BIOCHEMICAL AND MICROBIAL PROFILING OF YEASTS TO IDENTIFY THEIR ROLE IN WINE MAKING

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CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Biochemical and microbial profiling of yeasts to identify their role in wine making**" submitted by Ms. Pradnya S. Chavan was carried out under my supervision at the Biochemical Sciences Division, National Chemical Laboratory, Pune. Such material as has been obtained from other sources has been duly acknowledged in the thesis.

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Research Guide

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled " **Biochemical and microbial profiling of yeasts to identify their role in wine making**", submitted for the Degree of Doctor of Philosophy in Microbiology to the Savitribai Phule Pune University, is the record of work carried out by me at Biochemical Sciences Division, CSIR-National Chemical Laboratory, Pune - 411008, India, under the supervision of Dr. M. V. Deshpande. The work is original and has not been submitted in part or full by me for any other degree or diploma to any other University. I further declare that the material obtained from other sources has been duly acknowledged in the thesis.

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Abbreviations

A_{600}	Absorbance at wavelength 600 nm
BLAST	Basic local alignment search tool
bp	Base pair
CFU	Colony forming unit
DNase	Deoxyribo nuclease
dNTP	Deoxynucleotide triphosphate
EC	Enzyme commission
EDTA	Ethylene diamine tetra acetic acid
HPLC	High performance liquid chromatography
HR-LCMS	High resolution-liquid chromatography mass spectrometry
ITS	Internal transcribed region
IU	International unit
MGYP	Malt extract glucose yeast extract peptone
MIC	Minimum inhibitory concentration
ND	Not detected
NMR	Nuclear magnetic resonance
OD	Optical density
PCR	Polymerase chain reaction
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal RNA
S	Svedberg unit
SDS	Sodium dodecyl sulphate
TCA	Trichloro acetic acid
TE	Tris EDTA
U	Unit of enzyme activity
UV	Ultra violet
v/v	Volume by volume
w/v	Weight by volume
YNB	Yeast nitrogen base
ºBx	Degree brix

ABSTRACT

The biodiversity of yeasts present on berries, phylloplane and in soil is one of the important factors responsible for wine fermentation and quality. *Saccharomyces cerevisiae* and several non-*Saccharomyces* yeasts such as *Brettanomyces bruxellensis, Candida stellata, Candida valida, Hanseniaspora guillermondii, Issatchenkia orientalis, Kluveromyces marxianus, Pichia anomala*, *Pichia membranifaciens*, *Rhodotorula minuta, Torulaspora delbrueckii*, *Zygosaccharomyces* sp. have been reported to be present on the grape. The yeast flora on the surface of grape berries depends on the geographic location, climatic conditions (rainfall, temperature), soil type, berry maturity, damage due to birds, insect and mould attack, application of fungicides, insecticides etc. There are no reports on the natural flora associated with grape varieties in India. Therefore present study was initiated to systematically study yeasts diversity associated with different vine varieties grown in India and to understand the role of non-*Saccharomyces* yeasts in wine-making.

The literature survey on *Saccharomyces* and non-*Saccharomyces* yeast flora present on grapes and its relation to the quality of wine is comprised in the Introduction (**Chapter I**). The role of these yeasts in production of different enzymes i.e. β-glucosidases, β-1-3-glucanase, protease, pectinase and to release flavor and phenolic compounds have been described. A wide variety of contaminating yeast species implicated in wine spoilage and preservation strategies to control wine spoilage yeasts are reviewed in this chapter.

Materials and Methods (Chapter II) describes different sources of chemicals, equipments and protocols used during the investigation. The details of media used for the growth, maintenance and enzyme production have been given. Isolation of the yeasts present on the grapes and identification by different biochemical and molecular techniques have been described. Enzyme assays for β-glucosidases, β-1-3-glucanase, protease and pectinase and phenolics detection by HR-LCMS (high resolution mass spectrometry) are briefly described. Effect of natural preservatives on natural flora and wine spoilage yeasts was studied by antimicrobial susceptibility testing by CLSI method. Mechanism of action was studied by propidium iodide staining, ergosterol synthesis depletion. In situ use of carvacrol and thymol as wine preservative was checked in Shiraz red wine.

Yeast diversity associated with grapes from different varieties from different regions (Chapter 3, A) was studied. Total seventy eight yeasts isolates were isolated from varieties, namely, Bangalore Blue, Cabernet, Shiraz, Sauvignon Blanc and Zinfandel. The numbers of isolate from each variety were: 24, Bangalore Blue; 19, Zinfandel; 7, Cabernet; 17, Shiraz and 11, Sauvignon Blanc. The isolates were identified on the basis of morphology, cultural characteristics, biochemical tests and molecular techniques.

The isolates were belonging to eight genera and fifteen species. The isolates were 2; *C. azyma,* 3; *C. quercitrusa,* 20; *H. guilliermondii*, 9; *H. uvarum,* 5; *H. opuntiae,* 6; *I. orientalis*, 10; *I. terricola,* 2; *P. membranifaciens,* 2; *P. manshurica,* 13; *S. cerevisiae*, 2; *Z. steatolyticus* 1; *C. diversa,* 1; *T. delbrueckii,* 1; *P. kluyveri* and 1; *P. fermentans.*

To understand the role of non-*Saccharomyces* in production of enzymes of enological interest all the seventy eight yeasts were screened for the hydrolytic enzymes and results are described in **Chapter 3- Section B**. *I. terricola* I68, *H. guilliermondii* I5*, I. terricola* I57, and *H. guilliermondii* I56 were highest glucosidase (1010 nmol/mL/h), pectinase (27458 nmol/mL/h), glucanase (27279 nmol/mL/h) and protease (10810 nmole/mL/h) producers, respectively. The major contributors for enzyme production were: *C. azyma* and *H. guilliermondii*; protease (3512 and 10810 nmol/mL/h), *H. uvarum* and *I. terricola*; β-1, 3-glucanase, (14271 and 27353 nmol/mL/h); *I. terricola*; β- glucosidase (624-1010 nmol/mL/h).

Fermentation of Shiraz variety was done and microbial succession during fermentation was monitored for 15 days. *Hanseniaspora* sp., *Issatchenkia* sp., *Pichia* sp. and *Torulaspora* sp. were found to be present in the grape juice and during early days of fermentation. From five yeasts on 0 d, *Torulaspora* sp. could not be detected on 3rd d, *Pichia* sp. disappeared on 3rd d, *Issatchenkia* disappeared on 9th d whereas only *S. cerevisiae* was present in the sample from 9th d of fermentation. The count of *Hansenispora* sp. during fermentation was $1.6x10^4$ cells/mL; 0 d, $2x10^6$; 3 d and 10^7cells/mL; 6 d, whereas count of *Issatchenkia* sp. and *Pichia* sp. was $3x10^4$; 0d, $7x10^6$; 3 and $4x10^4$ cells/mL; 0 d; $5.2x10^5$ cells/mL; 3 d respectively. Inoculum of *S. cerevisiae* was $1x10^6$ cells/mL on 0 d which first increased and then decreased to $8.1x10^6$, $4x10^8$, $1.6x10^8$, $1.6x10^7$ and $8x10^5$ cells/mL on 3rd, 6th, 9th, 12th, and 15th d, respectively.

It was essential to study the enzymatic activities during winemaking. The levels of protease, pectinase and glucosidase decreased in late stage of fermentation. Pectinase levels were high (>4500 nmol/mL/h) and constant till 6th d, which then dropped by >60% from 9th d. Glucanase activity was not detected at 0 d, which then increased gradually and was maximum, i.e. 122 nmol/mL/h on 6th d. Glucanase activity was not detected in samples from 9, 12 and 15th d. Protease level was also maximum (568 nmol/mL/h) on 6th d and decreased in late stage of fermentation. Maximum level of β-glucosidase (36 nmol/mL/h) was on 0 d.

In grape juice tartaric and malic acid concentration were 13310 mg/L and 10140 mg/L, respectively, which reduced to 1620 mg/L and 2340 mg/L, respectively in test wine. Catechin (5.97 mg/L), P-coumaric acid (8.23 mg/L), and resveratrol (18.28 mg/L) were detected in test sample. Other parameters were also determined for the test wine like residual sugar (4.19 g/L), ethanol (12.8%), titrable acidity (6.34 g/L), total phenolic content (1340 mg/L), total flavonoids (462 mg/L), tannin content (1159 mg/L), glycerol (4.9 g/L), pH 3.7. All these values were comparable with marketed wine.

The antifungal activities of microbial secondary metabolites from *Pseudomonas aeruginosa* and plant essential oils, namely, carvacol and thymol was evaluated against natural yeast flora of grapes and wine spoilage yeasts and discussed in **Chapter 3, Section C - natural products for the control of wine spoilage yeasts.** Total three compounds i.e dipeptide cyclo (-Ile-homoVal), Phenazine-1-carboxamide and Phenazine-1-carboxylic acid methyl ester were extracted, purified from *Pseudomonas* sp. MCC 2142. KMS exhibited MIC in the range 64-256 µg/mL at pH 3.5. Comparatively, PC (32-128 µg/mL), and PCME (16-64 µg/mL) exhibited better activity. Essential oil components carvacrol and thymol (16-128 µg/mL) showed comparable or better antifungal activity at pH 3.5 against all the tested strains except *S. cerevisiae* (MIC 256 µg/mL). Carvacrol and thymol exhibited MIC in the range of 16-64 µg/mL and 16-32 µg/mL against spoilage yeasts at pH 3.5. The potential of carvacrol and thymol as a natural preservative for the control of spoilage yeasts was evaluated in wine. Both the compounds inhibited all the spoilage yeasts at 64 µg/mL.

The mode of antifungal action of carvacrol and thymol was investigated in *D. hansenii.* Treatment with carvacrol and thymol at 256 µg/mL resulted in >80% PI positive cells indicating membrane damage. Quantitation of sterols showed dose dependent decrease in ergosterol levels after treatment with 32 and 64 µg/mL

carvacrol or thymol. Hemolysis was not observed for carvacrol and thymol at concentrations upto 256 μg/mL. Even at higher concentrations of carvacrol and thymol (512 μg/mL) hemolysis was negligible (<5%). Thus, carvacrol, thymol and phenazine-1-carboxamide may have potential as alternative to the currently used chemical preservatives in wine industry. All the results and findings of present investigation are summarized in **Chapter IV - summary and conclusion**.

Chapter 1

Introduction and review of literature

"Nothing more excellent or valuable than wine was ever granted by the gods to man."

- Plato

as

"It is the only beverage that feeds the body, soul and spirit of man and at the same time stimulates the mind..."

- Robert Mondavi, Famous wine developer

Archaeological evidences suggest that the earliest known wine was being made in Mesopotamia around 3500 BC (Robinson, 2006). However, chemical analyses of organic residues on ancient pot sherds indicated that grape juice was deliberately being fermented in China as early as 7000 BC (McGovern et al., 2004). According to historical mural paintings and ancient pottery, the Egyptians, Phoenicians and Greeks were also quite willing winemakers and consumers. The Romans are assumed to have acquired the ability for cultivating grapes and winemaking from the Greeks and spread it into central and northern Europe. European pioneers in the 16th and 17th century introduced the grape vine into South, Middle and North America (Bassermann-Jordan, 1923).

1.1 Present scenario of wine production

1.1.1 International

In year 2014 the expected annual global wine production is ~16.7 billion litres. Italy, France, and Spain are the largest wine producing countries, representing 80 % of total output, followed by Germany, Portugal, Romania, Greece, and Austria. Currently, Italy is the world's largest wine producer with 17% of the world market share (4.49 billion litres), followed closely by Spain (4.46 billion litres), France (4.41 billion litres), Germany (0.85 billion litres), Portugal (0.67 billion litres), Romania (0.54 billion litres), Greece (0.37 billion litres), and Austria (0.22 billion litres). Wine production increased by 18.7 % from previous year (2013) as notable increases occurred in Spain (43 %), Italy (12 %), and Portugal (10 %). Significant increases were also seen in France (8.6 %), Romania (32 %), Greece (17.5 %), Hungary (9 %),

and Austria (5 %). Only Germany's production is estimated to decrease by 6 % from previous year as a result of unfavorable weather conditions during flowering (Bettini, 2014).

1.1.2 Indian

The British planted vineyards in India during year 1608 in Surat and Kashmir and since 1612, wine began to become more and more familiar throughout India (Singh and Bagul, 2005). Commercial wine grape production in India has only been in existence since 1980 (Kadam and Kadam, 2012). India's first winery was established during the 1980s and by 2000 there were just six operating wineries. However, the industry has expanded significantly over the past decade and there are now an estimated 60 wineries with up to 30 additional companies registered to start winery. These wineries are located mainly in three regions - A) Himachal region (Himachal Pradesh 30˚ 22' to 33˚ 12' N; 75˚ 47' to 79˚ 04' E) B) Bangalore region (Karnataka state 12˚ 8' N; 77˚ 37' E) C) Maharashtra region (19˚ 33' to 20˚ 53' N; 73˚ 16' to 75˚ 6' E) (Figure 1.1) (Singh and Baghul, 2005).

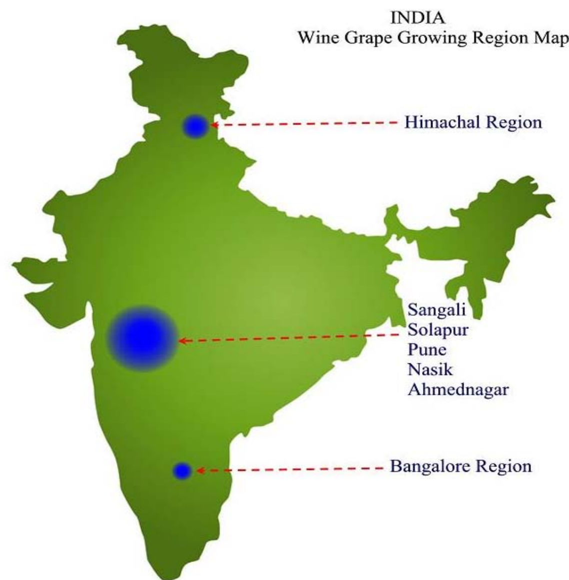


Figure 1.1 Wine grape growing regions in India. Source: ttp://indianwine.com/cs/blogs/about_wine/archive/2006/10/28/1138.aspx

Maharashtra accounts for 85% wineries with ~97% of India's total wine production. Most of these are located in the Nasik, Pune and Sangli district. Wine production in India increased rapidly from 2.3 million liters (2003) to 13.5 million

liters in 2010 and total overall sales were around 2.6 million liters per year (Williams and Mishra, 2011).

1.2 Grape varieties used for wine making

Worldwide different grape varieties are used for wine production. Based on the color wine is differentiated in to two types - red wine which is produced from grape varieties such as- Barbera, Black Rieslin, Cabernet Franc, Cabernet Sauvignon, Carignan, Cinsaut, Dornfelder, Gamay, Grenache, Malbec, Merlot, Muscat, Montepulciano, Pinot Noir, Pinotage, Portugieser, Riesling, Sangiovese, Saperavi, Shiraz, Syrah, Trollinger and Zinfandel; and white wine made from grape varieties- Aligote, Chardonnay, Chenin Blanc, Clairette, Feteasca Alba, Feteasca Regala, Prosecco, Muscat, Mueller-Thurgau, Pinot Blanc, Pinot Grigio, Sauvignon Blanc, Semillon, Silvaner Garganega, Ugni Blanc, Vermantino and Viognier. White wines are made without skin and are much lower in phenolics than red wines (Jackson, 2008a). Other regionally important and aromatically distinctive varieties are Arinto (white) and Ramisco (red) from Portugal; Corvina, Dolcetto, Negro Amaro (red), Fiano, Garganega, and Torbato (white) from Italy; Rhoditis (white) from Greece; Furmint (white) from Hungary; and Malvasia, Parellada (white), and Graciano (red) from Spain.

Red grape varieties used for wine making in India are Cabernet Sauvignon, Carignan, Grenache, Merlot, Pinot Noir, Saperavi, Shiraz, and Zinfandel; whereas, white varieties include Chardonnay, Chenin Blanc, Clairette, Garganega, Sauvignon Blanc, Ugni Blanc and Vermantino.

Wines are also classified as dry wines (up to 4 g/L residual sugar), Semi sweet wines (up to 12 g/L residual sugar) and dessert wine (wines containing more than 45 g/L residual sugar). Based on manufacturing practices, wines are termed as sparkling wine (dissolved carbon dioxide in the wine held under pressure), fortified wine (wine blended with liquor) and spicy wine (Herb-flavored fortified wine).

Grape variety used is an important factor determining wine quality as it imparts the "varietal character" to the wine, which is mainly because of the presence of different secondary metabolites responsible for the principal flavor compounds in grape must (Lambrechts and Pretorius, 2000). For instance, the varietal differences impart characteristic flavor and aroma to the wine like reminiscent of blackcurrants or cedar wood and firm tannins for Cabernet Sauvignon, herbal for Sauvignon Blanc,

spicy with pepper and wild berry flavors for Zinfandel and soft and rich wine characterized by smoky and chocolaty aromas in case of Shiraz.

1.3 Health benefits of wine

Moderate wine consumption (250-300 mL/d) has distinct health benefits (Doll et al., 2005; Thun et al., 1997). Multiple epidemiological studies suggest that daily, moderate consumption of wine is associated with a reduction in mortality from cancer and coronary heart disease as it reduces the cholesterol levels (Booyse and Parks, 2001; German and Walzem, 2000; Gronbaek et al., 2000; Renaud et al., 2004; Rimm et al., 1999). Consumption of red wine alleviates certain conditions associated with breast cancer, osteoporosis and cardiovascular diseases (Longnecker et al., 1988; Rotondo and Gaetano, 2000). Catechins in the wine reduce the risk from heart failure, cancer, hypertension, diabetes and related complications (Jackson, 2008b).

Wine has antioxidant properties (Rivero-Perez et al., 2008), which prevent the oxidation process in which “free radicals” cause damage to healthy cells. Red wine is a rich source of antioxidants such as polyphenols like resveratrol and therefore has anti-ageing properties (Pallas et al., 2010; Micallef et al., 2007). Resveratrol is also known to protect the skin from ultraviolet radiation (Aziz et al., 2005).

1.4 Wine making process

Grapes harvesting, crushing, fermentation, aging and bottling are different important processes in wine making (Figure 1.2). Sugar concentration and acidity are standard indicators of fruit maturity and harvesting time. Grapes with sugar concentration 20-24 ºBrix and acidity less than 3.3 for white variety and 3.5 for red variety are generally used for fermentation. Stems and leaves are separated from grapes and the grapes are used for crushing. In case of white wine, crushing is followed by limited maceration, pressing and extraction of juice which is used in primary fermentation. Whereas, for red wine must obtained by crushing which includes skin and seeds of red grapes along with the juice is directly fermented and macerated during fermentation to extract the phenolics, tannins, anthocyanins from skin and seeds into the must (Pretorius and HoJ, 2005). Primary fermentation is carried out by added starter culture *S. cerevisiae* and other non-*Saccharomyces* yeasts coming from the vines and often takes ~15 days. After the primary fermentation of red grapes the wine is pumped off into tanks and the skins are pressed to extract the wine. White wines are

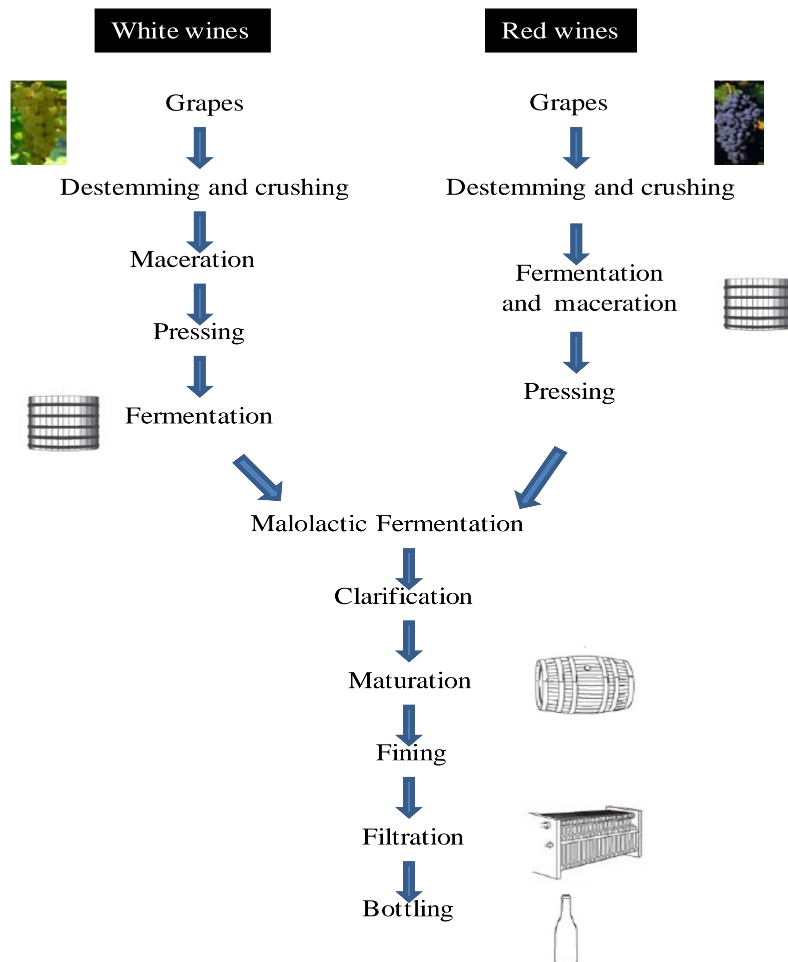


Figure 1.2 General scheme for red and white wine making.

generally fermented at 10ºC-18ºC to improve the retention of aromas; whereas red wines are fermented at higher temperatures between 18ºC-29ºC to achieve good extraction of phenolic compounds (Fugelsang and Edwards, 2007). Initial fermentation temperature of 20ºC is recommended for both wines in order to stimulate initiation of yeast growth (Jackson, 1994). For certain stylistic wines, secondary/malolactic fermentation is carried out in which lactic acid bacteria converts malic acid to lactic acid. The process decreases acidity of the wine and softens the taste. The wine is then clarified, allowed to mature/age (for certain wines), filtered and bottled.

1.5 Grape juice fermentation by yeasts

Wine making is complex biochemical process of alcoholic fermentation wherein sugars are transformed anaerobically into ethanol and carbon dioxide by the action of yeast. Besides ethanol, other compounds such as volatile fatty acids, higher alcohols, esters, carbonyl compounds, volatile phenols are produced during alcoholic

fermentation. Traditionally wine was prepared by spontaneous fermentation but presently commercially available *S. cerevisiae* is used as inoculums. Grape musts are typically inoculated with 10^6 yeast cells/mL. This cell number increases up to 10^9 cells/mL during fermentation. The fermentation rate increases during the exponential growth phase of the cells and diminishes gradually with rising alcohol level and decrease in sugar, nutrient levels. Most of the sugars are metabolized during stationary phase of growth. Measuring the development of CO_2 and/or the decrease in sugar level during fermentation allows an exact monitoring of the fermentation progress as CO_2 release (weight loss) and sugar decrease (density change of the must) are directly correlated with the production of ethanol. Fermentation is complete, when the residual sugar concentration becomes less than 2 g/L. Final ethanol concentrations can vary from 12.5% (v/v) to 14.5% (v/v).

