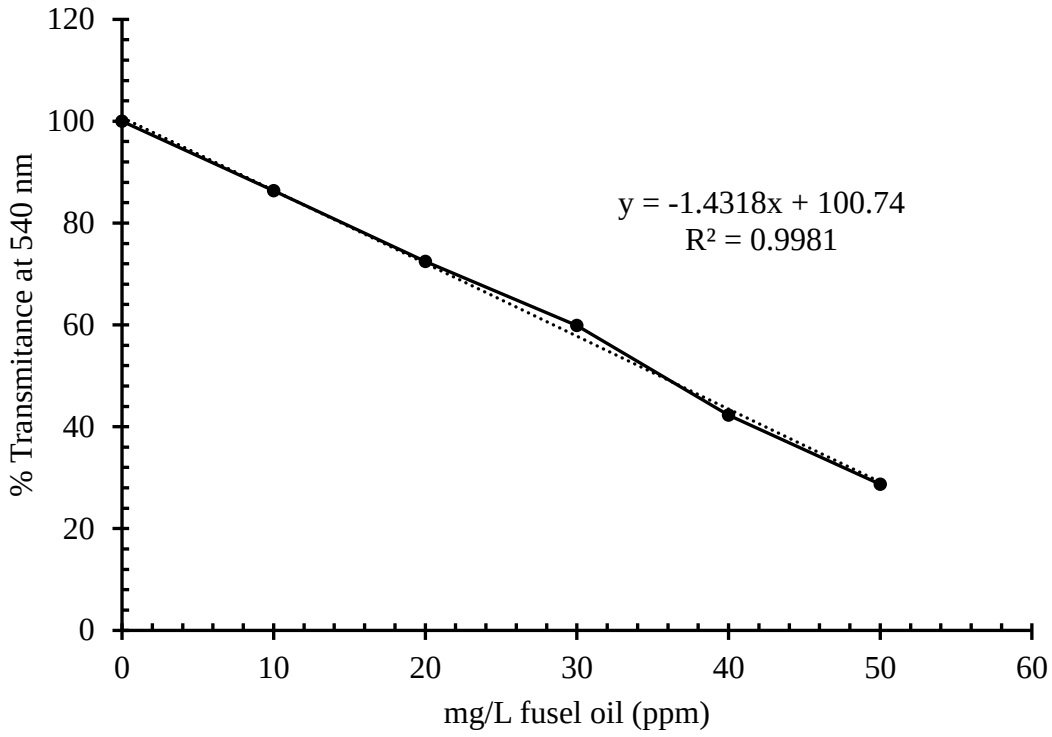


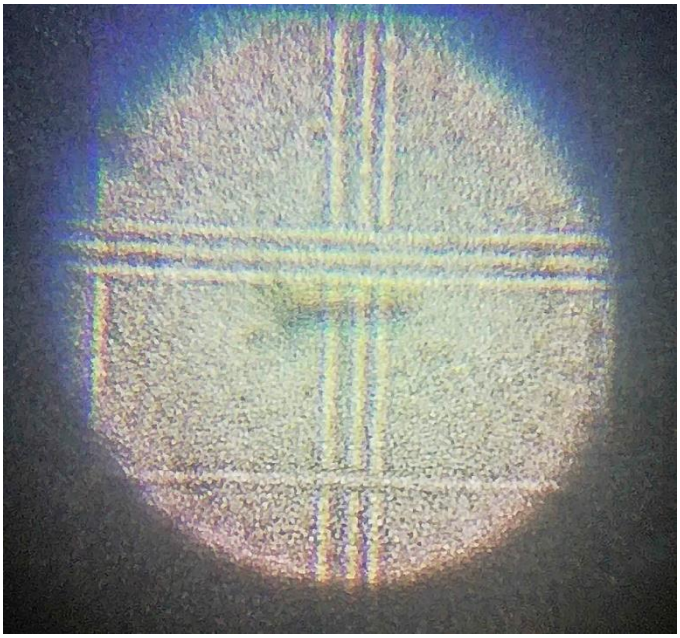
Fig. D.3 Standard curve for fusel oil content determination

Photo Gallery

Fermentation of honey must with different proportions of *B. ciliata*

Pasteurization of bottled metheglins

Setup for yeast cell count using hemocytometer



Microscopic view of hemocytometer

Distillation of metheglins to collect distillate for analysis

Top view of petri dish after 24 hrs, for a study on antimicrobial activity (zone of inhibition) of metheglins on *S. aureus*