1.6 An overview of microbes present from grapes to wine

The microbial populations (bacteria, yeasts and fungi) have different counts on grapes. Bacterial species found to be associated with grapes are *Bacillus* sp., *Burkholderia* sp., *Enterobacter* sp., *Enterococcus* sp., *Serratia* sp. and *Staphylococcus* sp., but they do not grow in wines due to high acidity and ethanol concentration (Barata et al., 2012). Lactic acid bacteria (*Lactobacillus, Leuconostoc, Oenococcus* and *Pediococcus,* count 10^2-10^4 CFU/mL in must) (Lonvaud-Funel, 1999; Osborne et al., 2005) are mainly involved in malolactic fermentation and acetic acid bacteria (*Acetobacter aceti A. orleanensis*, *A. pasteurianus*, *A. syzygii, Acidomonas*, *Asaia*, *Gluconobacter oxydans, G. cerinus G. hansenii. G. saccharivorans, G. intermedius, Granulibacter, Kozakia*, *Neoasaia, Saccharibacter, Swaminathania,* count 10^5 CFU/mL in must) are acetic acid producers during wine making (Barata et al., 2011; Gonzalez et al., 2005; Nisiotou et al., 2011).

In case of filamentous fungi, eleven species, namely, *Alternaria alternata*, *Aspergillus carbonarius*, *A. carbonarius*, *A. niger*, *A. japonicuss, Cladosporium herbarum*, *Eurotium amstelodami*, *Penicillium janthinellum*, *P. decumbens* and *Trichoderma harzianum* have been reported to be present on grapes (Valero et al., 2007). These fungi do not have the ability to grow in wines and their effect on wine quality is due to the damage of grape. *Cladosporium cladiosporioides, Erysiphe nigrum, Penicillium spinolusum, P. diplodiella, Trichoderma koningiopsis, T. cucumeris* and *F. oxysporum* were also reported on grapes (Diguta et al., 2011).

Plasmopara viticola, Erysiphe necator and *Botrytis cinerea* are main grapes spoilage organisms and cause downy mildew, powdery mildew and grey rot, respectively (Barata et al., 2012). However, one of the most important factors contributing to the quality of wine is the yeast diversity associated with the grapes and must.

1.7 Yeasts diversity

1.7.1 Non-Saccharomyces yeasts in vineyards and on grapes

Saccharomyces cerevisiae is primarily used for fermentation of grape juice but it has been found that there is low occurrence of *S. cerevisiae* among the natural yeast populations found on grape berries (Fleet, 2003). Grape berry surface provide physical environment suitable for the growth of microorganisms. Unripe grapes harbor predominantly species of genera *Rhodotorula*, *Cryptococcus* and *Candida* at concentration of 10-10^3 CFU/g. During maturation, the sugars increase from 10 mg g^{-1} to 150-300 mg g^{-1}, acids decrease from 20-30 mg g^{-1} to 5-10 mg g^{-1} (Fleet, 2001). Leaching or diffusion of the sugars to the grape skin surface causes the yeast numbers to increase to 10^4-10^6 CFU/g at maturity (Fleet, 2003). At the time of harvest, *Kloeckera apiculata/Hanseniaspora uvarum* is the most dominant species on the surface of mature grape berries, accounting for more than 50% of the total yeast flora (Jolly et al., 2006).

Other species of obligate aerobic or weakly fermentative yeasts with low alcohol tolerance are present in lesser proportions. These belong to the genera *Candida, Cryptococcus, Debaryomyces, Hansenula, Issatchenkia, Kluyveromyces, Metschnikowia, Pichia, Rhodotorula, Hanseniaspora, Saccharomyces, Torulaspora and Zygosaccharomyces* (Chavan et al., 2009; Ciani and Maccarelli, 1998; Fleet, 2003; Li et al., 2010; Loureiro and Malfeito-Ferreira, 2003). Most of these yeasts belong to ascomycetes and may exist on the grapes as sexual (ascospore producing, teleomorphic) or asexual (non-spore forming, anamorphic) or both the forms depending on the environmental conditions. Hot regions, cooler regions and moderate climate regions favor growth as teleomorphic, anamorphic and both types, respectively.

1.7.2 Non-Saccharomyces yeasts associated with fermenting must

During crushing, the non-*Saccharomyces* yeasts on the grapes, on the cellar equipment and in the cellar environment are carried to the must (Fleet, 2003). Non-*Saccharomyces* species that have been isolated from cellar surfaces include *Candida*

sp., *Cryptococcus* sp., *Pichia anomala, P. membranifaciens,* and rarely, *Debaryomyces hansenii*, *K. apiculata, Metschnikowia pulcherrima* and *Rhodotorula* sp. (Loureiro and Malfeito-Ferreira, 2003). The yeasts associated with fermenting must are divided into three groups i.e. yeasts that are largely aerobic (*C. pulcherrima, C. stellata*, *Cryptococcus albidus, Debaryomyces* sp*.*, *Pichia* sp. and *Rhodotorula* sp.), low fermentative (*K. apiculata,* and *K. javanica*) and fermentative (*Kluyveromyces marxianus, Torulaspora globosa, T. delbrueckii* and *Zygosaccharomyces* sp.) (Combina et al., 2005; Fleet et al., 1984).

The specific environmental conditions in grape must i.e. low pH, high sugar (high osmotic pressure), mixture of glucose and fructose, presence of SO_2 and temperature during cold settling are limiting factors for the yeasts growth (Pretorius, 2000). Moreover, anaerobic environment, with increasing levels of ethanol becomes toxic to the yeasts. *K. thermotolerans* and *T. delbrueckii* are less tolerant to low oxygen levels than ethanol toxicity, which leads to their death during fermentation (Hansen et al., 2001). Certain non-*Saccharomyces* yeasts disappear during the early stages of fermentation (Fleet et al., 1984; Henick-Kling, 1998) due to their slow growth and inhibition by the combined effects of SO_2, low pH, oxygen deficiency and increasing ethanol content (Combina, Elia et al., 2005; Jackson, 1994). Nitrogen levels are usually sufficient at the start of fermentation, but may become limiting towards the end of fermentation unless supplemented. The clarification of white must (centrifugation, enzyme treatments, cold settling) also reduces the initial population of yeasts (Fleet, 1990; Lonvaud-Funel, 1996; Pretorius, 2000).

The non-*Saccharomyces* yeast population undergoes constant change throughout cold maceration and alcoholic fermentation. Cold maceration seems to favour the presence of *H. osmophila*, *C. tropicalis* and *Z. bisporus* species (Hierro et al., 2006). The highly diverse non-*Saccharomyces* microflora has been reported to be present at 10^4-10^5 CFU/mL during cold maceration and the population increases to a maximum of 10^6-10^7 CFU/mL at the beginning of alcoholic fermentation, which then declines to ~10^3-10^4 CFU/mL at the end of fermentation (Zott et al., 2008). Non-*Saccharomyces* yeasts have also been observed to grow to levels upto 10^4cells/mL during malo-lactic fermentations.

1.7.3 Non-Saccharomyces yeasts associated with wine

Non-*Saccharomyces* yeasts in wine are usually associated with wines in barrels and post-fermentation spoilage (Loureiro and Malfeito-Ferreira, 2003).

Table 1.1 Diversity of grape associated yeasts in different regions

Country	Grape variety (red/white)	Associated yeast genera	References
India	Banglore Blue (red)	*Candida, Hanseniaspora, Issatchenkia, Pichia*	(Chavan et al., 2009)
	Cabernet Sauvignon (red)	*Candida, Hanseniaspora, Issatchenkia, Saccharomyces*	
	Zinfandel (red)	*Hanseniaspora, Issatchenkia, Saccharomyces, Zygoascus*	
	Shiraz (red)	*Debaryomyces, Hanseniaspora,, Saccharomyces*	
	Chenin Blanc (white)	*Hanseniaspora, Issatchenkia, Saccharomyces*	
	Sauvignon Blanc (white)	*Hanseniaspora, Issatchenkia, Pichia*	
South Africa	Chardonnay (white)	*Kluyveromyces, Debaryomyces Candida, Pichia, Kloeckera, Saccharomyces, Zygosaccharomyces, Rhodotorula,*	(Jolly et al., 2003)
China	Cabernet Sauvignon (red)	*Hanseniaspora, Cryptococcus, Pichia, Candida*	(Li et al., 2010)
	Merlot (red)	*Hanseniaspora, Cryptococcus, Pichia, Candida, Zygosaccharomyces, Issatchenkia, Metschnikowia, Pichia*	
	Chardonnay (red)	*Hanseniaspora, Candida*	
Canada	Icewine (red)	*Sporobolomyces, Cryptococcus, Rhodotorula, Hanseniaspora*	(Subden et al., 2003)
Argentina	Malbec (red)	*Pichia, Kloeckera, Saccharomyces, Zygosaccharomyces, Rhodotorula, Metschnicowia, Issatchenkia, Kluyveromyces*	(Combina et al., 2005)
France	Chardonnay (white)	*Candida, Rhodotorula, Pichia, Sporidiobolus, Cryptococcus, Hanseniaspora, Rhodosporidium*	(Renouf et al., 2005)
Brazil	Isabeal (red)	*Hanseniaspora, Saccharomyces, Issatchenkia, Sporidiobolus, Candida, Cryptococcus*	(Baffi et al., 2011)
North Spain	Folle Blanche and Hondarrabi Zuri (white)	*Candida, Cryptococcus, Kloeckera, Rhodotorula, Saccharomyces*	(Rementeria et al., 2003)
Southern Slovakia	Frankovka (red)	*Pichia, Candida, Metschnikowia, Hanseniaspora, Issatchenkia, Saccharomyces*	(Brezna et al., 2010)
	Veltlin (white)	*Saccharomyces, Metschnikowia, Hanseniaspora*	
Slovenia	Žametovka, Modra Frankinja (red)	*Cryptococcus, Debaryomyces, Hanseniaspora, Metschnikowia, Pichia, Rhodotorula, Sporobolomyces*	(Raspor et al., 2006)

	and Kraljevina (white)	*Rhodotorula, Cryptococcus, Sporobolomyces*	
Spain	Shiraz, Grenache, Barbera (red)	*Metschnikowia, Kluyveromyces, Candida, Pichia, Hanseniaspora, Torulaspora, Saccharomyces*	(Cordero-Bueso et al., 2011)
	Abarino, Godello (white) and Mencia (red)	*Candida, Pichia, Rhodotorula*	(Longo et al., 1991)
Portugal	Periquita (red)	*Metschnikowia, Kluyveromyces, Candida, Pichia, Hanseniaspora, Saccharomyces, Issatchenkia, Zygosaccharomyces, Zygoascus, Torulaspora*	(Barata et al., 2008)
Australia	Cabernet Sauvignon (red)	*Cryptococcus, Rhodotorula, Sporobolomyces, Hanseniaspora, Metschnikowia, Kluyveromyces, Torulaspora, Saccharomyces*	(Prakitchaiwattana, 2004)
Japan	Niagara (white)	*Kloeckera, Candida*	(Yanagida et al., 1992)
	Chardonnay (white)	*Cryptococcus, Rhodotorula*	
	Zenkoji (white)	*Cryptococcus, Rhodotorula, Candida*	
	Koshu (white)	*Kloeckera, Cryptococcus*	
Greece	Mavroliatis, Sefka (red)	*Aureobasidium, Candida, Hanseniaspora, Issatchenkia, Metschnikowia, Zygosaccharomyces*	(Nisiotou and Nychas, 2007)
Italy	Sangiovese (red)	*Aureobasidium, Metschnikowia*	(Guerzoni and Rosa, 1987)
	Rossiola (red)	*Candida, Kloeckera, Issatchenkia, Pichia*	
	Catarratto(white)	*Candida, Hanseniaspora, Issatchenkia, Kluyveromyces, Metschnikowia, Zygoascus, Zygosaccharomyces*	(Romancino et al., 2008)
	Muscat (white)	*Candida, Hanseniaspora, Kluyveromyces, Saccharomyces, Torulaspora*	
	Frappato (red)	*Hanseniaspora, Kluyveromyces, Metschnikowia, Zygosaccharomyces*	
	Nerodavola (red)	*Candida, Hanseniaspora, Issatchenkia, Metschnikowia, Zygoascus, Zygosaccharomyces*	

However, only a small number are able to tolerate the adverse conditions in wine. These include *Dekkera* sp., *C. krusei, C. valida, P. membranifaciens* and *Z. bailii* (Fleet et al., 1984). *Brettanomyces* sp. and *Zygosaccharomyces* sp. are ethanol tolerant like *S. cerevisiae* and may be found in bottled wine. *Dekkera bruxellensis* was often found to be associated with wineries and less commonly on grape berries (Fugelsang, 1997; Ibeas et al., 1996; Martorell et al., 2006).

1.7.4 Region specific non-Saccharomyces yeasts

The number and population of the microflora also varies with the geographical locations or regions (Barata et al., 2012). Higher numbers of yeast species have been reported with vineyards from Italy, Spain and China followed by France, India, Argentina and Portugal, whereas low species diversity was reported for vineyards from Australia, Brazil, Canada, Greece and Japan (Table 1.1).

1.8 Microbial succession during wine fermentation

The microorganisms present on the surfaces of berry are mainly non- *Saccharomyces* yeasts which dominate the early stages of alcoholic fermentation (Fleet, 1990). Yeasts with low fermentation activity, such as *Candida* sp., *Hanseniaspora* sp., *Pichia* sp., *Rhodotorula* sp. and *Kluyveromyces* sp. are dominant in grape musts and during the early stages of fermentation. Subsequently, *S. cerevisiae* proliferates, becomes dominant and completes the wine fermentation (Fleet, 2003; Fleet and Heard, 1993).

Initially species of *Candida* (*C. stellata, C. pulcherrima* and *C. sake*), *Cryptococcus, Kloeckera, Pichia* and *Rhodotorula* are found in low numbers in the fresh must (Combina, et al., 2005; Fleet, 2003; Granchi et al., 1998). Of these, *K. apiculata* is usually present in the highest numbers, followed by various *Candida* sp. Non-*Saccharomyces* yeasts die due to added SO_2 and increasing ethanol concentration during fermentation. However, some non-*Saccharomyces* yeast can survive till later stage of fermentation (up to 12 days) (Fleet, 1990; Fleet et al., 1984; Heard and Fleet, 1985). Fleet and Heard (1993) had reported the tolerance of non-*Saccharomyces* yeasts towards adverse conditions (ethanol, pH, organic acids) during fermentation. Whereas, the early death of two wine-related yeasts *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii* in mixed cultures with *S. cerevisiae* was attributed to the presence of ethanol, lack of oxygen, nutrient depletion or the presence of toxic compounds and cell-to-cell contact mechanism (Holm Hansen et al., 2001). *Saccharomyces cerevisiae* strains are also reported to secrete peptides that inhibit the

growth of some non-*Saccharomyces* (Albergaria et al., 2010; Nissen and Arneborg, 2003).

Ethanol production by *S. cerevisiae* appears to be a major factor that governs the growth and influence of non-*Saccharomyces* species during fermentation. The species of *Candida, Hanseniaspora, Issatchenkia, Kluyveromyces, Metschnikowia* and *Pichia* found in grape juice do not tolerate ethanol concentrations exceeding 5-7% (Gao et al., 2002; Heard and Fleet, 1988). However, low temperatures decrease the sensitivity of these species to ethanol and consequently, wine fermentations conducted at temperatures less than 15-20ºC may show a greater contribution of *Candida* and *Hanseniaspora* species. In such case, these species may equal to *S. cerevisiae* as the predominant species at the end of fermentation and accordingly, would have a greater impact on wine flavor (Heard and Fleet, 1988). Some strains of *Candida* sp. that has ethanol tolerances similar to *S. cerevisiae* (Cocolin et al., 2001; Mills et al., 2002). *Schizosaccharomyces pombe, Zygosaccharomyces bailii* and *Zygosaccharomyces fermentati* are well known for their tolerance of high ethanol concentrations (>10%) (Fleet, 2000; Romano and Suzzi, 1993). Strains with higher ethanol tolerance are more likely to dominate at later, rather than earlier stages of fermentation.

1.9 Factors affecting microflora from grapes to wine

The microflora of grapes is affected by a number of factors such as vineyard altitude, grape variety (cultivar, thickness of grape skin), developmental stage of grapes, health of grapes (physical damage to berries, insect pests), climatic conditions (temperature, rainfall, humidity), viticultural practices (fertilization, irrigation, canopy management, use of fungicides, use of elemental sulphur), ripening stage of grapes and winery waste-disposal practices (Bisson and Kunkee, 1991; Pretorius and Bauer, 2002; Raspor et al., 2006). The numbers of yeast cells are greater close to the peduncle than it is at the centre and lower part of the bunch (Rosini, 1984). The manner in which grapes are sampled (e.g. the berries or bunches) and processed (washing vs. crushing) also determine the yeasts diversity in must (Martini et al., 1996).

The availability of high sugar concentrations explains the higher populations on damaged grape berries. At the time of harvesting, grape temperature, method of harvesting (manual vs. mechanical), method of transport to the cellar (picking crates/baskets, tipsters), time of transport to the cellar, time lapse before crushing,

sulphite and enzyme addition can also affect yeast populations (Pretorius, 2000; Pretorius et al., 1999). The specific environmental conditions in the must (high osmotic pressure, presence of SO_2 and temperature) play a role in determining species which can survive and grow (Bisson and Kunkee, 1991).

Excessive rainfall during grape ripening, also contribute to greater numbers of non-*Saccharomyces* yeasts in the initial stages and later in the fermentation (Querol et al., 1990). *Botrytis cinerea* infection to grapes influences the non-*Saccharomyces* populations (*C. krusei* and *K. apiculata* numbers increased and *R. glutinis* decreased) (Le Roux et al., 1973). The range of non-*Saccharomyces* species isolated often depends on the place from which, and the stage in the winemaking process at which, the samples are taken. The methods of isolation and enumeration can also impact on the type of yeasts that are isolated. Methods include shaking grape berries in a broth or crushing whole berries and plating on nutrient agar media. The technique of crushing berries before plating is closer to practical winemaking protocols than shaking in a broth. The type of growth medium used can also play an important role by limiting the growth of specific yeasts (e.g. Lysine Medium does not allow the growth of *S. cerevisiae* due to the inability of this yeast to utilize lysine as the sole carbon source) (Heard and Fleet, 1986). However, some non-*Saccharomyces* yeast might also not be able to utilize lysine.

The fermentation rapidly becomes anaerobic and the alcohol level increases, which affects yeast populations. Pesticides and other chemical sprays used in the vineyard can also affect yeast populations (Guerra et al., 1999). The species of *Candida, Hanseniaspora, Issatchenkia, Kluyveromyces, Metschnikowia* and *Pichia* are not tolerant to ethanol concentrations exceeding 5-7% (Heard and Fleet, 1988).

1.10 Production of enzymes of enological interest by non-*Saccharomyces* yeasts

Grapes are the raw material for wine making, containing numerous different compounds, notably phenolics, aromatic precursors, enzymes, and structural components. The structural components include pectins, cellulose, glycans, hemicelluloses, proteins and lignin. Enzymatic degradation of these structural components can improve different stages of vinification, like enhancing the yield and clarification of the must, increasing color extraction, improving filtration of the wine and enhance the characteristics of the wine, especially the aroma (Charoenchai et al., 1997). Although *Saccharomyces cerevisiae* is responsible for the alcoholic

fermentation, the presence of non-*Saccharomyces* species could be important since they produce different enzymes and secondary metabolites, which contribute to the final taste and flavor of wines (Esteve-Zarzoso et al., 1998).

During the early stages of wine making there is substantial growth of non-*Saccharomyces* yeasts, which produce extracellular enzymes such as esterases, lipases, pectinases, proteases, β-1, 3 glucanase and β-glycosidases (Strauss et al., 2001). These enzyme activities improve the process of winemaking and enhance wine quality. Pectinases and β-glucanases increase juice extraction from grapes, improve wine clarification and facilitate wine filtration (Canal-Llauberes, 1993; Villettaz and Duboudieu, 1991). Haze formation from proteins in the finished wine may be decreased by the use of proteolytic enzymes (Waters et al., 2005). The aroma and flavor properties of wine can be enhanced by glycosidases that hydrolyse non-volatile glycosidic precursors of the grape (Pombo et al., 2011). *S. cerevisiae*, the principal wine yeast, is not recognized as a significant producer of extracellular proteases, lipases or proteolytic enzymes, although a few strains have been reported to degrade polygalacturonate (Gainvors et al., 1994; McKay, 1990) and β-glucosidase producers (Hernandez et al., 2003).

1.10.1 β-Glycosidase

Glycosidase has the potential to enhance wine flavor and aroma by releasing glycosidic bond of terpenes from sugar and these terpene compounds contribute to the varietal character of wines (Ferreira et al., 2001). Glycosidases such as β-glucosidase, β-xylosidase, β-apiosidase, α-rhamnosidase and α-arabinofuranosidase have been described as being involved in flavor releasing processes. Glycosides are present in all grape varieties, and their concentrations vary according to variety (500- 1700 g/L) (Gunata et al., 1985). Several non-*Saccharomyces* sp., e.g. *Candida, Dekkera*, *Debaryomyces*, *Hanseniaspora* and *Metschnikowia* are able to synthetize β-glucosidase. However, only *Candida* and *Debaryomyces* species produce and secrete β-glucosidase to the periplasmic space (Pogorzelski, 2007).

Glycosidase activity is frequent in the yeast species such as *Aureobasidium, Brettanomyces*, *Candida*, *Cryptococcus, Hanseniaspora*, *Hansenula, Kloeckera, Metschnikowia, Pichia, Saccharomyces, Saccharomycodes*, and *Torulaspora* whereas it is quite rare in *Saccharomyces cerevisiae* (McMahon, 1999; Palmeri and Spagna, 2007). *S. cerevisiae* strains isolated from winemaking habitats are capable of breaking down β-glucoside bonds in grape must. These strains are found to expresses activities

at low pH level and in the anaerobic conditions of winemaking (Hernandez et al., 2003).

1.10.2 Proteases

Extracellular protease activity enhances wine clarity by clearing the turbidity in wine. Yeast proteases hydrolyse the peptide linkages between amino acid units of proteins, improving the clarification process. These enzymes play a major role during the autolysis process in wines kept on yeast lees during ageing and have potential for use in protein haze reduction (Nelson and Young, 1986). Protease activity has been reported in strains of *Candida pulcherima, Kloeckera apiculata* and *pichia anomala* (Charoenchai et al., 1997; Fernandez et al., 2000). The addition of proteases from *K. apiculata* has been used successfully to degrade some of the protein in Chenin Blanc and Chardonnay wines. It has been demonstrated that proteases from *Candida olea, Candida lipolytica, Candida pulcherrima* and *K. apiculata* can produce a moderate reduction in wine turbidity (Lagace and Bisson, 1990). Dizy and Bisson (2000) demonstrated that species belonging to the genus *Kloeckera*/*Hanseniaspora* are the highest producers of proteolytic activity in the must and affect the protein profile of the finished wines.

1.10.3 β-1, 3Glucanase

The main polysaccharides responsible for turbidity, viscosity and filter stoppages are pectins, glucans, and to a lesser extent, hemicellulose (mainly xylans) (Pretorius, 2000). High molecular weight β-glucans produced by *Botrytis cinerea* may come in the must from infected grapes. Wine spoilage lactic acid bacterium *Pediococcus parvulus* produces slimes consisting of β-1, 3 glucan (Dols-Lafargue and Lonvaud-Funel, 2009) making must and wine filtration difficult or impossible. Therefore, β-1, 3 glucanases are important to improve the filterability of wine. Glucans from *Saccharomyces cerevisiae* (yeast responsible of alcoholic fermentation) are mainly β-D-1, 3 linked glucose units with β-D-1, 6-linked lateral glucose chains. Some branched β-1, 6 glucans with some β-1, 3-links are also present (Manners et al., 1973). The commercial β-glucanase preparations authorised for use in winemaking are produced by *Trichoderma* (e.g. *T. harzianum*) species. They are primarily used for clarification, filtration and maturation of wines (Canal-Llauberes, 1998). *S. cerevisiae* secretes several β-1, 3 glucanases (Canal-Llauberes, 1998). Strains of *Candida stellata, Kloeckera apiculata,* and *Pichia membranifaciens* also produce glucanase enzyme (Strauss et al., 2001). β-1, 3 glucanases from *Delftia tsuruhatensis* strain

MV01 has been reported to be useful tool to prevent slime production and undesirable yeast growth during vinification (Blattel et al., 2010).

1.10.4 Pectinase

Pectinases improve the process of winemaking by increasing juice extraction from grapes by dissolution of pectin in the must and also by clarifying wine. Pectinases are classified in two main groups, namely pectin esterases (PE) (able to de-esterify pectin by removal of methoxyl residues) and depolymerases (which readily split the main chain). The depolymerising enzymes are divided into polygalacturonases (PG), enzymes that cleave the glycosidic bonds by hydrolysis, and lyases (PL), which break the glycosidic bonds by L-elimination. Pectinases producing yeasts belonged to the genera *Candida, Pichia, Saccharomyces*, *Zygosaccharomyces* (Roelofsen, 1953) with significant activity reported in *Saccharomyces fragilis* (*Kluyveromyces fragilis*) and *Candida tropicalis.*Several *Saccharomyces* species including *S. carlsbergensis, S. chevalieri, S. cerevisiae, S. oviformis, S. pastorianus S. uvarum* and *S. vini* were reported to have polygalacturonase activity (Fernandez-Gonzalez et al., 2004; Kotomina and Pisarnitskii, 1974; Sanchez et al., 1984). Polygalacturonase activity was also found in *Candida silvae, C. norvegensis, Geotrichum candidum, Kluveromyces marxianus, Pichia guilliermondii, P. membranifaciens, Saccharomycopsis buligera, Torulopsis candida* and *Trichosporum cutaneum* (Blanco et al., 1999; Fellows and Worgan, 1984) whereas, pectin methylesterase activity was reported in *Candida, Debaryomyces* and *Pichia* sp.

1.10.5 Lipases

Lipases can degrade lipids from the grape or from autolytic reactions of yeasts, releasing free fatty acids into the wine, which may potentially affect wine quality. There is scanty information on lipase production by non-*Saccharomyces* yeasts. Strains of *C. stellata, C. pulcherrima, C. krusei, T. delbrueckii* have the potential of extracellular lipolytic activities (Charoenchai et al., 1997).

1.10.6 Esterases

Ester formation by yeast plays an important role in secondary flavors. Extracellular esterases are known to present in *S. cerevisiae* (Ubeda Iranzo et al., 1998). Yeast esterases studied include those from *Brettanomyces* (Spaepen and Verachtert, 1982), *S. cerevisiae* (Suomalainen, 1981), *Rhodotorula mucilaginosa* (Lee et al., 1987), and *Debaryomyces hansenii* (Besançon et al., 1995). Enzymes of enological interest found in different non-*Saccharomyces* wine yeasts are presented in Table 1.2.

Table 1.2 Enzymes of enological interest found in non-*Saccharomyces* wine yeasts*

Yeast	β- Glucosidase	Protease	β-1, 3 Glucanase	Pectinase	Esterase	Lipase
Brettanomyces	+	-	-	-	+	-
Candida famata	+	-	-	-	-	-
C. pulcherima	+	+	-	+	-	+
C. stellata	+	+	+	+	-	+
C. guilliermondii	+	+	-	-	-	-
C. valida	-	-	-	-	-	+
Debaryomyces hansenii	+	+	-	-	-	-
Hanseniaspora/Kloeckera	+	+	+	+	-	-
Hanseniaspora uvarum	+	+	+	+	-	+
Hansenula anomala	+	-	-	-	-	-
Issatchenkia orientalis	+	-	-	-	-	+
I. terricola	+	-	-	-	-	-
Metchnikowia pulcherima	+	+	+	+	-	+
Pichia anomala	+	+	-	+	-	+
Pichia fermentans / C. lambica	-	-	+	-	-	-
P. membranifaciens	+	+	+	+	-	-
P. kluyveri	+	-	+	+	-	-
Rhodotorula glutanis	-	-	-	+	-	+
Saccharomyces cerevisiae	+	+	-	+	+	+
Torulaspora delbrueckii	+	-	-	-	-	+
Schizosaccharomyces pombe	-	-	+	-	-	-
Zygoascus hellenicus / Candida hellenica	+	-	+	-	-	-
Zygosaccharomyces bailli	+	-	-	-	-	-

*Data compiled from - Barbagallo et al., 2004; Charoenchai et al., 1997; Esteve-Zarzoso et al., 1998; Fleet and Phaff, 1974; Gonzalez et al., 2004; Jolly et al., 2006; Lagace and Bisson, 1990; Otero et al., 2003; Rosi et al., 1987; Rosi et al., 1994; Strauss et al., 2001.

1.11 Enhancement of wine flavor by non-*Saccharomyces* yeasts

Non-*Saccharomyces* yeasts positively contribute to the analytical and sensorial composition of wine with production of hundreds of flavor active secondary metabolites (e.g. acids, alcohols, esters, phenolic compounds, aldehydes, ketones, volatile sulphur compounds) (Lambrechts and Pretorius, 2000). Various factors such as viticultural practices (Chone et al., 2006), pre-fermentation operations like skin contact (Maggu et al., 2007; Peyrot et al., 2000), fermentation conditions (Masneuf-Pomarede et al., 2006) affect the amount of aromatic precursors in grape must and in the wines.

The primary flavor of wine is derived from the grapes. However, secondary flavors are derived from ester formation by yeasts during wine fermentation (Lambrechts and Pretorius, 2000; Nykanen, 1986). *P. anomala* (*Hansenula anomala*), *K. apiculata* and *C. pulcherrima* are known to be high producer of esters (Bisson and Kunkee, 1991; Clemente-Jimenez et al., 2004). NS yeasts are dominant during pre-fermentation stages, e.g. cold maceration, early fermentation, while they represent 0.1% to 10% of the total community during fermentation (Zott et al., 2008).

Phenolic compounds contribute to the color, flavor, bitterness and astringency of wine. These compounds also contribute to the sensory and chemical qualities of wine because of their interaction with proteins, polysaccharides or other polyphenols. The main types of phenolic compounds found in wine are phenolic acids (hydroxybenzoic and hydroxycinnamic acids), stilbenes, flavones, flavonols, flavanonols, flavanols, and anthocyanins (Monagas et al., 2007). The free forms of *trans*-caffeic acid, *p*-coumaric acid, *trans*-ferulic acid and the derivatives were reported in young Riesling wine (Baranowski and Nagel, 1981; Somers et al., 1987). Apart from hydroxycinnamic and benzoic acids, flavan-3-ols and flavonols have been reported in white wines (10.38 mg/100 mL), depending on grape variety and enological factors (Neveu et al., 2010). The wine yeast strains have the ability to release or synthesize varietal volatile compounds from grape precursors (Papathanasiou et al., 2006; Ugliano et al., 2006).

In mixed culture fermentation, the concentration of 2-phenylethyl acetate was approximately 3-9 folds greater than that produced by *S. cerevisiae* pure culture. Sensory evaluation revealed a stronger fruity character in wines fermented with mixed cultures than in control wines (Viana et al., 2009). The concentration of the 2-phenylethyl acetate and isoamyl acetate in wines were increased as *H. guilliermondii*

and *H. uvarum* were grown as mixed cultures with *S. cerevisiae* respectively (Moreira et al., 2008; Rojas et al., 2003). Yeast strains in the *Hanseniaspora* genus produce high levels of phenylethyl acetate and phenyl ethanol that contribute to the complexity of wine aroma (Moreira et al., 2005; Rojas et al., 2001; Viana et al., 2008).

Different non-*Saccharomyces*/*Saccharomyces* multi starter cultures (mixed or sequential) have been investigated for improving the complexity and enhancing characteristics of wines (Ciani et al., 2010) i.e. *Issatchenkia orientalis* and *Schizosaccharomyces pombe* caused degradation of malic acid (Seo et al., 2007; Snow and Gallander, 1979), *P. fermentans* in sequential mixture with *S. cerevisiae* increased the acetaldehyde, ethyl acetate, 1-propanol, n-butanol, 1-hexanol, ethyl caprilate, 2,3-butanediol and glycerol contents in the wine both qualitatively and quantitatively (Clemente-Jimenez et al., 2005; Moreira et al., 2005) and *T. delbrueckii* with *S. cerevisiae* produced lower acetic acid content in high-sugar fermentations (Bely et al., 2008).

1.12 Enhancement of aroma

Volatile fatty acids, higher alcohols, carbonyl compounds, volatile phenolics, esters and sulfur compounds are principle aroma compounds in wine. Volatile acid content of wine lies between 500-1000 mg/L. Acetic acid concentration in wine is generally 0.2-0.7 g/L and beyond 0.7 g/L the wine quality gets affected. The short chain fatty acids, acetic acid, propanoic acid and butanoic acids are by products of wine fermentation. Studies have shown the potential of acetic acid production by different non-*Saccharomyces* yeasts including *C. krusei* (1 g/L), *C. stellata* (1-1.3 g/L), *H. anomala* (1-2 g/L), *K. apiculata* (1-2.5 g/L), *Metchnikkowia pulcherima* (0.1-0.15 g/L) and *S. cerevisiae* (0.3-1.2 g/L). Apiculate yeasts are also known as high producers of acetic acid (Ciani and Picciotti, 1995). However, large strain variability exists and all strains of *Kloeckera* sp. do not produce high levels of acetic acid (Romano et al., 1992). Some strains of *Kloeckera* sp. produce less than 1 g/L and are comparable to *S. cerevisiae*.

Higher alcohols are the largest group of aroma compounds in alcoholic beverages and are secondary products of alcoholic fermentation. They can be recognized by their strong, pungent smell and have significant influence on taste of wine. Concentration of higher alcohols upto 300 mg/L contribute to the aromatic properties whereas the quality gets affected when the concentration exceeds 400 mg/L

(Rapp and Mandery, 1986). Romano et al. (2003) reported that apiculate yeasts were low producers of higher alcohols, when compared to *S. cerevisiae*. Pure cultures of *H. uvarum* and *H. guilliermondii* contribute to wines with a total content in aliphatic higher alcohols of 143 mg/L and 257 mg/L, respectively. These values are significantly lower than those obtained in wines produced by a pure culture of *S. cerevisiae* (574 mg/L); furthermore, growth of apiculate yeasts with *S. cerevisiae* decreased the total content of higher alcohols in wines (Moreira et al., 2008)

2- Phenylethanol plays a positive role in the aroma of wines, bringing fruity and flowery flavors (Rapp and Mandery, 1986). Wines produced by *S. cerevisiae* with mixed cultures of apiculate yeasts produced between 43.1 and 58.1 mg/L of 2-phenylethanol, whereas lower concentration of 2-phenylethanol (32.5-34.5 mg/L) was observed in wines produced by pure cultures of apiculate yeasts. Similarly, highest level of 2-phenylethanol (82.1 mg/L) was observed in wines produced by *S. cerevisiae*, whereas 14.7 mg/L was present in wine from *H. guilliermondii*; however, a concentration of 29.2 mg/L was obtained in wines with mixed cultures of *S. cerevisiae* and *H. guilliermondii* (Rojas et al., 2003).

Esters are group of volatile compounds that impart pleasant smell. Most esters are produced by yeasts as secondary product of sugar metabolism and constitute one of the largest and most important groups of compounds affecting flavor. Esters of alcoholic beverages are of three types i.e. ethyl esters, isobutyl esters and isoamyl esters. Nykanen et al. (1986) showed that *H. anomala* and *C. krusei* yeasts produce more ethyl acetate than *P. membranifaciens, S. cerevisiae* and *S. pombe* (Nykanen, 1986). Apiculate yeasts elevated amount of residual sugars and esters similar to those wines inoculated with *S. cerevisiae* (Gil et al., 1996).

Volatile aldehydes are also important to the flavor of beverages. Acetaldehyde is a normal product of alcoholic fermentation and its amount can vary from 10 mg/L to 300 mg/L. *S. cerevisiae* produce relatively high levels from 50-120 mg/L, where as other non-*Saccharomyces* such as *C. krusei*, *C. stellata, K. apiculata* and *M. pulcherima* produce low levels of acetaldehyde (Fleet and Heard, 1993). Wine contains 0.2-0.3 mg/L of diacetyl produced by yeast metabolism. This compound with buttery aroma becomes objectionable at 1-4 mg/L. These levels are due to the growth of lactic acid bacteria (Sponholz, 1993). Some yeasts are able to utilize benzaldehyde and transform in to the benzyl alcohol and benzoic acid (Nykanen, 1986). *Schizosacchromyces* and *Zygosaccharomyces* were strong producers of benzaldehyde

(1200 mg/L) and benzyl alcohol (523 mg/L) (Delfini and Cervetti, 1991). *Zygosacchromyces* also produced notable amount of benzoic acid (536 mg/L) followed by *Cryptococcus, Kloeckera, Torulaspora* and *Saccharomyces*.

The volatile phenols are a group of compounds with a strong influence on wine flavor. Main molecules in the red wines are 4-ethylphenol and 4-ethylguaiacol, whereas 4-vinylphenol and 4-vinylguaiacol are present the white wines (Chatonnet et al., 1992). High amounts of 4-ethylphenol in the red wines causes unpleasant taint described as phenolic odour, leather, horse sweat, stable or varnish taint (Chatonnet et al., 1992). Deterioration of wine caused by the formation of volatile ethylphenols (metabolic products of *Brettanomyces* and *Dekkera* species) is a serious problem with respect to high quality wines (Suarez et al., 2007).

D. bruxellensis shows hydroxycinnamate decarboxylase and vinyl reductase activity under oenological conditions. Hydroxycinnamate decarboxylase converts hydroxycinnamic acids (ferulic, p-coumaric or caffeic acid) into hydroxystyrenes (vinylphenols) (Edlin et al., 1998) which is then reduced to 4-ethylphenol by vinylphenol reductase (Dias et al., 2003b). The sensorial threshold of this compound is 620 µg/L (Chatonnet et al., 1992). The enzyme that facilitates decarboxylation is present in a large number of bacteria, fungi and yeasts but the reduction step is only performed by the species *Dekkera bruxellensis, Candida versatilis, Candida halophil, Candida mannitofaciens, Dekkera anomala* and *Pichia guillermondii* (Chatonnet et al., 1995; Chatonnet et al., 1997). Yeasts present in wine such as *Pichia* sp., *S. cerevisiae, Torulaspora* sp. and *Zygosaccharomyces* sp. can produce 4-vinylphenol but do not reduce it to 4-ethylphenol (Dias et al., 2003a).

1.13 Spoilage of wine

The concept of spoilage yeast has a more complex meaning, where yeast able to change food sensorial characteristics can be regarded as a ‘‘spoilage yeast.’’ Yeast activity is essential during the fermenting process therefore detrimental and beneficial activity must be distinguished. It is very difficult to draw a line between beneficial fermenting activity and spoilage activity in the wine industry where alcoholic fermentation occurs in the presence of many yeast species and bacteria (mainly lactic and acetic bacteria). Bacterial wine spoilage imparts mousy taint, bitterness, geranium notes, volatile acidity, oily and slimy-texture, and overt buttery characters to the wine (Bartowsky and Henschke, 2008). whereas, the common spoilage effects due to yeasts

are off odours, off-tastes, film formation, cloudiness or haziness, sediments and gas production in bottled wines (Loureiro and Malfeito-Ferreira, 2003; Toit and Pretorius, 2000).

The yeasts involved in wine spoilage mainly are from genera *Brettanomyces, Candida, Debaryomyces, Hanseniaspora, Hansenula*, *Kluveromyces, Metschnikowia, Pichia, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* (Loureiro and Malfeito-Ferreira, 2003) whereas, lactic acid bacteria and acetic acid bacteria also contribute to wine spoilage. *Lactobacillus* and *Pediococcus* induce spoilage due to frequent predominance in this condition. Lactic acid bacteria can spoil wine during winemaking, maturation and aging (Lonvaud-Funel, 1999). Acetic acid bacteria (AAB) from genera *Acetobacter, gluconobacter* and lactic acid bacteria (LAB) from *Leuconostoc, Lactobacilli* and *Pediococcus* genera are major contributors to wine spoilage (Bartowsky and Henschke, 2008).

Spoilage yeasts are rarely found during wine fermentation and more often during storage/aging and the bottling process. However, many detrimental effects of yeasts occur before fermentation e.g., ethyl acetate produced by *Pichia anomala* (Plata et al., 2003) or the early stage of fermentation e.g., acetate production by *Kloeckera apiculata/Hansaniaspora uvarum* (Romano et al., 1992). Sponholz (1993) highlighted the problems due to ester production and film formation by species of *Brettanomyces, Hansenula, Candida*, *Pichia* and *Zygosaccharomyces*. The spoilage abilities of *Brettanomyces* and *Z. bailii* sp. have been reported (Fugelsang, 1997). Although *Schizosaccharomyces pombe* and *Saccharomycodes ludwigii* are dangerous spoilers, they are not regarded as common contaminants (Fugelsang, 1997; Kunkee and Bisson, 1993). Few yeasts such as *Brettanomyces/Dekkera bruxellensis, Dekkera anomalus* affect the wine quality due to production of volatile phenols such as 4-ethyl phenol that gives off-flavor at levels > 620 μg/L (Chatonnet et al., 1992).

1.14 Wine preservation

1.14.1 Chemical preservatives

Use of preservatives after fermentation stabilizes the wine and protects it from any microbial contaminations that would cause spoilage.

Sulfur dioxide is one of the most common preservative having antioxidant properties and stabilizes the wine; it has some effects on the overall taste of the wine. The concentration of SO_2 required to conrol yeasts such as *Pichia, Saccharomyces,*

Schizosaccharomyces and *Zygosaccharomyces* is 2 mg/L. (Ough and Were, 2005). Many wine makers attempt to maintain 0.4 to 0.6 mg/L concentration after fermentation to control *Brettanomyces.* The reported MIC of Potassium metabisulphite for lactic acid bacteria, acetic acid bacteria and yeast were 400 µg/mL, 50 µg/mL and 200 µg/mL, respectivtly (Rojo-Bezares et al., 2007).

Vitaman K_5 along with sulfur dioxide showed distinct advantages over the vitamin used alone. Apart from its own preserving effects, sulfur dioxide stabilized vitamin K_5, thus increasing its effectiveness (Yang and Orser, 1962). Sulfur dioxide has adverse effects if used in high concentration during the wine making process. Sulfite treatment produced an assortment of significant sensory differences in the finished wines, but the additions of SO_2 to the must had no significant effect on indigenous yeast populations or on flavor (Henick-Kling, 1998).

Dimethyl dicarbonate (Velcorin^{TM}) is effective against *Saccharomyces, Brettanomyces* and *Schizosaccharomyces* at 25 mg/L concentration (Ough, 1983). DMDC was found to be more effective than the combination of SO_2 and sorbic acid (Threlfall and Morris, 2002). Renouf et al. (2008) studied effectiveness of DMDC to prevent *Brettanomyces bruxellensis* growth in wine. MIC of DMDC for yeasts species *D. bruxellensis, S. cerevisiae, S. pombe* and *P. guilliermondii* was 100 mg/L. The most sensitive strains were *Zygosaccharomyces bailii, Zygoascus hellenicus* and *Lachancea thermotolerans* with MIC of 25 mg/L (Costa et al., 2008).

Sorbic acid concentration used to control yeast species is between 100-200 mg/L. Inhibition of yeast growth was found to be at relatively low levels of sorbic acid 80 mg/L (Ough and Ingraham, 1960). However, bacteria are not affected by sorbic acid (Neves et al., 1994; Ough and Ingraham, 1960).

Fumaric acid inhibits growth of LAB. It is useful to reduce initial bacterial flora such as *Lactobacillus* but its application is limited due to fumarase activity of *Lactobacilli, Oenococci* and *Pediococci* (Ough and Kunkee, 1974).

Brettanomyces and *Hansenula* are controlled at 90 mg/L of concentration of carbon monoxide, while *Kloeckera* requires 250 mg/L and *Zygosaccharomyces bailli* at 400 mg/L. *Saccharomyces cerevisiae* is resistant up to 1000 mg/L. There has been reluctance to consider carbon monoxide for microbiological control because of the inherent toxicity as well as the public perception to use toxic compounds in wine.

1.14.2 Microbial metabolites

Many microbial metabolites were investigated as preservative in wine. Lysozyme, a low molecular weight protein (14500 Da), brings lysis of cell of *Oenococcus, Lactobacillus* and *Pediococcus* and can be used to inhibit LAB and production of volatile acids (Gao et al., 2002). Lysozyme does not exhibit activity against yeasts and molds. Lysozyme is also affected by presence of phenolics therefore it is less active in red wine (Daeschel et al., 2002).

Nisin is a bacteriocin produced by *Lactobacillus lactis*. Like lysozyme it is a inhibitor of gram positive bacteria (Radler, 1990). Most of LAB inhibited at low concentration of nisin, whereas, yeasts and sensory quality of wine are not affected. Nisin MIC values for *Oenococci, Lactobacilli, Pediococci* and *Leuconostoc* were from 0.024 to 12.5 µg/mL, whereas, MIC for acetic acid bacteria and yeasts were 200 and ≥400 µg/mL, respectively (Rojo-Bezares et al., 2007).

Natamycin is used in South Africa to control the wine spoilage yeasts and mold. Addition of 40 µg/mL concentrations was found effetctive in controlling *Brettanomyces* (Thomas et al., 2005).

Commercial *β*-glucanase preparation had been evaluated against wine spoilage yeasts such as *Cryptococcus albidus*, *Dekkera bruxellensis*, *Pichia membranifaciens*, *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, and *Zygosaccharomyces bisporus* (Enrique et al., 2010).

Killer Proteins, also called as mycocins/zymocins/killer toxin, are produced by >90 yeasts (Golubev, 1998) including species of the genera *Debaryomyces, Hanseniaspora, Cryptococcus, Kluyveromyces, Rhodotorula, Pichia, Saccharomyces,* and *Williopsis* (Buzzini et al., 2004; Ciani and Fatichenti, 2001; Comitini et al., 2004a, 2004b; Izgu et al., 2006). All mycocins are either proteins or glycoproteins. Most yeasts secrete mycocins with a molecular mass of about 10-30 kDa, whereas mycocins of *Kluyveromyces lactis*, *Picha acaciae*, *P. anomala* and *P. inositovora* are much higher, about 100 kDa or greater (Sawant et al., 1989; Stark et al., 1990).

Novel killer toxin, labelled as KT4561, secreted by *Williopsis saturnus* DBVPG 4561, was found to possess a wide antimycotic activity against 310 yeast strains belonging to 21 food spoilage species of 14 genera. Minimum inhibitory concentration (MIC) determinations showed that over 65% of the target strains were susceptible to concentrations ≤32 µg/mL of KT4561 (Goretti et al., 2009). Among the species with killer character, *Pichia anomala*, NCYC 432 was extensively studied for

various potential applications. Killer strains are considered useful in biological control of spoilage yeasts in the preservation of food and beverages (Palpacelli et al., 1991).

1.14.3 Plant extracts

Plant essential oils as an alternative to synthetic fungicides for the preservation, storage and for maintaining postharvest quality of different table grapes have been suggested by Abdolahi et al. (2010, 2012). Carvacrol and thymol are two main constituents of these essential oils. Production of carvacrol vapour atmosphere inside packages have been shown to control growth of *Botrytis cinerea* inoculated in grape berries and thereby preventing fruit decay (Martinez-Romero et al., 2007). Based on the in vitro growth inhibition of different foodborne pathogens by antimicrobial wine formulations and marinades containing carvacrol and thymol, their use as antimicrobial marinades for meat, poultry products, salad dressings, and rinses and sprays for contaminated surfaces of fruits, vegetables, meat, poultry has been proposed (Friedman et al., 2006; Friedman et al., 2007). However, the use of carvacrol and thymol for control of wine spoilage has not been explored.

1.15 Objectives of the present investigation

Literature review presented here indicates that biodiversity of yeasts associated with grapes has been studied in different regions in various countries. Most of the species associated with the wine environment are similar whereas, some species are specifically associated with specific regions. There is need to be acquainted with the yeast diversity associated with the different wine grape varieties commonly used in different locations in India. The potential of associated non-*Saccharomyces* yeasts with respect to production of different enzymes of enological interest needs to be understood for the benefit of good quality Indian wine production. The profiling of non-*Saccharomyces* yeast flora and enzyme during fermentation becomes important, and will be helpful to the wine industry to control the fermentation with respect to quality and spoilage. Secondly, due to drawbacks and growing consumer bias against chemical preservatives, there is a need to identify natural antimicrobial compounds obtained from plants, animals and microorganisms as wine preservatives.

Based on the background, the objectives of the present investigation were decided as-

1) Isolation and identification of yeast flora of grapes from different varieties from different regions and its qualitative and quantitative changes during fermentation.

2) Profiling of enzymes, flavor and phenolic compounds produced by yeasts and its correlation with wine quality.
3) Effect of known preservatives, plant and microbial metabolites on the wine quality.

Chapter 2

Materials and methods

2.1 Chemicals

The chemicals used in the present study and their sources are listed in Table 2.1.

Table 2.1 List of chemicals and source

Name	Source
Ferrous sulphate	BDH, India
Haemoglobin	MP Biomedicals, USA
Glass beads	BioSpec Products, USA
PCR primers	IDT, USA
PCR purification kit	Quiagen, India
DNA ladder mix	Promega, UK
Peptone (Bacto)	Difco Industries, USA
RNAase	Promega, UK
Sodium citrate	Sarabhai Chemicals, India
Tetracyclin	Aventis, India
Trichloro acetic acid	Qualigenes, India
Bromothymol blue, Citric acid, Glycin, $MnSO_4.4H_2O$, Sodium acetate, Sodium carbonate, Sodium phosphate dibasic	Loba Chemie, India
Ethanol, Acetic acid, Methanol	Merck, India
Phenol, Chloroform	Fisher Scientific Co., UK
dNTP, HF PCR buffer, Phusion HF DNA polymerase	Finnzyme, Finland
Acetonitrile, Cupric sulphate, di-Potassium hydrogen orthophosphate, Magnesium sulphate, Manganous sulphate, Mannitol, Potassium dihydrogen orthophosphate, Potassium sodium tartarate	S D fine-chemicals, India
Beef extract, Cellobiose, 3, 5-dinitrosalicylic acid (DNS), Galactose, Gallic acid, Lactose,	Himedia, India

Malt extract, Maltose, Peptone, Raffinose, Salicin, Sucrose, Yeast extract	
Glycerol	Ranbaxy Fine Chemicals Limited, India
EDTA, Folin-Ciocalteau reagent, Isoamyl alcohol, para-nitrophenyl-β-D-glucanopyroside, Sodium hydroxide, Tween 80	Sisco Research Laboratories, India
Agarose, Bovine serum albumin, Caffeic acid, Carvacrol, Coumaric acid, D-galacturonic acid, Ethidium bromide,Glass beads, Laminarin, Polygalacturonic acid, Propidium iodide, Quercetin, Syringic acid, Thymol, trans-resveratrol, Triton X-100, Tyrosin, Yeast Nitrogen Base, 2'-7'-dichlorodihydrofluorescein diacetate (DCFH-DA)	Sigma–Aldrich, Germany

2.2 Buffers and solutions

The buffers and solutions used in present study are listed in Table 2.2. All percentages are weight by volume (w/v) unless otherwise stated.

Table 2.2 Composition of buffers and solutions

Name	Composition
Breaking buffer	2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8
TE buffer	10 mM Tris, 1 mM EDTA, pH 7.6
TAE buffer	40 mM Tris-acetate, 1 mM EDTA, pH 8.0
Citrate phosphate buffer	0.1 M citric acid, 0.1 M sodium citrate, pH 3.2
Acetate buffer	0.2 M acetic acid, 0.2 M sodium acetate, pH 4.5 and pH 4.0
Phosphate Buffer Solution (PBS)	0.8% NaCl, 0.02% KCl, 0.144% Na_2HPO_4, 0.024% KH_2PO_4, pH 7

2.3 Collection of grapes

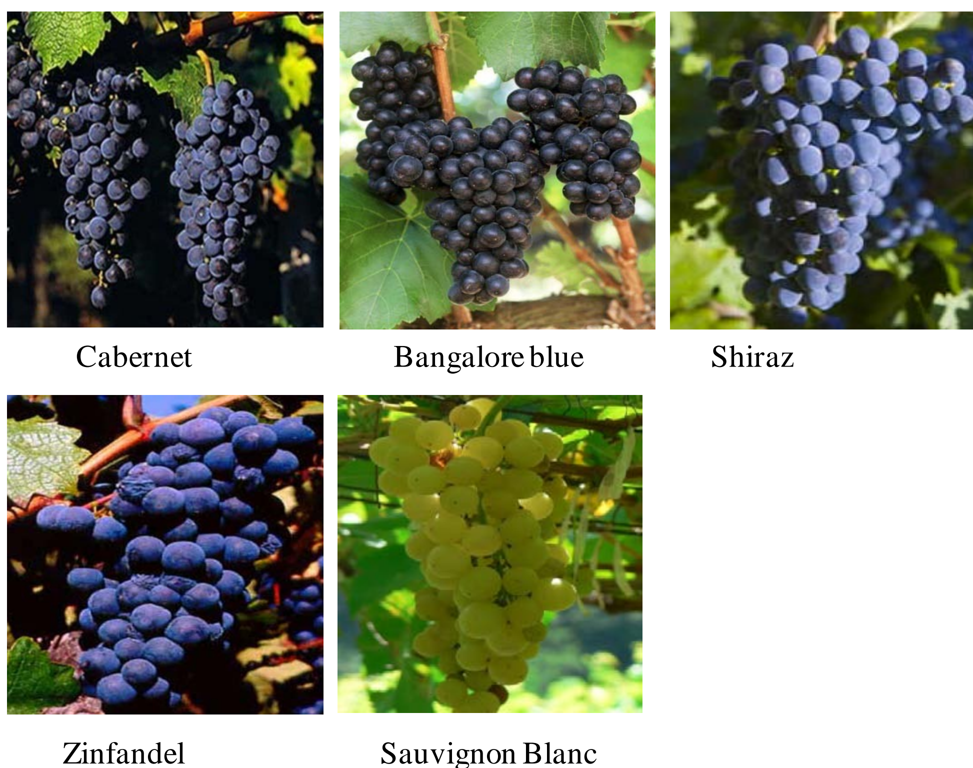


Figure 2.1 Five different grape varieties used for isolation of yeasts

Wine grapes of five varieties, namely, Cabernet, Bangalore Blue, Shiraz, Zinfandel and Sauvignon Blanc were collected from different vine yards located in Pune and Nashik regions of Maharashtra, India (Figure 2.1). Bangalore Blue, Cabernet, Shiraz and Zinfandel were collected from Pune district (18° 31´ N, 73° 55´ E) whereas, Cabernet, Sauvignon Blanc and Shiraz were collected from Nashik district (19° 99´ N, 73° 78´ E). For each variety, 15 kg healthy grapes with 20-22° Brix maturity level were collected.

2.4 Isolation of the natural yeast flora

Grapes (1.5 kg) from each variety were separately mixed and crushed. The grape juice was serially diluted and 0.1 mL from each dilution was spread on malt extract-glucose-yeast extract-peptone (MGYP; composition in g/L: malt extract, 3; glucose, 10; yeast extract, 3; peptone, 5, agar, 20; pH 6.5) agar plates containing 0.025% tetracycline for isolation of the yeasts. The plates were incubated at 28ºC for 48 h. Individual isolated colonies with different morphologies were picked and streaked on MGYP plates to obtain pure cultures.

2.5 Maintenance of yeasts

The pure cultures obtained were maintained on MGYP agar slats at 4ºC till further use. Sub-culturing of the isolates was done every 30 days. Spoilage yeasts - *Dekkera bruxellensis* NCIM 3534, *Debaromyces hansenii* NCIM 3146, *Metchnikowia pulcherima* NCIM 3109, *Schizosaccharomyces pombe* NCIM 3457, *Torulaspora delbrueckii* NCIM 3295, *Zygosaccharomyces rouxii* NCIM 3460 were procured from National Collection of Industrial Microorganisms (NCIM), Pune, India and maintained on MGYP agar slants at 4ºC.

2.6 Identification of the yeasts based on biochemical characteristics

2.6.1 Colony characteristics and morphology

Identification of the yeast isolates was done based on colony characteristics, morphology and carrying out different biochemical tests as described by Kurtzman and Fell (Kurtzman and Fell, 1998). The cultures were streaked on MGYP agar plates and colony characteristics, namely, size, shape, color, margin, consistency, opacity, elevation and appearance were recorded. The morphology of the yeast cells were observed by wet mount under light microscope (40 x).

2.6.2 Biochemical tests

In biochemical tests, fermentation and assimilation of different sugars as sole carbon source were studied (Kurtzman and Fell, 1998).

2.6.2.1 Fermentation of sugars

For sugar fermentation tests, sterile basal medium containing 4.5 g/L yeast extract, 7.5 g/L peptone and bromothymol blue indicator was dispensed in test tubes. Filter sterilized sugars, namely, glucose, galactose, sucrose, maltose, lactose, raffinose were added separately to these tubes to get 2% final concentration. Inverted Durham's tubes were put to collect the gas formed during fermentation. The tubes were inoculated with the cultures and incubated at 28ºC. After 48 h incubation, the tubes were observed for acid (bromothymol blue indicator color changes from dark green to yellow) and gas production (accumulated in Durham's tube). Depending on the amount of gas collected and time taken, the results were scored as positive (+), weak positive (w) and negative (-).

2.6.2.2 Assimilation of sugars

Assimilation tests for sugars (0.5%) such as glucose, galactose, sucrose, maltose, lactose, cellobiose and salicin were carried out in 0.6% yeast nitrogen base (YNB, 5

mL) as basal liquid medium. After inoculation of the cells ($1x10^6$ CFU/mL), the tubes were incubated at 28ºC for 48 h in a rotary shaker incubator (180 rpm). Tubes with glucose and without any carbon source were considered as positive and negative control. After incubation, growth in the liquid medium was observed visually, compared with the controls and recorded as positive (+)/negative (-).

2.6.3 Cluster analysis

For identification of the different yeast isolates based on the results of biochemical tests, cluster analysis using SPSS 11 software program was carried out. The biochemical and physiological characters scored as positive (+), weak (w) and negative (-) were given an ordinal scale of 1, 2 and 3 for all the 78 isolates. This data for isolates from Pune and Nashik regions along with comparable standard strains from Centraalbureau voor Schimmelcultures (CBS) fungal biodiversity center and Kurtzman and Robnett was used for the cluster analysis (Kurtzman and Robnett, 1998).

2.7 Identification of the yeasts by molecular techniques

For isolates which remained unidentified or identified till genus level (based on biochemical tests) as well as confirmation of identified cultures, further molecular identification was carried out by amplification and sequencing of ITS1-5.8S-ITS2 and 26S rDNA region.

2.7.1 DNA isolation

For DNA extraction, procedure described by Cocolin et al. (2000) was used with some modification. Briefly, yeast cells overnight grown in MGYP broth (5 mL) were harvested by centrifugation at 16,000 g, 4ºC for 10 min. The cell pellet (~100 µL) resuspended in a solution containing 200 µL breaking buffer, 200 µL of phenol/chloroform/isoamyl alcohol (50:48:2) and 0.3 g of glass beads (0.5 mm diameter) was homogenized by vortexing. After homogenization, 200 µL TE buffer was added and the mixture was centrifuged at 16,000 g, 4ºC for 10 min. Aqueous phase was collected and 2.5 volumes of 100% ethanol was added to it. The precipitated DNA was separated by centrifugation at 16,000 g, 4ºC for 10 min. The pellet was washed with 70% ethanol, dried and resuspended in 50 µL sterile distilled water containing 2 IU RNase. The sample was incubated at 37ºC for 30 min. Purity and quantity of the DNA in the samples was determined using the A_{260}/A_{280} ratio

measured on Nanodrop 1000 UV-visible spectrometer. A_{260}/A_{280} ratio ≥ 1.8 was considered as pure DNA.

2.7.2 Amplification of ITS region

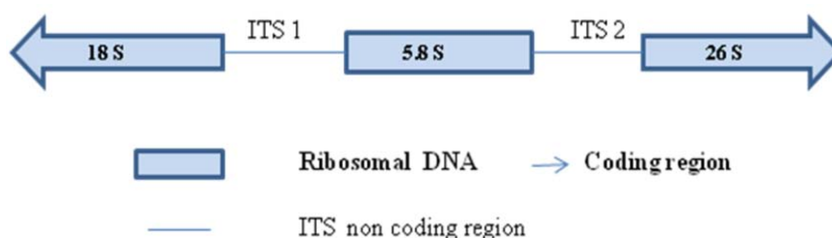


Figure 2.2 ITS region of yeast ribosomal DNA

ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') forward and ITS4 (5'-TCCTCC GCTTTATTGATATGC-3') reverse primer were used for amplification of the ITS1, 5.8S and ITS2 regions of rDNA (Figure 2.2) (White et al., 1990). Polymerase chain reaction (PCR) was performed in a final volume of 20 µL containing 1X water, 1X HF PCR buffer, 1 µL dNTP (0.2 mM), 1 µL each of the reverse and forward primers (0.5 mM), 0.4 U Phusion HF DNA polymerase and 1 µL of the extracted DNA (~15 ng). The PCR programme conditions were: initial denaturation at 98ºC for 30 sec, 32 cycles of: denaturation at 98ºC for 10 sec, annealing at 58°C for 20 sec, extension at 72°C for 90 sec; followed by a final extension at 72°C for 7 min.

2.7.3 Amplification of 26S rDNA

Figure 2.3 26S region of yeast ribosomal DNA

For amplification of the 26S rDNA (Figure 2.3), the primer pair used were forward - NL1 (5'-GCATATCAATAAGCGGAGGAAAAG) and reverse - NL4 (3'GGTCCGTGTTTCAAGACGG) (O'Donnell, 1993). PCR programme conditions used were same as described for amplification of ITS region.

2.7.4 Purification of PCR product

PCR products were purified with QIAquick PCR purification kit (Qiagen, Germany). One volume of the PCR sample was mixed with five volumes of PB Buffer and transferred to the QIAquick column placed in a provided 2 mL collection tube. The

QIAquick column was centrifuged for 60 sec and flow-through was discarded. The QIAquick column was washed with 750 µL PE Buffer by centrifugation for 60 sec. After discarding the flow-through, residual wash buffer was removed by further centrifugation for an additional 2 min. The QIAquick column was then placed in a clean 1.5 mL microcentrifuge tube. EB Buffer (10 mM Tris·Cl, pH 8.5) (50 µL) was added and the column was centrifuged for 1 min to elute the DNA. Purified PCR products were analyzed on 1.5 % agarose gel with 100 bp-10 kb gene ruler DNA ladder mix as marker and visualized using UV transilluminator.

2.7.5 Sequencing and data analysis

Purified PCR products were sequenced using the ABI prism 3730 DNA analyzer (Applied Biosystems, Foster city, USA).

Chromas 2.1 software was used for reading and editing the DNA sequences. Blast searches of sequences were performed at the National Centre for Biotechnology Information (NCBI) GenBank data library. Sequence alignments were performed with type strains using ClustalW in the Bioedit program to obtain the percentage identity (Thompson et al., 1994). The phylogeny was estimated by the neighbor joining method the dendrogram was constructed using Treeview program. All the sequences were deposited in GenBank.

2.8 Enzyme assays

All the isolates were inoculated ($1x10^6$ CFU/mL) separately in a series of tubes containing MGYP broth (5 mL) and the tubes were incubated at 28°C and 180 rpm for 48 h. After incubation, the medium was centrifuged at 16,000 g, 4°C for 10 min, the supernatant was collected and used for the estimation of different enzyme activities, viz., pectinase, protease, β-glucosidase and β-1, 3 glucanase.

2.8.1 β-Glucosidase assay

Araujos et al. (1983) method with a modification was used for β-glucosidase estimation. The reaction mixture consisting of 0.2 mL supernatant and 1.8 mL substrate (0.1 % para-nitrophenyl-β-D-glucanopyroside in 0.1 M acetate buffer, pH 4.5) was incubated at 37°C for 15 min. Reaction was terminated by adding 2 mL of 2% Na_2CO_3. Released para-nitrophenol was determined by measuring absorbance at 405 nm.

One unit of enzyme activity was defined as the amount of enzyme that liberates one nmole of paranitrophenyl/mL/h, under the given assay conditions.

2.8.2 Protease assay

Acid proteases activity was determined using haemoglobin as substrate (Nelson and Young, 1986). Reaction was started by adding 2 mL substrate (1% haemoglobin in 0.1M citrate phosphate buffer, pH 3.2) to 0.5 mL of the supernatant and the mixture was incubated at 37°C for 30 min. After incubation, 3 mL trichloroacetic acid (5% TCA in 3.3 N HCl) was added to stop the reaction. The reaction mixture was filtered through Whatmann filter paper No. 1 and absorbance was measured at 280 nm. Tyrosine in the range of 50 - 500 µg/mL was used as standard. One unit of acid protease activity was defined as the amount of enzyme that liberates one nmole of tyrosine/mL/h.

2.8.3 β-1, 3 Glucanase assay

For β-1, 3 glucanase activity, DNSA sugar estimation method was used (Miller, 1959). Supernatant (0.1 mL) was added to 0.5 mL of substrate (1% laminarin in 0.05 M sodium acetate buffer, pH 4.5) and incubated at 37°C for 30 min. Reaction was stopped by addition of 1 mL DNSA reagent. The tubes were boiled for 5 min, cooled and 8 mL distilled water was added. Absorbance of the developed orange color was measured at 540 nm. The amount of released reducing sugars was calculated using D-glucose (100 µg/mL -1000 µg/mL) as a standard. A unit of enzyme activity was defined as the amount of enzyme that liberates one nmole of glucose/mL/h from laminarin.

2.8.4 Pectinase assay

Pectinase activity was determined by dinitrosalicylic acid (DNSA) method (Miller, 1959). To 0.1 mL enzymatic solution/supernatant, 0.9 mL substrate (0.5% pectin in 0.05 M sodium acetate buffer, pH 5) was added and the reaction mixture was incubated at 50°C for 30 min. The reaction was stopped by addition of 1 mL DNSA reagent. For preparing DNSA reagent, 150 mL of 10.66% NaOH was added drop wise with stirring to 3, 5 dinitrosalicyclic acid solution (10 g of 3, 5-dinitrosalicyclic acid in 200 mL water). Potassium sodium tartarate (300 g) was added in above solution and volume was adjusted to 1 L with distilled water. Solution was filtered with sintered glass filter.

After DNSA addition, the tubes were boiled for 5 min. After cooling 8 mL distilled water was added. Absorbance of the developed color was measured at 540 nm. The amount of released reducing sugars was calculated using D-galacturonic acid

(10 µg/mL -100 µg/mL) as a standard. One unit of pectinase activity was defined as nmole of reducing sugars released/mL/h under the given assay conditions.

2.9 Analysis of grape juice

The grape juice of all varieties were used for estimation of sugars and pH, whereas, grape juice of Shiraz variety was used for determination of color, total phenolics content, total flavonoids content and tannins.

2.9.1. Estimation of reducing sugars

Reducing sugars were estimated by DNSA method. The reaction mixture containing 1 mL sample and 1 mL DNSA reagent was boiled for 5 min in water bath. Tubes were allowed to cool and 8 mL of distilled water was added. The absorbance of the developed color was measured at 540 nm. The amount of released reducing sugars was calculated using D-glucose (100 µg/mL -1000 µg/mL) as a standard.

2.9.2 Color and phenolic measures

Four test tubes were set for 1) sample, 2) sample + acetaldehyde, 3) sample + sodium metabisulfite and 4) sample + HCl. Grape juice sample, 2 mL and 100 µL were added in first three and 4th test tube, respectively. Acetaldehyde solution (20 µL, 10% w/v) was added to the 2nd test tube and mixed thoroughly. Absorbance was measured after 45 min incubation. Sodium metabisulfite (30 µL; 25% w/v) was added to 3rd test tube. Mixed gently and spectral measures were taken. In the 4th test tube, 10 mL of 1 M HCL solution was added, mixed thoroughly by inverting several times and kept for 3 h incubation.

Absorbance of the solutions from test tube 1, 2 and 3 were measured at 520 and 420 nm and for test tube no. 4 at 520 and 280 nm. Different parameters were calculated using following formulas (Iland et al., 2004).

Wine color density (a. u.) = $A_{520} + A_{420}$

Wine color hue = A_{420} / A_{520}

$$\text{Estimation of } SO_2 \text{ resistant pigments (a. u.)} = A_{520}^{SO_2}$$

$$\text{Total red pigments (a. u.)} = A_{520}^{HCl}$$

$$\text{Modified wine color density} = A_{520}^{CH_3CHO} + A_{420}^{CH_3CHO}$$

$$\text{Modified wine color hue} = A_{420}^{CH_3CHO} \;/\; A_{520}^{CH_3CHO}$$

2.9.3 Total phenolics content (TPC)

Grape juice sample (diluted 1:50 times with distilled water), 1 mL, was mixed with 2.5 mL of 2% sodium carbonate solution and 2.5 mL of 10 % Folin-Ciocalteu reagent. The reaction mixture was incubated for 30 min at room temperature. After incubation, the absorbance of the blue colored complex was measured at 765 nm (Khatoon et al., 2013). A stock solution of gallic acid (1 mg/mL) was prepared in methanol, and standard solutions were prepared by dilution with distilled water to plot a standard curve (10 µg/mL-100 µg/mL). The total phenolics content was expressed as milligrams of gallic acid equivalent (GAE)/L.

2.9.4 Total flavonoids content (TFC)

A stock solution of catechin (1 mg/mL) was prepared in methanol, and calibration solutions between 10 - 100 µg/mL were prepared by dilution with distilled water. To this, 2 mL of deionized water and 150 µL of 5% sodium nitrite were added. After 5 min at room temperature, 150 µL of 10% aluminium chloride was added and further incubated for 6 min. After incubation 1 mL of 1 M sodium hydroxide was added. Deionized water was used to adjust the total volume to 5 mL and absorbance was read at 510 nm. Similar procedure was adopted for the grape juice sample (diluted 1:50 times with distilled water). The total flavonoids content was expressed as mg catechin equivalents (CE)/L (Feliciano et al., 2009).

2.9.5 Estimation of total tannins

Tannins were estimated by protein tannin precipitation method as described by Hagerman and Butler (1978). Sample or the aqueous tannin (1 mg/mL) solution (1 mL) was added to 2 mL bovine serum albumin (BSA, 1 mg/mL in 0.2 M acetate buffer, pH 5.0 containing 0.17 M sodium chloride). The reaction mixture was mixed properly by vortexing and allowed to stand at room temperature for 15 min followed by centrifugation at 5000 g for 15 min. The supernatant was discarded and pellet was washed with 0.2 M acetate buffer. The precipitate was dissolved in 4 mL of SDS-triethanolamine (1% SDS and 5% triethanolamine). One mL of $FeCl_3$ reagent (0.01 M $FeCl_3$ in 0.01 N HCl) was added and the absorbance was read at 510 nm after 30 min.

2.10 Ethanol tolerance

The experiment was carried out in 96 well microtiter plates. YPG broth supplemented with 1% - 13% absolute ethanol (Merk, India) were added (200 µL/well) separately to the wells of the plate. The yeast cells (~$1x10^6$ CFU/mL), freshly grown in YPG broth

in logarithmic phase, were inoculated in the wells. The plate was incubated for 48 h, growth was checked visibly and by measuring absorbance at 600 nm. Ethanol tolerance was defined as the highest percentage of ethanol exhibiting visible growth as compared to the growth of the control (wells with 0% ethanol).

2.11 Wine fermentation

The grapes of Shiraz were crushed and the juice was used for fermentation. Fermentations were carried out in 2 L bottles sealed by standard home-brewing fermentation locks which were filled with 25 mL of sterile water. Volume of the grape juice was 1.5 L. Dry *S. cerevisiae* yeast powder (25 mg/L) and $K_2S_2O_5$ (50 mg/L) were added to the juice and the fermentors were kept at 20ºC for 15 d.

Sugar concentration, pH was monitored daily. Samples (50 mL) were withdrawn on 0, 3, 6, 9, 12 and 15th d for determination of enzyme activities and microflora. The samples (1 mL) were diluted serially and plated on MGYP agar plates. After 24-48 h incubation, colonies were counted and cells were observed microscopically.

The experiment was carried out in 96 well plates. The yeast cells (~1x10^{6} CFU/mL), freshly grown in YPG broth in logarithmic phase, were suspended in the medium and inoculated (200 µL) in the wells of the plate. Absolute ethanol (Merk, India) was added to well containing YPG with cells from 1% to 13%. The microtiter plate was incubated for 24-48 h, and growth was checked visibly and measuring absorbance at 600 nm using microtiter plate reader (Bio-Rad, India). Tolerence was defined as the highest percentage of ethanol exhibiting visible growth compared to growth of the control.

The samples (50 mL) were centrifuged at 5000 rpm, 4ºC for 15 min. Supernatant was mixed with equal volume of acetone : ethanol (1:1) and precipitated overnight at -20ºC. After centrifugation at 5000 rpm, 4ºC for 15 min, the pellet was dissolved in 5 mL sodium acetate buffer (0.05 M, pH 5) and used as enzyme sample for the estimation of pectinase, acid protease, β-glucosidase and β-1,3 glucanase as described in section 2.8.

2.12 Analysis of wine

Residual sugar, color, phenolic measures, TPC, TFC, total tannin were determined for the wine sample as described in section 2.9.

2.12.1 Estimation of ethanol

Final concentration of ethanol in the fermented wine was determined for the 15th d sample. Ebulliometer was used to calculate alcohol percentage in wine. Ebulliometer involves measurement of decrease in the boiling point caused by the total amount of alcohol in the wine sample. Boiling point of a sample is measured relative to the boiling point of pure water. Ebulliometer condenser was filled with cold tap water. Distilled water/sample (50 mL) was added to the boiling chamber and heated with a spirit lamp. Boiling point was recorded after the thermometer showed constant temperature. The ethanol v/v % was determined by using calculating dial.

2.12.2 **Analysis of glycerol content by High Performance Liquid Chromatography (HPLC)**

Glycerol content of the wine sample was determined by HPLC (Waters, Milford, MA, USA) with Waters Sugar Pak-1 column (300 x 6.5 mm). The mobile phase used was 0.01 mM Ca-EDTA at 0.4 mL/min flow rate. The temperature of the column oven was set at 80°C and injection volume was 30 µL. Glycerol content of the samples was calculated from the peak area for sample using glycerol (150 µg/mL) as a standard.

2.12.3 Analysis of phenolic compounds by High Resolution-Liquid Chromatography/ Mass spectrometry **(HR-LCMS)**

Qualitative and quantitative estimation of phenolic compounds present in the grape juice, wine prepared in the laboratory and wine procured from market was done by HR-LCMS. The samples were centrifuged at 5000 rpm for 5 min, filtered through 0.45 µm nylon membrane (Millipore, Bedford, MA, USA). Stock standard solutions of polyphenols, viz., catechin, caffeic acid, p-coumaric acid, trans-resveratrol, syringic acid, quercetin, gallic acid, tartaric acid and malic acid were prepared by dissolving these compounds in methanol (1 µg/L).

The analysis was performed on Q-Exactive hybrid quadropoule orbitrap mass spectrometry (Thermo scientific, Germany). The injection volume was 5 µL. Liquid chromatographic separation was achieved on Thermo hypersil gold C-18 (150 mm × 4.6 mm, 8 µm) reverse phase column. The column and auto sampler tray temperature were kept constant at 25ºC and 8ºC, respectively. The mobile phase consisted of methanol (A) and 0.1% formic acid aqueous (B). The gradient program used was given by Cui et al., 2012 (Table 2.3).

The analytes were ionized with an electrospray ionisation (ESI) source in negative ion mode under the following source parameters: sheath gas 45; auxillary gas

10; sweep gas 2; RF value 50; spray voltage 3.60 kv; auxillary gas heating temperature 350ºC; capillary temperature 320ºC. MS analysis was carried out by selected ion monitoring (SIM) in the negative mode. Chromatographic peaks were identified by comparing their retention times and spectral data with those of the pure standards. Calibration curves were obtained by plotting the peak areas against different concentrations of standard phenolic compounds.

Table 2.3 Gradient programme for High Resolution-Liquid Chromatography / Mass spectrophotometry

Time	A%	B%	Flow (mL/min)
0	04	96	0.5
6	04	96	0.5
7	20	80	0.5
15	30	70	0.5
27	80	20	0.5
35	90	10	0.5
40	04	96	0.5

2.13 Microbial metabolite for the control of wine spoilage yeasts

2.13.1 Production, extraction and purification of microbial metabolites

The *Pseudomonas* strain MCC 2142, isolated previously from garden soil, was grown in a inhibitor production medium containing (g/L)- soyabean meal, 10.0; mannitol, 15.0; yeast extract, 10.0; starch (soluble), 5.0; under shaking conditions (180 rpm) at 28ºC for 96 h. The cells were harvested by centrifugation at 10000 g for 15 min and the cell free broth (9 L) obtained was evaporated at 60ºC till dryness to yield yellow brown residue 49.7 g. The residue was extracted with chloroform and solvent was evaporated under vacuum to yield chloroform extract 5.5 g. The extract 5.5 g was separated by column chromatography using silica gel 200-300 mesh as stationary phase and methanol: chloroform (5:95) as eluting system into 10 fractions. From fraction 12 and fraction 9 compounds **1** (50 mg) and **2** (22 mg) were crystallized. Fractions 4-8 (560 mg) were combined and separated using methanol: chloroform (5:95). Out of 10 fractions, fractions 4 and 5 (419 mg) were mixed and subjected to coloum chromatography using methanol: chloroform (2:98) as mobile phase. Again 10 fractions were collected and preperative Thin Layer Chromatography (TLC) of

fractions 5-9 (315 mg) in methanol: chloroform (2:98) was carried out to isolate compound, **3** (5 mg). It was further purified by crystallization.

2.13.2 Structure elucidation of the compounds

Spectroscopic analyses were performed to determine the structure of the purified metabolite. Melting point was recorded using a BÜCHI B 540 apparatus (BUCHI Labortechnik AG, Flawil, Switzerland). Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Ultrashield 500 MHz for protons and 125 MHz for carbon-13. Spectra were measured in deuterated chloroform ($CDCl_3$) at room temperature. Mass spectrum was recorded with electrospray ionization mass spectrometer, ESI-MS (API-QSTAR-PULSAR, applied Biosystems), in positive ion mode.

2.13.3 Antimicrobial activity against natural yeast flora and spoilage yeasts

In vitro antifungal activity of extracts and fractions was evaluated against wine yeasts by Kirby Bauer disc diffusion assay. The purified final compounds were evaluated for antifungal susceptibility testing against all the cultures under study (isolated natural yeast flora and wine spoilage yeasts, section 2.5) by Clinical Laboratory Standards Institute's broth microdilution method (CLSI, 2008). Appropriate amount of the compound was dissolved in dimethyl sulfoxide to get 100X final strength. The stock is then diluted 1:50 in YPG (Glucose 1%, Yeast extract 0.3% and Peptone 0.5%) medium and 200 µL from this is added to the first row of a 96-well microtiter plate.

The compound was serially diluted two fold in successive wells to get a range of 2-256 µg/mL. The yeast cells (~1x104 100 CFU/mL), freshly grown in YPG broth in logarithmic phase, were suspended in the medium and inoculated (100 µL) in the wells of the plate. The microtiter plate was incubated for 24-48 h, and growth was checked visibly and measuring absorbance at 600 nm using microtiter plate reader (Bio-Rad, India). The Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration exhibiting >90% inhibition of visible growth compared to growth of the control.

2.14 Carvacrol and thymol as natural wine preservative

2.14.1 Antimicrobial activity against natural yeast flora and spoilage yeasts

Pure carvacrol and thymol were evaluated for antifungal activity against natural yeast flora and spoilage yeasts by micro broth dilution method (section 2.13.3) according to the recommendations of the Clinical and Laboratory Standards Institute with a modification that the assay was performed by using YPG medium at pH 6.5 and 3.5.

2.14.2 Mechanism of antifungal action of carvacrol and thymol

2.14.2.1 Propidium iodide (PI) staining

Damage or permeabilization of fungal membrane after carvacrol and thymol treatment was checked using a membrane impermeable dye, propidium iodide. For this, actively growing *D. hansenii* (~1×10^6 CFU/mL) cells were suspended in YPG medium containing carvacrol or thymol (64, 128 and 256 μg/mL) and PI (3 μM). After incubation for 6 h at 28°C with constant shaking (180 rpm), cells were harvested by centrifugation and suspended in phosphate buffer saline (PBS, pH 7.2). The cells were then visualized by epifluorescence microscope (Leitz Laborlux S, Germany) using filter set (N 2.1 filter block with excitation filter BP 515-560, and an emission filter LP580).

2.14.2.2 Release of cellular contents

To check the leakage of cytoplasmic contents, *D. hansenii* (~$1\text{x}10^7$ CFU/mL) cells suspended in PBS were treated with different concentrations (64, 128 and 256 μg/mL) of carvacrol and thymol for 6 h. After incubation, cells were separated by centrifugation and absorbance of the supernatant at 260 nm was measured.

2.14.2.3 Quantitation of sterols

Extraction and quantitation of total sterols after the treatment of *D. hansenii* cells with carvacrol and thymol was done as described by Ahmad et al. (2011). Briefly, a single *D. hansenii* colony from an overnight MGYP plate culture was used to inoculate 50 mL of MGYP broth containing 0, 32 and 64 μg/mL of carvacrol and 0, 32 and 64 μg/mL of thymol along with positive control (without test compound) and negative control (fluconazole). The cultures were incubated for 16 h with shaking at 28°C. The stationary-phase cells were harvested by centrifugation at 2,700 rpm for 5 min and washed once with sterile distilled water. The net wet weight of the cell pellet was determined. Three millilitres of 25% alcoholic potassium hydroxide solution (25 g of KOH and 35 mL of sterile distilled water, brought to 100 mL with absolute ethanol) was added to each pellet and vortex-mixed for 1 min. Cell suspensions were transferred to 16 x 100 mm sterile glass screw-cap tubes and were incubated in an 85°C water bath for 1 h. After incubation tubes were allowed to cool to room temperature. Sterols were then extracted by addition of 1 mL of sterile water and 3 mL of n-heptane followed by vortexing for 3 min. The heptanes layer was scanned spectrophotometrically between 200-300 nm.

2.14.3 Toxicity testing by hemolysis assay

The toxicity of carvacrol and thymol were checked by the red blood cell (RBC) lysis assay as described by Khan and Ahmad (2011). The concentrations of the compounds tested were in the range of 2-1024 μg/mL. The freshly obtained RBCs of sheep blood were washed with 1 mL of PBS buffer (pH 7.0) and 4 mL was added to 5% (w/v) glucose solution to obtain 4% RBC suspension. Next, 750 μL of PBS buffer containing the desired concentration of compound was mixed with 750 μL of RBC suspension in Eppendorf tubes and incubated at 37°C for 2 h. Triton X-100 (0.1% (v/v) in PBS buffer was used as a positive control whereas 1% DMSO and PBS buffer were used as negative controls. Tubes were centrifuged at 2,000 rpm for 10 min and the absorbance of supernatant was measured at 540 nm. Percent haemolysis was calculated as: [(A-B)/(C-B) x 100], where A and B is the absorbance values of supernatant from the test sample and PBS buffer (solvent control), respectively, and C is the absorbance value of supernatant from the sample after 100% lysis. Each experiment was performed in triplicate and the mean values were considered for calculation of percent haemolysis.

2.14.4 Antimicrobial effect of carvacrol and thymol in wine

Effect of carvacrol and thymol on the growth of spoilage yeasts in red wine (Shiraz; pH 3.5; ethanol 12.0%) was checked. The experiments were carried out in 24 well plates containing 2 mL wine/well. Overnight grown yeast cultures (~$1x10^5$ CFU/mL) were inoculated in the wine along with 64 μg/mL of thymol or carvacrol. Plates were incubated at 20°C for 16 d without shaking. The tests were run in triplicate and appropriate controls were maintained. Growth was determined by measuring OD at 600 nm every 24 h using a microtiter plate reader.

Chapter 3

Results and discussion

A. Yeasts diversity of different grape varieties

The diversity of yeasts present on berries, phylloplane and in soil is one of the important factors responsible for wine fermentation (Clemente-Jimenez et al., 2005; Fleet, 2003; Sabate et al., 2002). Non-*Saccharomyces* yeast isolates such as *Candida, Debaryomyces, Dekkera Hansenula, Hanseniaspora, Kluyveromyces Metschnikowia, Pichia, Rhodotorula,* and *Saccharomyces* were present on the surface of grape berries (Renouf et al., 2005). *Dekkera bruxellensis/Brettanomyces* was often found to be associated with wineries and less commonly on grape berries (Fugelsang, 1997; Ibeas et al., 1996; Martorell et al., 2006). Different factors affect the yeast flora present on grapes such as rainfall, temperature, soil type, grape variety, berry maturity, damage due to birds, insects and fungi, mechanical damage, application of fungicides, insecticides and geographic location etc. (Combina et al., 2005; Guerra et al., 1999; Mortimer and Polsinelli, 1999; Torre et al., 2006).

As there are no reports on the yeast flora associated with Indian wineries, the focus of the present study was to get acquainted with the yeast diversity associated with the different wine grape varieties commonly used in India. This section describes the isolation and identification of yeast flora from grape juice by morphological, cultural, biochemical methods and molecular methods (5.8S and ITS rRNA) from five varieties from two regions.

3.1 Grape varieties

The grape varieties such as Bangalore Blue, Cabernet Sauvignon, Shiraz, Zinfandel and Sauvignon Blanc were crushed and used for the isolation of natural yeast flora. The sugar concentration of grape juice from different varieties was between 163 g/L (Zinfandel) and 270 g/L (Sauvignon Blanc) and acidity was between 3.5 and 4.5. The sugar content (ºBrix) and pH for the five varieties were: Bangalore Blue (20.49 ºBrix, pH 3.64), Zinfandel (16.1 ºBrix, pH 4.15), Cabernet-Sauvignon (19.3 ºBrix, pH 3.54), Shiraz (19.7 ºBrix, pH 3.99) and Sauvignon Blanc (26.3 ºBrix, pH 4.5). Preferred sugar content at the time of harvesting is between 19-26 ºBrix.

Grape variety used is the most important factor in wine quality as it imparts the "varietal character" due to the presence of different secondary metabolites responsible for the principal flavor compounds in grape must (Lambrechts and Pretorius, 2000).

3.2 Isolation of natural yeast flora

Total seventy eight yeasts isolates were obtained from five different varieties of grapes collected from Nashik (Lat. 19º 99' N; Long. 73º 78' E) and Pune (Lat. 18º 32' N; Long.73º 52' E) region. The number of isolates from each variety is listed in table 3.1.

Table 3.1 Isolates obtained from five different grape varieties

Region	Type	Variety	No of isolates	Isolates designated as
Pune	Red	Bangalore Blue	24	I1,I2, I3, I4, I5, I6, I7, I8, I9, I10, I11, I12, I13, I14, I15, I16, I17, I18, I19, I20, I21, I22, I23, I24
		Zinfandel	19	I25, I26, I27, I28, I29, I30, I31, I32, I33, I34, I35, I36, I37, I38, I39, I40, I41, I42, I43
		Shiraz	5	I44, I45, I46, I47, I48
		Cabernet Sauvignon	3	I49, I50, I51
Nashik	Red	Shiraz	12	I52, I53, I54, I55, I56, I57, I58, I59, I60, I61, I62, I63
		Cabernet Sauvignon	4	I64, I65, I66, I67
	White	Sauvignon Blanc	11	I68, I69, I70, I71, I72, I73, I74, I75, I76, I77, I78

The numbers of yeasts isolated from the five vine varieties were: Bangalore Blue, 24; Zinfandel, 19; Cabernet Sauvignon, 7; Shiraz, 17 and Sauvignon Blanc, 11. Sauvignon Blanc, Shiraz and Bangalore Blue grapes were more ripen than Zinfandel

and Cabernet-Sauvignon. Yeasts and yeasts-like population increase during the grape ripening process due to sugar exudation (Martins et al., 2014).

3.3 Identification of natural yeast flora

Identification of natural yeasts was carried out on the basis of morphology, colony characteristics as well as corresponding tests for biochemical characterization and similarity in nucleotide sequence with the closest species.

3.3.1 Morphology and colony characteristics

Five different types of colony morphologies were observed in the 78 isolates. Thirty four isolates had a creamy and glossy colony appearance (Figure 3.1A) typical of *Hanseniaspora* species. Twenty one colonies were with white dry (Figure 3.1B) appearance; among these 21 isolates four isolates showed pseudomycelium formation. Four pale brown colonies (Figure 3.1C) showed pseudomycelium formation characteristic to *P. membranifaciens*. Creamy, smooth and butyrous colony type (Figure 3.1D) was displayed by 17 isolates and two colonies were with yellowish mucoid morphology (Figure 3.1E).

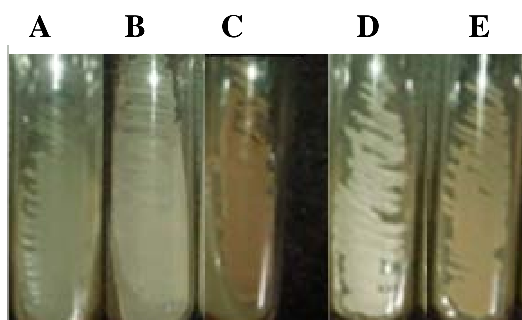


Figure 3.1 Colony morphologies of non-*Saccharomyces* yeasts. **A:** creamy, smooth and glossy**;** *Hanseniaspora* sp., **B:** white and dry; *Issatchenkia* sp., **C:** pale brown and dry; *Pichia* sp., **D:** creamy, smooth and butyrous**;** *Saccharomyces* sp., **E:** yellowish mucoid; *Candida* sp.

In light microscopy, cells having creamy, smooth, glossy appearance showed apiculate cell morphology (Figure 3.2 A, B, C). Globose to ovoidal morphology was observed in the isolates of white dry appearance (Figure 3.2 D, E, F, G). Pseudomycelium was observed in isolates no I12, I13, I14, I71 and I75 (Figure 3.2 H, I, J, K). Cells from cream smooth and butyrous group observed ovoidal to elongated (Figure 3.2 L M, N). Small elongated cells were observed for yellowish mucoid

colony type (Figure 3.2 O). *Hanseniaspora* sp. and *Pichia* sp. could be identified on the basis of their morphology and colony characters.

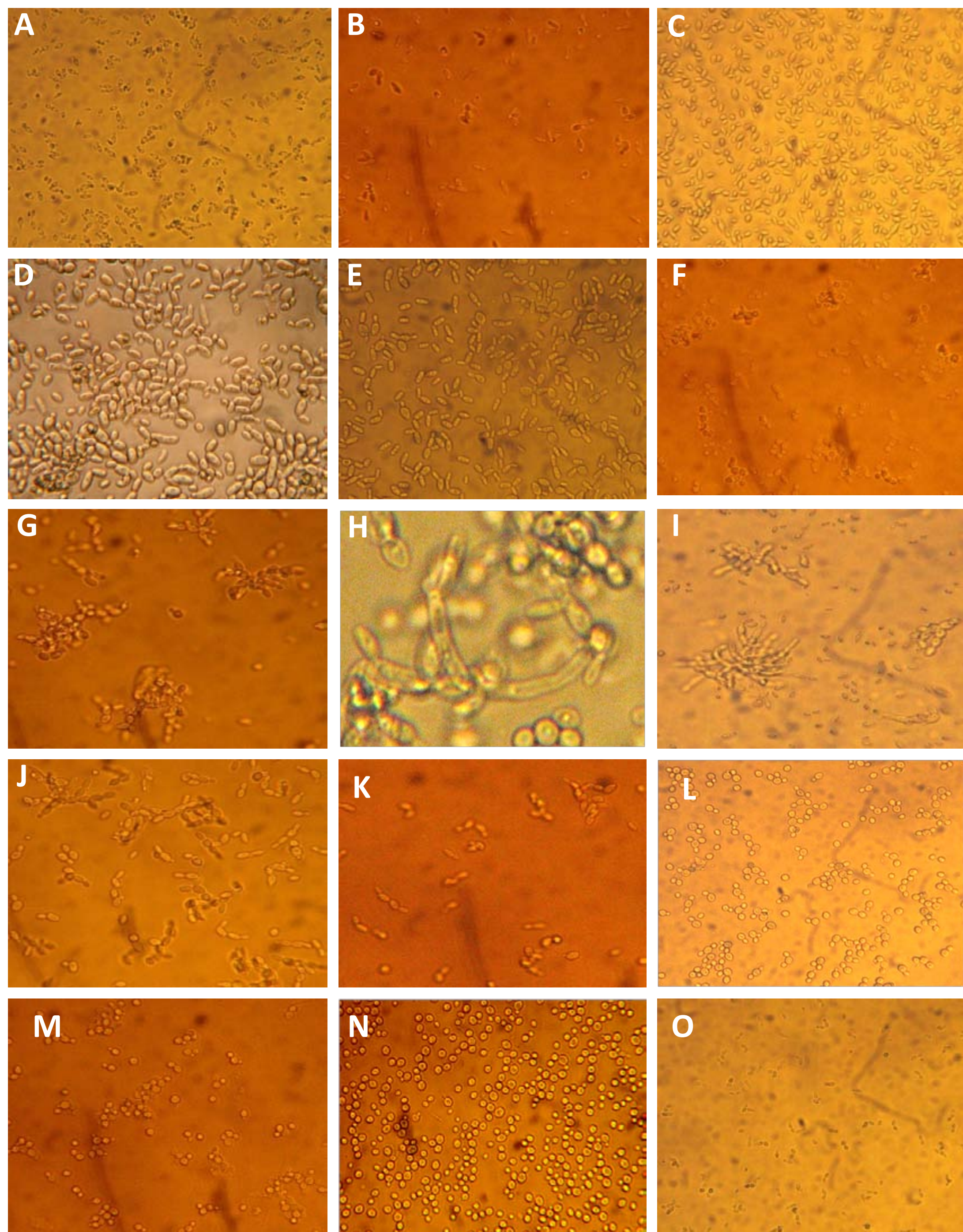


Figure 3.2 Different cell morphologies of isolates

3.3.2 Biochemical tests

In biochemical tests employed for identification, the ability of the isolates to ferment and assimilate different sugars was studied. Based on Lodder (1970) and Kurtzman

and Fell (1998), 14 assimilation and fermentation tests were selected for the identification of yeasts.

3.3.2.1 Fermentation and assimilation of sugars

Fermentation was observed according to two parameters, acid production and acid-gas production. All 51 isolates from Pune region (Table 3.2) fermented glucose. Few isolates from colony type A and from colony type D fermented galactose whereas all isolates from colony type D assimilated galactose. Colony type D and E isolates fermented and assimilates sucrose and maltose. Cellobiose and salicin sugars were assimilated by the isolates belonging to *Hanseniaspora* sp. (colony type A). Among the colony type B and C isolates, 13 isolates could not assimilate any other sugar while few isolates fermented galactose and sucrose.

All 27 isolates from Nashik variety (Table 3.3) were grouped in to the four different colony morphologies. Glucose was fermented and assimilated by all isolates. Assimilation of cellobiose and salicin was observed in all isolates of colony type A and D except two isolates from colony type D (I52, I74). Cellobiose and Salicin assimilation as in colony type D isolates was not observed in similar (type D) isolates from Pune region. The pattern of fermentation and assimilation of sugar was similar in the case of isolates from colony type B and C. Lactose and rhamnose was not assimilated by any isolate from both regions. Though colony type D fermented and assimilated glucose, galactose, sucrose and maltose, isolate I52 did not assimilated maltose. Raffinose was fermented by most of the isolates from colony type D except two isolates (I6, I7).

3.3.2.2 Cluster analysis

Cluster analysis of the isolates was carried out on the basis of comparison of results for 15 biochemical tests with 11 comparable standard isolates reported in the literature (Kurtzman and Fell, 1998; Lodder, 1970). In cluster from pune region two major branches (I and II) with seven clusters were generated. In the first branch 14 isolates were grouped in clusters 1, 2, and 3. In cluster 1, ten isolates were grouped with *Saccharomyces cerevisiae* and two isolates with *Zygoascus steatolyticus.* In cluster 2, two isolates were grouped with *C. azyma.* Two isolates were grouped with *C. quercitrusa* in cluster three. In second branch twenty three isolates were grouped in cluster four with *H. guilliermondii, H. uvarum* and *H. opuntiae* (Figure 3.3).

Table 3.2 Fermentation and assimilation of carbon compounds by 51 yeasts with different colony types isolated from Pune region

		Fermentation[a]										Assimilation							
Colony Type[b] Source[c]	Isolate No	D-Glu		D-Gal		Suc		Mal		Raff		D-Glu	D-Gal	L-Rha	Mal	Suc	Cello	Lact	Sali
		a	g	a	g	a	g	a	g	a	g								
A, I	I2	+	+	+	-	w	-	-	-	-	-	+	-	-	W	-	+	-	+
A, I	I5	+	+	-	-	+	+	-	-	-	-	+	-	-	-	-	+	-	-
A, I	I8	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	I9	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
A, I	I10	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	I18	+	+	+	+	-	-	-	-	W	-	+	-	-	-	-	+	-	+
A, I	I21	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	+	-	+
A, I	I22	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	I23	+	+	+	-	-	-	-	-	-	-	+	-	w	-	-	+	-	+
A, II	I26	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I28	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I30	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I35	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I38	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+

A, II	I39	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I41	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	I44	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	I46	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	I47	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	I48	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, IV	I49	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, IV	I50	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, IV	I51	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
B, I	I3	+	-	-	-	w	+	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I4	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I11	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I15	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I16	+	+	-	-	w	+	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I17	+	+	-	-	w	+	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I19	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I20	W	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, II	I27	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, II	I40	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C, I	I12	+	-	-	-	w	-	-	-	-	-	+	-	-	-	-	-	-	-

C, I	I13	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C, I	I14	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	-
D, I	I6	+	+	W	+	-	-	+	+	-	-	+	+	-	+	+	-	-	-
D, I	I7	+	-	-	-	+	-	-	-	-	-	+	+	-	+	+	-	-	-
D, I	I25	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I29	+	+	-	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I31	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I32	+	+	W	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I33	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I34	+	+	-	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I42	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I43	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D, 1	I37	+	+	W	-	+	-	+	-	+	-	+	+	-	+	+	-	-	-
D, III	I45	+	+	-	-	-	-	-	-	+	-	+	+	-	-	-	+	-	+
E, I	I1	+	+	-	-	w	+	+	+	-	-	+	+	-	+	+	-	-	-
E, I	I24	+	-	-	-	+	+	-	-	-	-	+	+	-	+	+	-	-	-

[a] Fermentation: D-Glu, D-Glucose; D-Gal, D-Galactose; Suc, Sucrose; Mal, Maltose; Raff, Raffinose;
a; acid production, g; gas production, +, Positive; –, Negative; w, Weak
Assimilation: L-Rha, Rhamnose; Cello, Cellobiose; Lact, Lactose; Sali, Salicin
[b] A: creamy, smooth and glossy; B: white and dry; C: pale brown and dry; D: creamy, smooth and butyrous; E: yellowish and mucoid
[c] I: Bangalore Blue, II: Zinfandel, III: Shiraz, IV: Cabernet

Table 3.3 Fermentation and assimilation of carbon compounds by 27 yeasts with different colony types isolated from Nashik region

		Fermentation[a]										Assimilation							
Colony Type[b] Source[c]	Isolate No	D-Glu		D-Gal		Suc		Mal		Raff		D-Glu	D-Gal	L-Rha	Mal	Suc	Cello	Lact	Sali
		a	g	a	g	a	g	a	g	a	g								
A, I	I53	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	I54	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	I56	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	I61	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	I69	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	I76	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I64	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I65	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I66	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	I60	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	I67	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
B, III	I78	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I55	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I57	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-

B, I	I58	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, III	I68	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, III	I70	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, III	I71	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	I59	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C, III	I75	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D, III	I74	+	+	+	w	+	+	+	-	+	-	+	+	-	+	+	+	-	-
D, I	I62	+	+	+	+	+	W	+	+	+	-	+	+	-	+	+	+	-	+
D, I	I63	+	+	+	+	+	W	+	-	+	-	+	+	-	+	+	+	-	+
D, III	I72	+	+	+	-	+	+	+	W	+	-	+	+	-	+	+	+	-	+
D, III	I73	+	+	+	+	+	W	+	+	+	-	+	+	-	+	+	+	-	+
D, III	I77	+	+	+	-	+	+	+	-	+	-	+	+	-	+	+	+	-	+
D, I	I52	+	+	+	-	+	+	+	-	+	-	+	-	-	-	+	-	-	-

[a] Fermentation: D-Glu, D-Glucose; D-Gal, D-Galactose; Suc, Sucrose; Mal, Maltose; Raff, Raffinose;
a, acid production; g, gas production; +, Positive; –, Negative; w, Weak
Assimilation: L-Rha, Rhamnose; Cello, Cellobiose; Lact, Lactose; Sali, Salicin
[b] A: creamy, smooth and glossy; B: white and dry; C: pale brown and dry; D: creamy, smooth and butyrous;
[c] I: Shiraz, II: Cabernet, III: Sauvignon Blanc

One isolate was grouped with *C. diversa* in cluster five and one isolate grouped with *P. manshurica* in cluster six. Isolates of *I. orientalis* (5)*, I. terricola* (5) *and P. membranifaciens* (2) were grouped together in cluster seven.

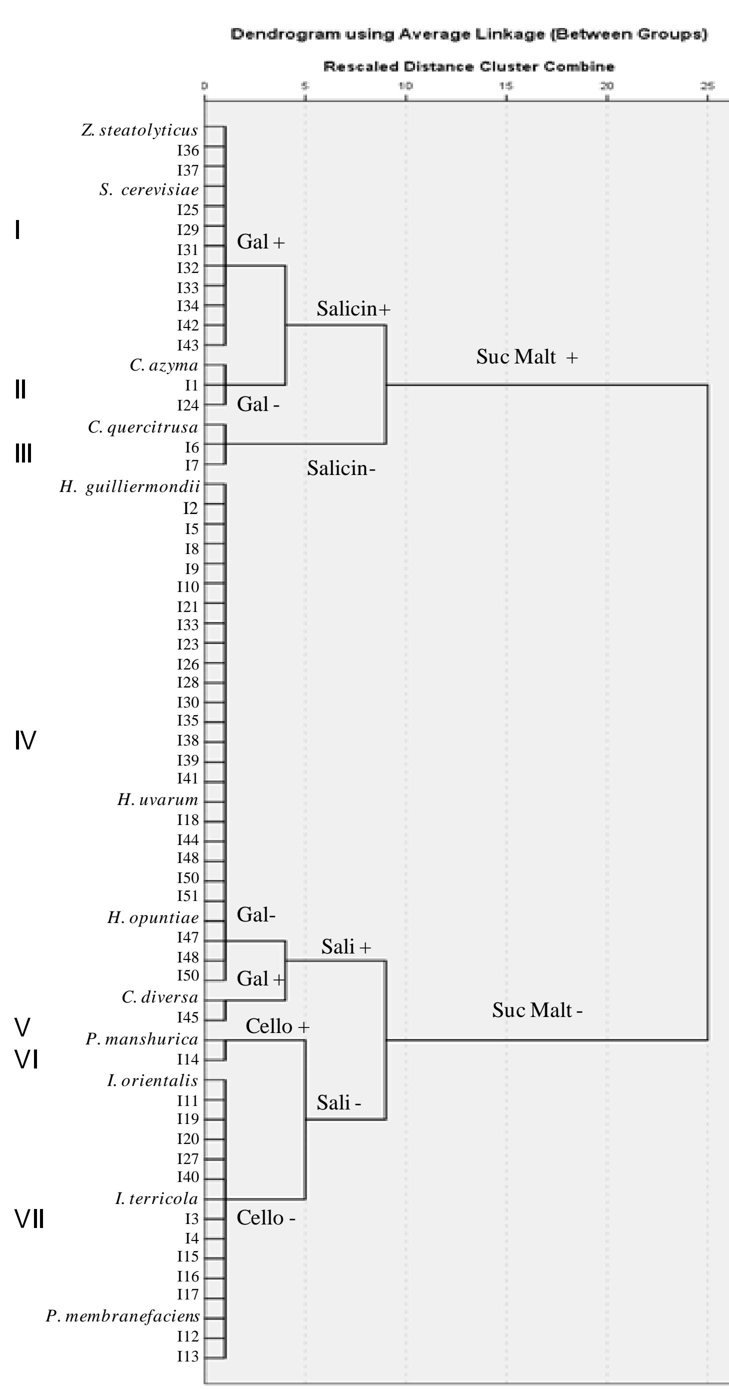


Figure 3.3 Cluster analysis of 51 isolates from Pune region with standard isolates - *Candida azyma* CBS 6826, *Candida quercitrusa* CBS 8602, *Candida diversa* CBS 4074, *Hanseniaspora uvarum* CBS 314, *Hanseniaspora guilliermondii* CBS 479, *Hanseniaspora opuntiae* CBS 8733, *Issatchenkia orientalis* CBS 5147, *Issatchenkia terricola* CBS 8131, *Pichia membranifaciens* CBS 107, *Pichia manshurica* CBS 7324, *Saccharomyces cerevisiae* CBS 1171.

Pseudomycelium formation differentiated *P. membranifaciens* isolates. Identification of *C. azyma, C. quercitrusa, C. diversa, P. manshurica* in the cluster 2, 3, 5 and 6 was possible using the biochemical tests (Figure 3.3).

In cluster from Nashik region two major branches (I and II) with six clusters

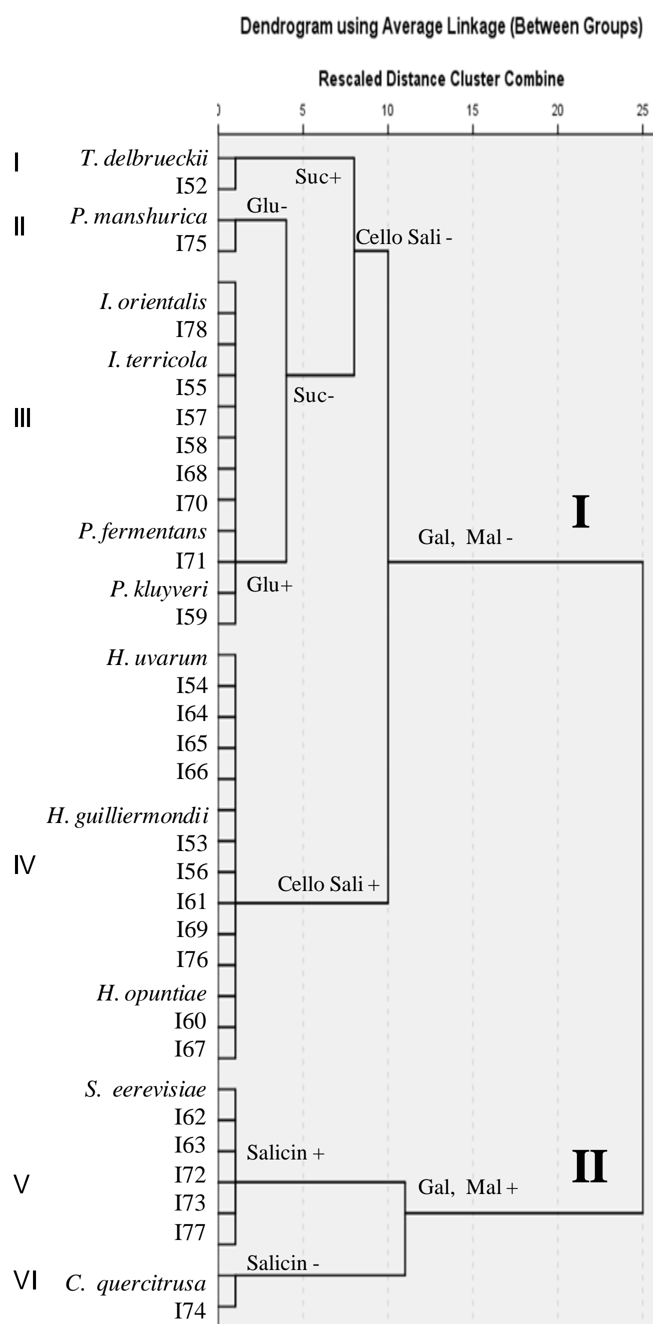


Figure 3.4 Cluster analysis of 27 isolates from Nashik region with standard isolates - *Candida quercitrusa* CBS 8602, *Hanseniaspora uvarum* CBS 314, *Hanseniaspora guilliermondii* CBS 479, *Issatchenkia orientalis* CBS 5147, *Issatchenkia terricola* CBS 8131, *Pichia fermentans* CBS 187, *Pichia manshurica* CBS 7324, *Saccharomyces cerevisiae* CBS1171, *Torulaspora delbrueckii* CBS 1146.

were generated (Figure 3.4). In the first branch 21 isolates were grouped in clusters 1, 2, 3 and 4. In cluster 1, one isolate was grouped with *T. delbrueckii* and in cluster 2 one isolate was grouped with *P. manshurica.* Six isolates of *Isaatchenkia* sp. and two isolates of *Pichia* sp. were grouped in cluster three. Eleven *Hanseniaspora* sp. were grouped together in cluster four. In second branch, five isolates were grouped with *S. cerevisiae* in cluster 5. One Isolate was grouped with *C. quercitrusa* in cluster six. Identification of *T. delbrueckii, P. manshurica, S. cerevisiae, C. quercitrusa* in the cluster 1, 2, 5 and 6 was possible based on the biochemical results. No clear grouping was observed in case of *Issatchenkia* sp., *Pichia* sp. and *Hanseniaspora* sp., as they had several similar tests used for identification and strain dependant variations among the isolates. Therefore, molecular identification was carried out to identify these isolates and confirm the results of cluster analysis.

3.3.3 Molecular identification

3.3.3.1 Amplification of ITS1-5.8S-ITS4 rRNA region

The ITS1-5.8S-ITS4 region of 78 isolates was obtained by amplification using ITS1 and ITS4 fungal primers (White et al., 1990).

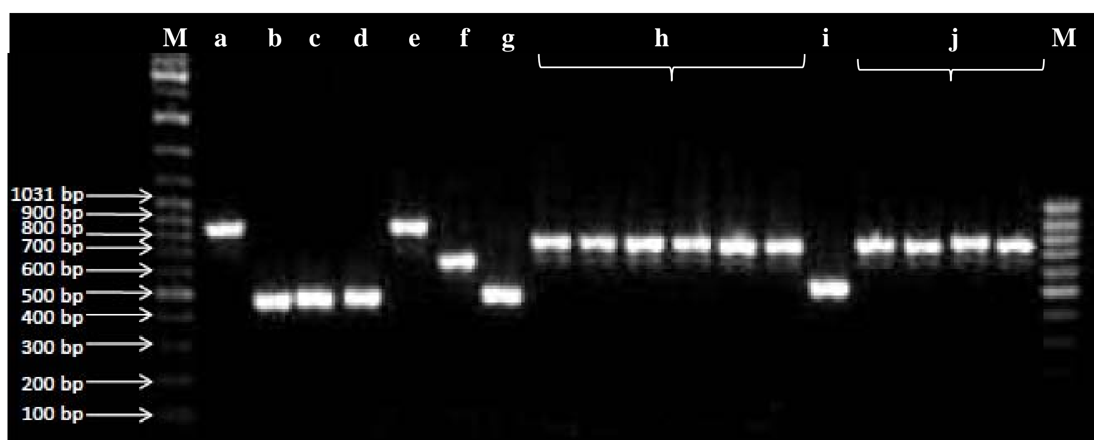


Figure 3.5 Electrophoretic analysis of PCR product of ITS1-5.8S-ITS4 region of rDNA **M**; marker, **a;** I25, **b;** I12, **c;** I13, **d;** I14, **e;** I29, **f;** I6, **g;** I18, **h;** I2, I5, I8, I9, I10, I21, **i;** I36, **j;** I26, I28, I30, I35, I38

Gel electrophoresis of purified PCR products showed differences in size of ITS1-5.8S-ITS4 region of different genera (Figure 3.5). All 78 isolates had amplicon size between 361-752bp. Two isolates had 361bp amplicon size*;* likewise 3; 548bp, 1; 460, 20; 679, 9; 535, 5; 577, 6; 440, 10; 339, 2; 372, 1; 387, 1; 369, 2; 361, 6; 752, 1; 478, 2; 511.

3.3.3.2 Sequencing and data analysis

Purified products were sequenced with ABI 3730 for rapid identification of the 78 isolates. The ITS1-5.8S-ITS4 sequences from the 78 isolates were used to perform BLAST. The sequence identity with the closest sequence was used to identify the isolates into seven different genera (Table 3.4).

Table 3.4 Percent identity of ITS1-5.8S-ITS4 region of rDNA of 78 isolates from five different grape varieties with type strain

Type strain	Isolate No	% identity	Variety*
C. azyma (EF533997)	1, 24	99 (all)	I
C. diversa (KC509573)	45	97	IV
C. quercitrusa (AM160627)	6,7,74	97,99,98	I,I,V
H. guilliermondii (EF449522)	2,5, 8, 9, 23	99 (all)	I
	10,21,22,	97,80,98	I
	26,28,30,35,	99 (all)	II
	38, 39, 41,		
	53,56,61,69,	98,99,95,99,	IV
	76	92	V
H. opuntiae (KF953902)	60, 46,44,47,48,54	93,99 (all)	IV
	67,49	99 (all)	III
H. uvarum (AM160628)	18,	80,	I
	64,65,66,50,51	96,99(all)	IV
I. orientalis (EF198013)	11,19,20	98,92,76	I
	27,40,78	97,99(all)	II,II,V
I. terricola (EF648009)	3,4,15,16,17	97,98,98,100,99	I
	55,57,58,68,	93,91,99,88,	IV
	70	99	V
P. membranifaciens (DQ198964)	12,13,	89,99	I
P. manshurica (FM199959)	14,75	92,96	I,V
P. fermentans (DQ674358)	71	99	V
P. kluyveri (JX188203)	59	97	IV
S. cerevisiae (AM262831)	25,29,31,32,33,34,42,	99(all)	II
	43,62,63,	98 (all)	IV
	72,73,77		V
T. delbrueckii (KJ160641)	52	90	IV
Z. steatotlticus	36,37	99(all)	II

*I, Bangalore Blue; II, Zinfandel; III, Cabernet; IV, Shiraz; V, Sauvignon Blanc

The isolate no those showed 99% similarity with type strains are I1, I24; *Candida azyma* (EF533997), I7; *C. quercitrusa* (AM160627), I2, I5, I8, I9, I23, I56, I69, I54, I44, I48; *H.* guilliermondii (EF449522), I46, I47; *H. opuntiae* (KF953902),

I50, I51, I65, I66; *H. uvarum* (AM160628), I40, I78; *I. orientalis* (EF198013), I17, I58, I70; *I. terricola* (EF648009), I71; *P. fermentans* (DQ674358), I25, I29, I31, I32, I33, I34, I42; *S. cerevisiae* (AM262831) and I36, I37; *Z. steatolyticus* (AY447033) whereas rest all showed less than 98 percent similarity with type strains. For the isolates that showed less than 98% similarity with standard type strain in ITS sequencing, amplification of another region i.e D1/D2 region of 26S rDNA was carried out. The use of this D1/D2 rDNA amplification has the potential to markedly increase the accuracy of yeast identification (Kurtzman and Robnett, 1998).

3.3.3.3 Amplification of 26S rDNA region

Amplification of D1/D2 region of 26S rDNA was carried out using NL1 and NL2 primers (O'Donnell, 1993). The PCR products were purified using the Qiagen purification kit and sequenced using NL1 and NL2 fungal primers with ABI 3730 for rapid identification of the 11 isolates. No difference in the size of the amplicon was observed for the PCR products by gel electrophoresis (Figure 3.6).

Figure 3.6 Electrophoretic analysis of PCR product of D1/ D2 region of 26S rDNA **a**; I3, **b**; I6, **c**; I12, **d**; I18, **e**; I19, **f**; I52, **g**; I55, **h**; I59

3.3.3.4 Sequencing and data analysis

Amplified sequences of 26S rDNA region from the selected isolates were used to for BLAST analysis. The identification was carried out on the basis of the significant sequence alignments in BLAST search. The sequence identity with the closest sequence was used to identify the isolates (Table 3.5). All 14 isolates whose

ITS1-5.8S-ITS4 region were previously showing less than 98% percent similarity with type strain is now showing 99-100% similarity of 26S rDNA region with the same type strains. Therefore, it can be emphasized that the amplification of the D1/D2 region of 26S rRNA provides greater resolution than ITS region. All the ITS1-5.8S-ITS4 region sequences for 78 isolates were submitted to NCBI and strains with accession number are listed in Annexure I.

Table 3.5 Percent identity of D1 and D2 region of 26S rDNA from selected isolates with type strains

Type strain	Isolate No	% identity	Variety
I. orientalis (EF550233.1)	I3	99	I
C. quercitrusa (AM160627.1)	I6	99	I
I. orientalis (FJ770558.1)	I11	99	I
P. membranifaciens (EU057561.1)	I12	99	I
H. uvarum (FJ871131.1)	I18	99	I
I. orientalis (EF585438.1)	I19	100	I
I. orientalis (FJ770478.1)	I20	99	I
T. delbrueckii (GU138404.1)	I52	99	IV
I. terricola (KJ506735.1)	I55	100	IV
I. terricola (KJ506735.1)	I57	99	IV
P. kluyveri (KC510043.1)	I59	99	IV
H .opuntiae (KC870065.1)	I60	99	IV
H. uvarum (HM627056.2)	I61	99	IV
H. guilliermondii (JQ707775.1)	I76	99	V

I, Bangalore Blue; II, Zinfandel; III, Cabernet; IV, Shiraz; V, Sauvignon Blanc

3.3.3.5 Phylogenetic analysis

ITS sequences were used to generate the phylogetic tree. The phylogenetic tree of Pune region representing all 51 yeast isolates showed 12 different yeast species (Figure 3.7). The phylogenetic tree was in accordance with the observations of Kurtzman and Robnett (1998). The members of the genus *Pichia* closely related to *Issatchenkia* species whereas species of *Hanseniaspora* and *C. quercitrusa* shared common branch point shared a common origin with *S. cerevisiae* and *Z. steatolyticus.*

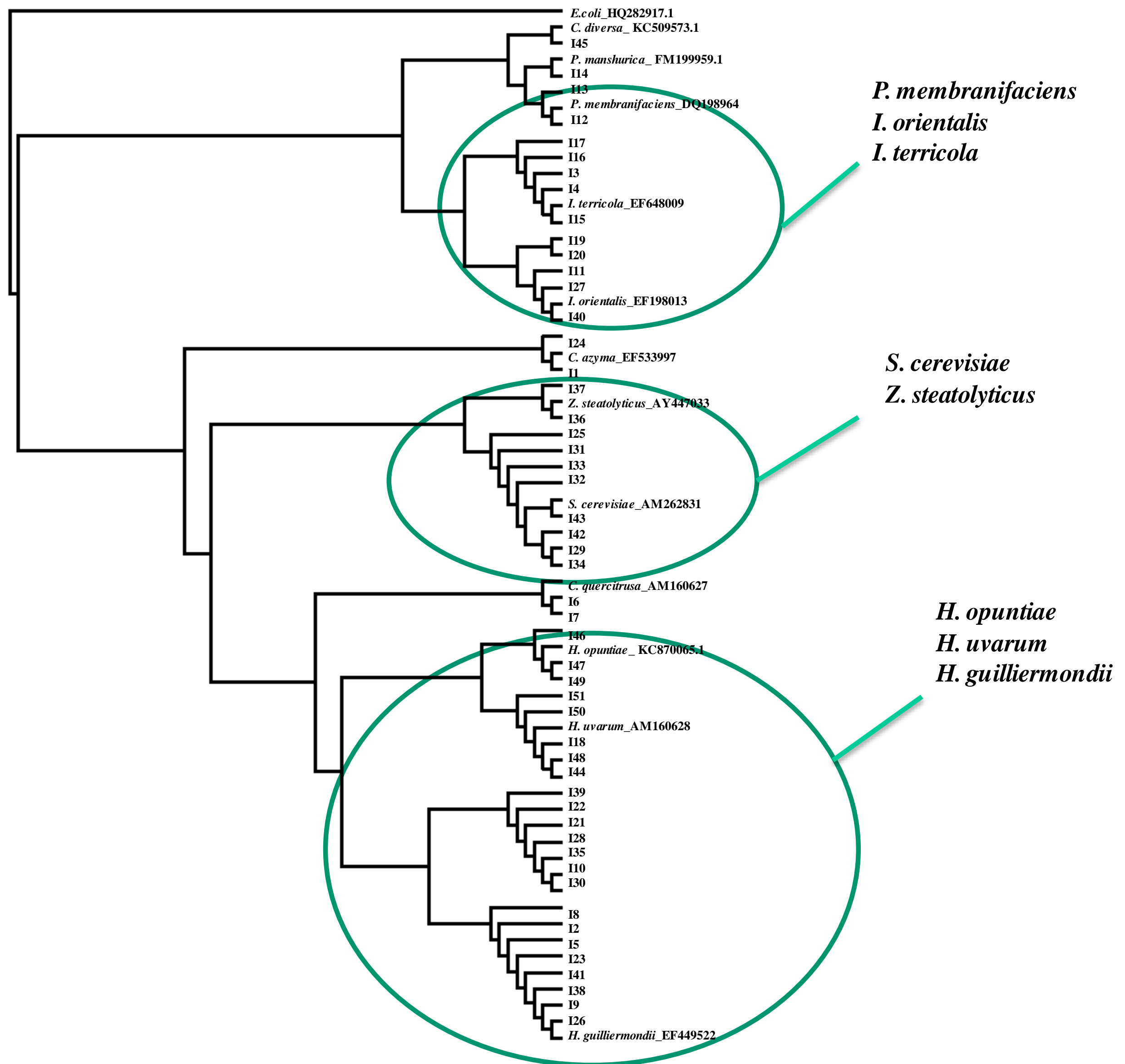


Figure 3.7 Dendrogram of 51 isolates from Pune region and their closest related species

Species such as *C. diversa*, *I. orientalis, I. terricola, H. guilliermondii, H. opuntiae, H. uvarum, P. membranifaciens, P. manshurica, P. fermentans, P. kluyveri, T. delbrueckii, Z. steatolyticus* and *S. cerevisiae* found in the present study are similar to those species studied on grapes and wines in other parts of the world (Barata et al., 2012; Jolly et al., 2013; Nisiotou and Nychas, 2007; Saez et al., 2011). *Hanseniaspora* sp. was predominantly present among the isolates obtained from the five vine varieties. Fleet, (2003) reported increased incidence of the apiculate yeast from the mature grape berries. *S. cerevisiae* isolates were detected in Shiraz,

Sauvignon Blanc and Zinfandel vine varieties. Few studies indicated low occurrence of *S. cerevisiae* in the grape juice and must (Mortimer and Polsinelli, 1999). Nurgel et al. (2005) reported high counts of *S. cerevisiae* in grape juice from white and black grapes grown in Anatolia due to excess use of sulfite in the vineyard.

C. quercitrusa and *C. azyma* were reported first time from Bangalore Blue variety grown in Pune region. While *C. quercitrusa* was reported to be associated with insects, *C. azyma* was reported to be associated with the sugarcane crop (Insuellas de Azeredo et al., 1998; Meyer et al., 1998). *C. azyma* has also been described on lichens and bees from Convolvulaceae (Lachance et al., 2001; Suzuki et al., 1999). This association may be attributed to the change in cropping pattern from sugarcane to viticulture in the vine growing regions and the known association of *C.*

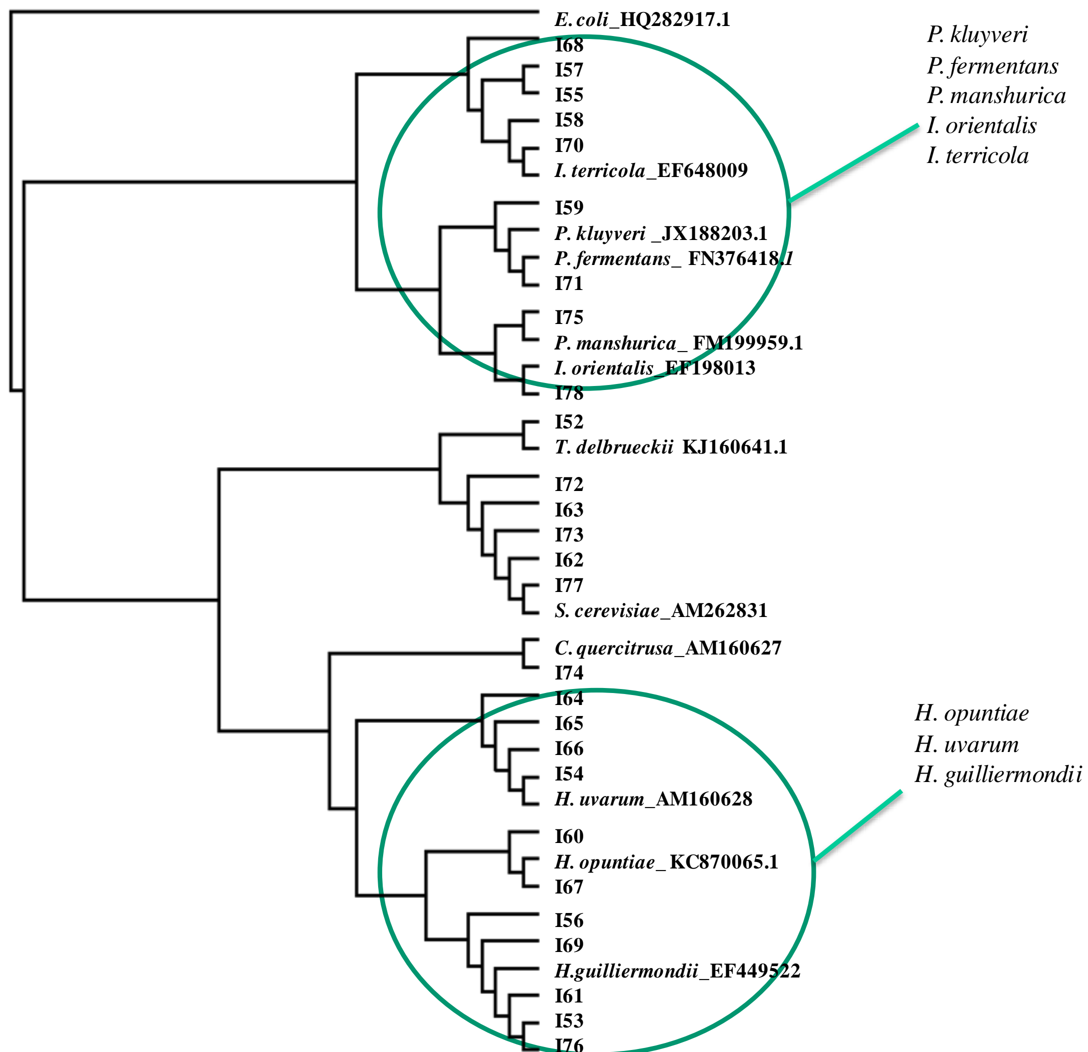


Figure 3.8 Dendrogram of 27 isolates from Nashik region and their closest related species

azyma with sugarcane phylloplane.

The phylogenetic tree of Nashik region for 27 yeast isolates showed 11 different yeast species (Figure 3.8). The topology of the phylogenetic tree for nine isolates showed that *I. orientalis*, *I. terricola* and *P. fermentans, P. kluyveri, P. manshurica,* shared common branch point. Likewise, *S. cerevisiae, T. delbrueckii, C. quercitrusa,* and *Hanseniaspora* sp. shared common branch point.

Among the 78 identified indigenous grape yeast isolates, the largest diversity of yeast species (eight) was found in the Bangalore Blue and Shiraz vine variety followed by Zinfandel (four), Sauvignon Blanc (seven), and Cabernet Sauvignon (Two). The phylogenetic tree topology was in accordance with the observations of Kurtzman and Robnett (1998). The genus *Pichia* was polyphyletic as its members are closely related to *Issatchenkia*, among others (Kurtzman and Robnett, 1998).

3.4 Variety and region specific analysis of the isolates

Table 3.6 Variety specific association of the yeasts

Sr. No	Species	Cabernet Sauvignon	Shiraz	Bangalore Blue	Zinfandel	Sauvignon Blanc
1	*C. azyma*	-	-	+	-	-
2	*C. quercitrusa*	-	-	+	-	+
3	*C. diversa*	-	+	-	-	-
4	*H. guilliermondii*	-	+	+	+	+
5	*H. opuntiae*	+	+	-	-	-
6	*H. uvarum*	+	+	+	-	-
7	*I. orientalis*	-	-	+	+	+
8	*I. terricola*	-	+	+	-	+
9	*P. fermentans*	-	-	-	-	+
10	*P. kluyveri*	-	+	-	-	-
11	*P. manshurica*	-	-	+	-	+
12	*P. membranifaciens*	-	-	+	-	-
13	*S. cerevisiae*	-	+	-	+	+
14	*T. delbrukii*	-	+	-	-	-
15	*Z. steatolyticus*	-	-	-	+	-
	No .of genera	1	5	4	4	4
	No. of species	2	8	8	4	6

+; present, -; absent

H. guilliermondii strains were found on almost all varieties except cabernet Sauvignon. Only two species of *Hanseniaspora* (*H. opuntiae* and *H. uvarum*) were found on Cabernet Sauvignon variety. *S. cerevisiae* were detected on Shiraz, Zinfandel and Sauvignon Blanc (Table 3.6). Species found to be associated with specific variety were - *C. diversa;* Shiraz, *P. fermentans;* Sauvignon Blanc, *P. kluyveri*; Cabernet Sauvignon, *T. delbrueckii;* Cabernet Sauvignon and *Z. steatolyticus;* Zinfandel.

Region specific difference was also observed in the diversity of the natural yeasts flora. Eleven species belonging to six genera were found in Nashik region and twelve species belonging to six genera were found in Pune region. Species such as *P. fermentans, P. kluyveri,* and *T. delbrueckii* were specifically associated with Nashik region whereas *C. azyma, C. diversa, P. membranifaciens* and *Z. steatolyticus* were only found from Pune region. *C. quercitrusa, H. opuntiae, H. guilliermondii, H. uvarum, I. terricola, I. orientalis, P. manshurica, S. cerevisiae* were found in both regions.

Thus, the 78 yeasts isolates obtained from the grape berries of five vine varieties from two regions of Maharashtra, India were identified by biochemical analysis and molecular techniques into 15 different species belonging to seven genera. The indigenous yeast flora associated with the grapes differed among vine varieties as well as sampling locations.

B. Non-*Saccharomyces* yeasts in wine fermentation

Wine fermentation by traditional method is a biochemical process carried out by *S. cerevisiae* and several non-*Saccharomyces* yeasts, which grow more or less in succession throughout the fermentation. These yeasts survive during early stage of fermentation and ethanol tolerant species take over fermentation (Fleet and Heard, 1993). The non-*Saccharomyces* yeasts are known to secret different enzymes of enological interest such as pectinases, protease, β-1, 3-glucanase and β-glucosidase (Esteve-Zarzoso et al., 1998) in grape juice; which extracts precursors, improves fermentation and produce aroma active compounds, thereby enhancing the quality of wine (Charoenchai et al., 1997).

For better understanding of the role of non-*Saccharomyces* yeasts, different enzyme activities *in vitro* in artificial medium were estimated. Also, fermentation with Shiraz variety grapes was carried out and parameters monitored including the succession of yeasts, enzyme levels in the fermenting must and chemical analysis of finished wine are discussed in following sections.

3.5 Potential of the yeast isolates for extracellular hydrolytic enzyme production

In order to ascertain the potential of individual species for production of different enzymes, all the 78 yeast isolates were grown in artificial media and the levels of hydrolytic enzymes were determined. From the four enzymes evaluated, pectinase activity was present in the all isolates. Isolates *I. terricola* - I3, I57; *C. quercitrusa* - I6, I7; *I. orientalis* - I9, I78; *H. guilliermondii* - I21, I22; *S. cerevisiae* - I31, I77; *H. uvarum* - I48, I51, I54; *H. opuntiae* - I49; *P. fermentans* I71 were found to be potential producers of all four enzymes. The remaining isolates exhibited either one or two activities from the enzymes; protease, β-1, 3 glucanase and β-glucosidase. Among the 78 isolates, highest glucosidase (1010 nmol/mL/h), pectinase (27458 nmol/mL/h), glucanase (27279 nmol/mL/h) and protease (10810 nmole/mL/h) activity were observed for isolates *I. terricola* I68, *H. guilliermondii* I5, *I. terricola* I57, and *H. guilliermondii* I56 respectively.

Issatchenkia sp. were found to be significant glucosidase producers. *Candida* sp., *Hansenispora* sp. and *Issatchenkia* sp. were significant β-1, 3 glucanase producers, whereas glucanase activity was not detected in *Pichia* sp., *Zygoascus* sp. and *Torulaspora* sp. *C. azyma* (2822-3512 nmol/mL/h), *H. opuntiae* (2398-2619

nmol/mL/h), and *Z. steatolyticus* (2278-2439 nmol/mL/h) were potential protease producers (Table 3.7).

Table 3.7 Enzyme activities (range) in isolated yeast strains of 15 species

Species	β-Glucosidase nmol/mL/h	β- 1,3 Glucanase nmol/mL/h	Pectinase nmol/mL/h	Protease nmol/mL/h
C. azyma	ND	1070-4162	11026-11097	2822-3512
C. diversa	216	8796	8216	ND
C. quercitrusa	64-568	713-911	6508-23101	701-1851
H. guilliermondii	17-154	317-8721	2205-27458	191-10810
H. uvarum	17-53	3865-14232	4054-15365	184-2619
H. opuntiae	26-220	3162-13181	3414-10883	2398-2619
I. orientalis	50-342	396-8697	9336-13480	295
I. terricola	298-1010	2656-27279	7860-18531	970-1180
P. fermentans	138	1779	17179	12544
P. kluyveri	141	ND	10350	ND
P. membranifaciens	35-201	ND	6989-15329	ND
P. manshurica	41-153	ND	4801-18531	221
S. cerevisiae	95-624	436-6128	2347-18282	110
T. delbrueckii	188	ND	11524	ND
Z. steatolyticus	99	ND	44335	2278-2439

ND-Not detected. Activities are given as nmole/mL/h, under the given assay conditions

The results showed potential of non-*Saccharomyces* yeast diversity for enzyme production and also highlighted the possibility of considering these autochthonous strains (*I. terricola* I68, *H. guilliermondii* I5, *I. terricola* I57, and *H. guilliermondii* I56) for the mixed culture fermentation along with starter strain. Ferreira et al. (2001) showed exocelluar β-glycosidase activity (8.40 nmol/mL/h) in *K. apiculata*, whereas Hernandez et al. (2003) reported that *S. cerevisiae* produced 98 nmol/mL/h β-glycosidase in YPG medium.

3.6 Ethanol tolerance of natural yeast flora and spoilage yeasts

The ability of non-*Saccharomyces* yeasts to influence wine quality depends on their ethanol tolerance. Therefore, fifteen natural yeasts species belonging to the seven genera were checked for in vitro ethanol tolerance (1-13% ethanol) in YPG medium. Most of the natural yeast flora could tolerate up to 6% of ethanol and their growth was not detected at and above 7% ethanol concentration. *I. orientalis, D. hansenii* and *S. cerevisiae* were found to grow in a medium containing 12 % of ethanol. In case of spoilage yeasts, growth of *S. pombe, D. bruxellensis, Z. rouxii* and *D. hansenii* was

observed till 12% and at 13% ethanol, growth of only *D. bruxellensis* was observed after 48 h.

The species of *Hanseniaspora, Candida, Pichia, Kluyveromyces, Metschnikowia* and *Issatchenkia* found in grape juice are not tolerant of ethanol concentrations exceeding 5-7%, and this explains their decline and death as the fermentation progresses beyond the mid-stage (Gao and Fleet, 1988; Heard and Fleet, 1988). Cells of *S. cerevisiae* showed no loss in viability when incubated for 12 d in the presence of 15% ethanol. Cells of *C. stellata* and *K. apiculata* were tolerant of 12.5% ethanol (Gao and Fleet, 1988). *Schizosaccharomyces pombe, Zygosaccharomyces bailii and Zygosaccharomyces fermentati* are well known for their tolerance of high ethanol concentrations (>10%) and occur in winery environments (Fleet, 2000; Romano and Suzzi, 1993).

3.7 Fermentation with Shiraz variety

Figure 3.9 Laboratory fermentation of Shiraz variety
Temperature; 15-20º C, Time; 15 d

In the laboratory, fermentations of Shiraz variety grapes were carried out with naturally present non-*Saccharomyces* on grapes and *S. cerevisiae* as an inoculums (Figure 3.9). During fermentation the succession of yeasts and enzyme activities were monitored.

3.7.1 Microbial succession during fermentation

Fermentation with *S. cerevisiae* (added as inoculum) was carried out for red Shiraz variety. A quantitative change of the flora during fermentation was monitored for 15 days. *Hanseniaspora* sp., *Issatchenkia* sp., *Pichia* sp. and *Torulaspora* sp. were found to be present on 0 d. The count of *Hansenispora* sp. was $1.6x10^4$ cells/mL in grape

juice and it reached to 2x10^6 cells/mL on 3rd d and further 10^7cells/mL on 6th d of fermentation. The cell concentration decreased with increase in ethanol concentration. From the five species present on 0 d, *Torulaspora* sp. could not be detected on 3rd d, *Pichia* sp. disappeared on 6th d, and *Issatchenkia* disappeared on 9th d whereas only *S. cerevisiae* was present in the sample from 9th d of fermentation. *Issatchenkia* sp. and *Pichia* sp. were present in grape juice at 3x10^4 cells/mL and 4x10^4 cells/mL, respectively. On 3rd d of fermentation, *Issatchenkia* sp. and *Pichia* sp. count reached to 7x10^6 cells/mL and 5.2x10^5 cells/mL, respectively. Inoculum of *S. cerevisiae* was 1x10^6 cells/mL on 0 d which first increased and then decreased to 8.1x10^6, 4x10^8, 1.6x10^8, 1.6x10^7 and 8x10^5 cells/mL on 3rd, 6th, 9th, 12th, and 15th d, respectively.

Beltran et al. (2002) reported that non-*Saccharomyces* yeasts, particularly *H. uvarum* and *C. stellata* dominated the first stages of fermentation. Non-*Saccharomyces* yeasts disappear as soon as alcohol concentration increases (Fleet, 2003). Fleet et al. (1984) isolated *H. uvarum* and *T. stellata* as dominant yeasts in musts. *Hanseniaspora, Rhodotorula, Cryptococcus* and *Debaryomyces* were reported by Moreira et al. (2011) during early days of fermentation. Several genera are occasionally present during different stages of fermentation, including *Candida, Zygosaccharomyces, Torulaspora, Metschnikowia* and *Pichia* (Fleet, 2003; Fleet et al., 1984; Heard and Fleet, 1985). In the initial phases of spontaneous fermentations, *Kloeckera* and *Candida* sp. mainly appear, followed by *Metschnikowia*, *Pichia* and occasionally *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula* and *Zygosaccharomyces* (Clemente-Jimenez et al., 2004).

3.7.2 Enzyme activity profile during fermentation

Protease**,** β-1, 3 glucanase, β-glucosidase and pectinase are important enzymes of enological interest. Pectinase and glucosidase isolated from different fungal sources are added externally during fermentation to improve wine quality. Mixed culture fermentation with *S. cerevisiae* inoculum and other non-*Saccharomyces* yeasts (added or from grape flora) as a possible natural source of these enzymes is a promising approach and will reduce the cost and improve the wine quality. Monitoring of these enzymes during fermentation could also be useful in preventing stuck fermentation.

The enzymatic activities levels are not necessarily constant throughout the fermentation process (Maturano et al., 2012). In present study, fluctuations in the enzyme levels were detected during alcoholic fermentation but all the four activities

were detected during early days of fermentation. The levels of protease, pectinase and glucosidase decreased in late stage of fermention (Figure 3.10). Pectinase levels were high (>4500 nmol/mL/h) and constant till 6th d, which then dropped by >60% from 9th d. Glucanase activity was not detected at 0 d, which then increased gradually and was maximum, i.e. 122 nmol/mL/h on 6th d. Glucanase activity was not detected in samples from 9, 12 and 15th d. Protease level was also maximum (568 nmol/mL/h) on 6th d and decreased in late stage of fermentation. Maximum level of β-glucosidase (36 nmol/mL/h) was on 0 d.

Glucosidase activity decreased as fermentation advanced and was absent in

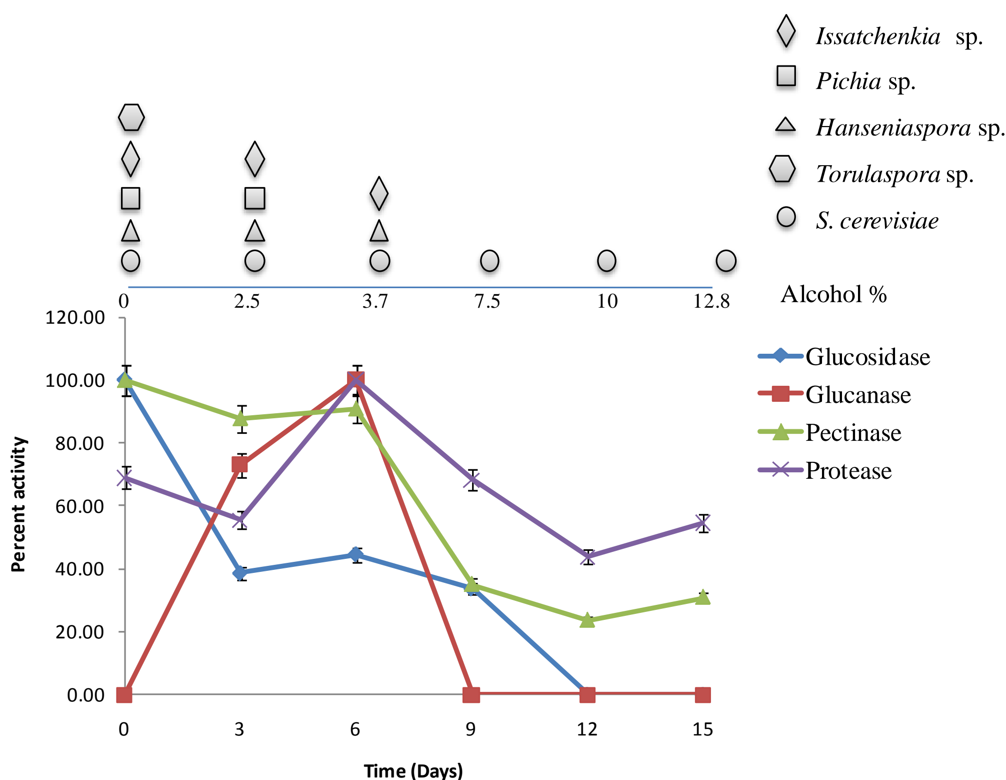


Figure 3.10 Microflora and enzyme activity profile during fermentation of Shiraz variety

last stage of fermentation. Increase (till 6th d) and fall (9 d onwards) in all the enzyme activities over the fermentation period was concurrent with increase in cell count and disappearance of non-*Saccharomyces* yeasts. These results indicated that non-*Saccharomyces* yeasts are important source for enzymes of enological interest.

The inference was also supported by enzyme activities exhibited by the isolates from fermentation in YPG medium. Three strains of *I. terricola* isolated on 0 d produced maximum levels of glucosidase (upto 904 nmol/mL/h), whereas, *S.*

cerevisiae isolated on 9th and 12th d produced comparatively far less i.e. 129 nmol/mL/h and 156 nmol/mL/h of glucosidase, respectively. *I. terricola* isolated on 6th d produced maximum glucanase (27279 nmol/mL/h), while, maximum protease (10810 nmol/mL/h) was produced by *H. guilliermondii* strain isolated on 3rd d.

Zamuz et al. (2004) reported the levels of different enzyme activities during fermentation. Initially pectinase activity was 40 nmol/mL/h (0 d), which increased to 60 nmol/mL/h on 5th d and later decreased to 20 nmol/mL/h on 7th day and remains the same till 26th d. β-1, 3 glucanase activities ~50 nmol/mL/h were detected during initial stage of fermentation, whereas, β-glucosidase was detected on 7th and 8th d (~10 nmol/mL/h).

3.7.3 Analysis of grape juice and wine

Table 3.8 Analysis of grape juice and test wine

Parameter	Grape juice	Test wine
Sugar (g/L)	261.1	4.19
Ethanol (%)	ND	12.8
Titrable acidity (g/L)	ND	6.34
Total phenolic content (mg/L)	566.66	1340
Total flavonoid content (mg/L)	407.29	462.5
Tannin content (mg/L)	896.87	1159.3
Glycerol content (g/L)	ND	4.92
pH	3.71	3.76
Wine color		
Wine color density (a. u.)	2.18	4.49
Wine color hue	1.00	1.17
Estimate of SO_2 resistant pigments (a. u.)	0.92	1.70
Total red pigments (a. u.)	0.06	0.10
Modified wine color density (a. u.)	3.74	4.49
Modified wine color hue	1.32	1.12

ND; Not detected

Grape juice and the Shiraz wine prepared through fermentation (test wine) were analysed for different parameters including initial sugar, total phenolic content, total flavonoid content, tannin content and color. Initial grape sugar was 261 g/L. Total phenolic contents and tannin contents were higher in test wine (1340 mg/L, 1159 mg/L, respectively) as compared to grape juice (566 mg/L, 896 mg/L, respectively).

Significant difference was not observed in pH of grape juice and wine. Wine color density was higher (4.49 a.u) than grape juice (Table 3.8). All these values were comparable with marketed wine.

3.7.4 Phenolic compounds in Shiraz variety

Various enzymes are responsible for efficient extraction of desirable red grape pigments and other phenolic compounds in grape pulp. These compounds can be faster released by the action of enzymes (Capounova and Drdak, 2002). Non-*Saccharomyces* yeasts have a prominent role in enzyme production. Therefore these non-*Saccharomyces* yeasts could positively contribute to the analytical and sensorial composition of wine with production of number of flavor active secondary metabolites (e.g. acids, alcohols, esters, phenolic compounds, aldehydes, ketones, volatile sulphur compounds) (Lambrechts and Pretorius, 2000).

By comparing the retention time and MS/MS spectra, total 14 polyphenols and organic acids were identified from both the samples (Figure 3.11). The organic acids

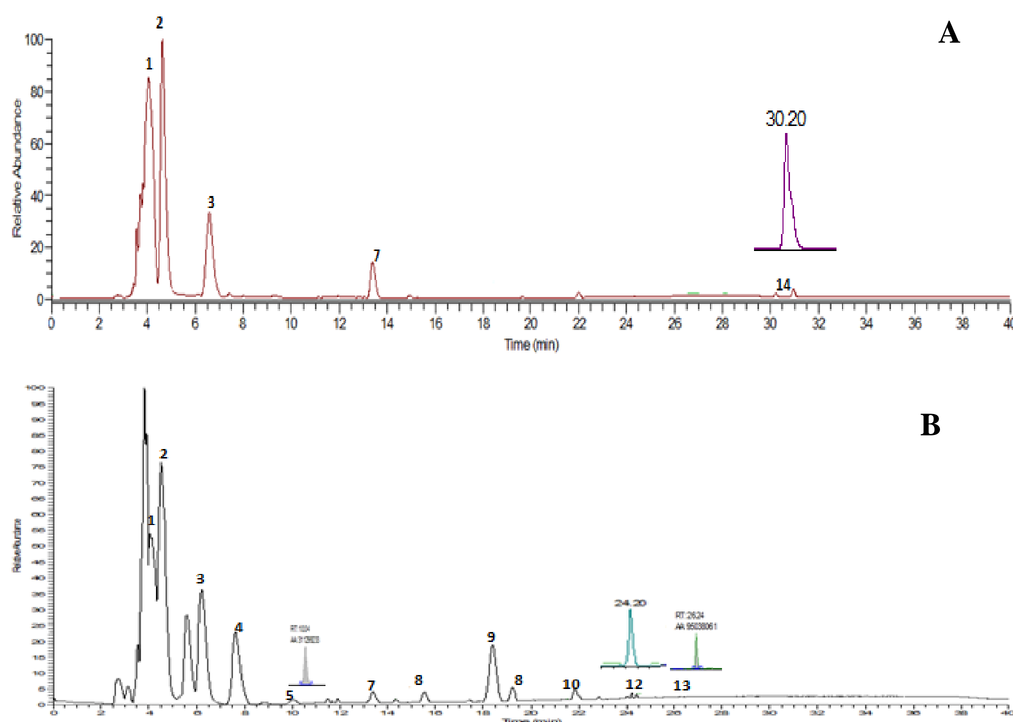


Figure 3.11 HR-LCMS chromatogram of A; Grape juice, B; laboratory wine prepared from Shiraz variety grapes. Peak no. 1; Tartaric acid, 2; Malic acid, 3; Citric acid, 4; Succinic acid, 5; Gallic acid, 6; Esculatin, 7; Glutationyl caffeoyl tartaric acid, 8; Catechin, 9; Fragment of piceatannol, 10; Syringic acid, 11; p-coumaric acid, 12; Quercetin-3-glucoside, 13; Resveratrol, 14; Vanilic acid β-d-glucopyranoside (mass peaks are listed in Annexure II)

included tartaric acid, malic acid, succinic acid, and citric acid. In polyphenols, hydroxybenzoic acids found were gallic acid, syringic acid and vanilic acid. p-Coumaric acid (a hydroxycinnamic acid) catechin (a flavanol) and resveratrol (a stillbene) were other polyphenolics.

Tartaric acid, malic acid and citric acid were detected in grape juice and wine. Gallic acid, syringic acid, succinic acid, fragment piceatannol, succinic acid and p-coumaric acid were detected in both marketed wine and test wine samples, whereas quercetin-3-glucoside, catechin, resveratrol and glutationyl caffeoyl tartaric acid were detected only in test wine (Table 3.9). Esculatin and vanilic acid β-d-glucopyranoside detected in marketed wine and grape juice, respectively, were not detected in test wine.

Phenolic compounds are mainly responsible for the astringency, color and bitterness of wine. The main type of phenolic compounds found in wine include hydroxybenzoic and hydroxycinnamic acids, stilbenes, flavones, flavonols, flavanonols, flavanols, and anthocyanins (Monagas et al., 2007). The free forms of *trans*-caffeic, *p*-coumaric acid, *trans*-ferulic acid and derivatives were reported in young Riesling wine (Baranowski and Nagel, 1981; Somers et al., 1987). Whereas, phenolic compounds mainly include hydroxycinnamic and benzoic acids, flavan-3-ols and flavonols are also reported in white wines (10.38 mg/100 mL), depending on the grape variety and enological factors (Neveu et al., 2010).

In grape juice, tartaric and malic acid concentration were 13310 mg/L and 10140 mg/L, respectively, which reduced to 1620 mg/L and 2340 mg/L, respectively in test wine. Acidity of wine is important for the quality and taste; organic acids like tartaric acid and malic acid impart sour taste to the wine. Gallic acid, succinic acid, fragments of piceatannol and syringic acid were not detected in grape juice because they may be bound to skin or present in seeds. These compounds got extracted in wine during fermentation by microbial action. Catechin (5.97 mg/L), p-coumaric acid (8.23 mg/L), and resveratrol (18.28 mg/L) were also detected in the test wine. Catechin reacts with tannin and is important to for primary flavor whereas resveratrol has antimicrobial, antioxidant and anticancer properties. Relative percentage of few compounds was calculated with respect to the concentration of tartaric acid (Table 3.9).

Table 3.9 Organic acids and polyphenols detected in grape juice and test wine of Shiraz variety

Compound	Peak No	RT (min)	Mass m/z	Molecular formula	Shiraz GJ mg/L	Test wine mg/L
Tartaric acid	1	4.05	149	$C_4H_6O_6$	13310	1620
Malic acid	2	4.50	133	$C_4H_6O_5$	10140	2340
Gallic acid	5	9.97	169	$C_7H_6O_5$	ND	19
Catechin	8	15.53/19.19	289	$C_{15}H_{14}O_6$	ND	5.9
P-coumaric acid	11	22.87	163	$C_9H_8O_3$	ND	8.2
Resveratrol	13	26.24	227	$C_{14}H_{12}O_3$	ND	18.2
					Relative percentage with respect to tartaric acid (%)	
Citric acid	3	6.22	191	$C_8H_8O_7$	33.99	62.44
Succinic acid	4	7.63	117	$C_4H_6O_4$	ND	39.78
Esculatin	6	11.68	177	$C_9H_6O_4$	ND	ND
Glutationyl caffeoyl tartaric acid	7	13.37	616	$C_{23}H_{27}N_3O_{15}S$	13.02	35.47
fragment piceatanol	9	18.38	175	-	ND	27.86
Syringic acid	10	21.84	197	$C_9H_{10}O_5$	ND	31.85
Quercetin 3-glucocide	12	24.20	463	$C_{21}H_{20}O_{12}$	ND	18.72
Vanilic acid β-d-glucopyranoside	14	30.24	329	$C_{14}H_{18}O_9$	2.02	ND

ND; Not detected

The levels of tartaric acid, gallic acid, catechin and p-coumaric acid were comparable, while, malic acid and resveratrol concentrations were higher than that reported by Ciu et al. (2012).

It has been hypothesized that the phenolic substances of wine might be responsible for potential health benefits through their antioxidant, anti-inflammatory properties, inhibition of platelet aggregation and antimicrobial activities (Goldberg et al., 1999; Wang et al., 2002). Organic acids, major sour substances and polyphenols which are most abundant and bioactive compounds play important roles in health-promoting properties and the taste of red wine (Waterhouse, 2002).

The primary flavor of wine is derived from the grapes. However, secondary flavors are derived from ester formation by yeasts during wine fermentation (Lambrechts and Pretorius, 2000; Nykanen, 1986). Flavor producing yeasts included *P. anomala* (*Hansenula anomala*) and *K. apiculata*. *C. pulcherrima* is also known to be a high producer of esters (Bisson and Kunkee, 1991; Clemente-Jimenez et al., 2004).

In conclusion non-*Saccharomyces* yeasts such as *H. guilliermondii, H. uvarum, H. opuntiae* and *Issatchenkia terricola* have the potential to produce different extracellular enzymes. Mixed culture fermentation with these non-*Saccharomyces* yeasts and *S. cerevisiae* can be used to develop better quality wine with regards to flavor, aroma and taste.

C. Natural products for the control of wine spoilage yeasts

Vinification is critically influenced by different steps from growing, harvesting vines in the vineyard to fermentation, aging and storage in the winery. Inadequate precautions or poor practice during any of these steps leads to growth of wine spoilage organisms and consequent wine fault. The yeasts involved in wine spoilage mainly are from genera *Brettanomyces, Candida, Debaryomyces, Hansenula, Kluveromyces, Pichia, Metschnikowia, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* (Loureiro and Malfeito-Ferreira, 2003).

Traditionally, sulphur dioxide (as potassium metabisulphite), sorbic acid, fumaric acid and dimethyldicarbonate (DMDC) are used for preservation of different wines in various countries. The added sulphite exists as molecular SO_2 at low pH values, as bisulphite ions at intermediate pH values, and as sulphite ions at high pH values (Romano & Suzzi, 1993). Molecular SO_2, the antimicrobial/active form from the three, is only around 5-10% in wines with pH 3, which further decreases with increase in pH and becomes negligible at pH 4. Though effective against LAB, some yeasts and AAB have been reported to proliferate at the maximum concentrations of SO_2 added. Health risks like allergic reactions in some individuals those with asthma and organoleptic changes are other problems associated with the use of SO_2. Sorbic acid, fumaric acid and DMDC are less commonly used due to their drawbacks like - sorbic acid is not effective in controlling LAB, AAB and yeasts *Brettannomyces*, *Zygosaccharomyces*, etc., fumaric acid gets inactivated by fumarase from LAB and yeasts, whereas though highly effective as antimicrobial, DMDC gets hydrolysed to CO_2 and methanol with no lasting activity in the bottled product.

Due to these drawbacks and growing consumer bias against chemical preservatives, research efforts are directed towards use of different physical methods and exploitation of natural antimicrobial compounds obtained from plants, animals and microorganisms for wine preservation (Toit and Pretorius, 2000). Pulsed electric fields technology (Marselles-Fontanet et al., 2009; Puertolas et al., 2009), high power ultrasonics (Jiranek et al., 2008), thermal inactivation (Couto et al., 2005) have also been suggested to prevent the growth of wine spoilage yeasts. Many studies have demonstrated the potential of natural products such as hydroxycinnamates and organic acids (Stead, 1993), chitosan (Gómez-Rivas et al., 2004), nisin (Rojo-Bezares, 2007),

lysozyme (Gerbaux et al., 1997), antimicrobial peptides (Bom et al., 2001; Enrique et al., 2007), killer toxins (Comitini et al., 2004a; Santos et al., 2009), natamycin (Thomas et al., 2005), β-glucanases (Enrique et al., 2010), bovine lactoferrin-derived peptides (Enrique et al., 2008) for the control of wine spoilage yeasts and bacteria.

In an attempt was to identify new natural products for wine preservation, the antifungal activity and mode of action of a microbial secondary metabolite from *Pseudomonas aeruginosa* and plant essential oils, namely, carvacol and thymol were evaluated against natural yeast flora of grapes as well as wine spoilage yeasts and discussed in this chapter.

3.8 Production, extraction and purification of a microbial metabolite

Pseudomonas strain MCC 2142 is a fluorescent pseudomonad. The isolate produced extracellularly yellowish-green pigment in soyabean meal medium after prolonged incubation (28° C for 4 d). Supernatant of the soyabean meal medium showed inhibition of wine spoilage yeasts growth in disc diffusion assay. Further the potential antifungal compounds were concentrated in chloroform. Yellow brown residue was extracted with chloroform and solvent was evaporated under vacuum to yield chloroform extract 5.5 g. The extract of chloroform was fractionated using methanol and the residue was purified using silica gel chromatography (200-300 mesh) with methanol: chloroform (5:95) mixture for elution.. From 5.5g chloroform extract, 3 compounds were extracted and purified, compounds **1** (50 mg), compound **2** (22 mg) and compound, **3** (5 mg). It was further purified by crystallization and characterized.

3.9 Physico-chemical characterization of microbial natural products

Compound **1** was isolated as white crystals. The HR-ESI-MS of **1** showed $[M + 1]^+$ peak at 227.1750 in agreement with molecular formula $C_{12}H_{22}N_2O_2$ (Annexure II, 1A, 1B) indicating three degrees of freedom. ^1H NMR revealed triplet methyl at δ 0.93 (t, 6.7 Hz, δ_C 12.0), secondary methyl at δ 1.01 (d, 7.0 Hz, δ_C 15.6) (Annexure II, 1C). ^{13}C NMR showed 12 resonances corresponding to 4 methyls, 2 methylenes, 4 methines and two carbonyl carbons (Annexure II, 1D). This along with methylene at δ_C 25.1 (δ_H 1.48 m, 1.20 m), methine carbons at δ_C 39.8 (δ_H 1.94 bs) and 60.5 (δ_H 3.83, bd, 3.1 Hz), carbonyl carbon at δ 168.8 towards isoleucine (Annexure II, Table 1). This was confirmed by analysis of H2BC spectra that confirmed isoleucine

structure. This indicated compound **1** to be cyclic dipeptide with isoleucine as one amino acid. ^{1}H NMR showed two methyl doublets at δ 0.93 (d, 6.4 Hz) and 0.95 (d, 6.4 Hz) indicating another amino acid to be valine or its congener. Methylene carbon at δ_C 45.2 indicated towards homovaline. Homovaline was confirmed by detailed analysis of H2BC spectrum that confirmed skeletal connectivity. Cyclic nature of the dipeptide was confirmed by observation of HMBC correlation of both protons at δ_H 3.83 (H-2) and δ_H 3.93 (H-3') with both carbonyl carbons at δ_C 170.4 and 168.8. Thus compound **1** was identified as new natural product cyclo (-Ile-homo-Val). To the best of our knowledge, this is the first report of a cyclic dipeptide having homovaline as one of the amino acid.

Compound **2** was yellow crystalline needles with a melting point 245°C, and was extensively characterized by ESI-MS, ^{1}H-NMR and ^{13}C NMR. ESI-MS spectrum showed $[M + H]^+$ peak at 224 (base peak) and $[M + Na]^+$ peak at 246, corresponding to molecular formula $C_{13}H_9N_3O$ (molecular weight 223) with 11 indices of hydrogen deficiency. Peak at *m/z* 207 due to loss of NH_3 [M+1-17] suggested a fragmentation typical of amide containing compound (Annexure II, 2A, 2B). ^{1}H-NMR revealed presence of three methane at δ 8.97 (dd, J = 7.0; 1.3 Hz), 8.54 (dd, J = 8.7; 1.3 Hz) and 8.02 (dd, J = 7.0; 8.7 Hz), corresponding to 1, 2-3 trisubstituted benzene ring. ^{1}H-NMR also exhibited four peaks in the range of δ 8.34-7.97 (4H, m), indicating towards disubstituted ring (Annexure II, 2C). ^{13}C NMR revealed 13 resonances which included 7 methine and 6 quaternary carbons. Peak at δ 165.93 was indicative of amide carboxyl group (Annexure II, 2D). By comparing the data with previous report (Chin-A-Woeng et al., 1998), compound **2** was identified as phenazin-1-carboxamide (PC).

Compound **3** was isolated as pale yellow crystals with melting point 243°C - 245°C and characterized by ^{1}H-NMR, ^{13}C NMR, ESI-MS. The ESI-MS of **3** showed $[M + 1]^+$ peak at 238.97 in agreement with molecular formula $C_{14}H_{10}N_2O_2$ (Annexure II, 3A, 3B) with 11 indices of hydrogen deficiency. ^{1}H-NMR revealed presence of one methoxy at δ_H 3.51 (s). ^{1}H-NMR also exhibited four peaks in the range of δ_H 8.37 ddd (*J*= 7.5;1.3;0.5), 8.33 ddd (*J*= 8.0; 1.5;0.5), 8.04 ddd (*J*= 7.5; 7.5;1.5) and 7.99 ddd (*J*= 8.0; 7.5;1.3), indicating towards disubstituted ring and three peaks at δ_H 8.55 dd (*J*= 8.7; 1.25), 8.01 dd (*J*= 8.7; 7.0) and 9.00 dd (*J*= 7.0; 1.25) indicating presence of trisubstituted ring (Annexure II, Table 3A). ^{13}C NMR revealed

11 resonances which included 7 methine at δ_C 133.22(C-3), 127.97 (C-4), 135.11(C-5), 137.43(C-6), 130.07 (C-7), 130.29 (C-8) and 131.74 (C-9) and 6 quaternary carbons including Peak at δ_C 165.97 indicating presence of ester carboxyl carbon (Annexure II, Table 3B). By comparing the data with previous report (Nansathit et al., 2009), compound **3** was identified as phenazin-1-carboxylic acid methyl ester (PCME).

Pseudomonas sp., in general, were reported to produce extracellularly different secondary metabolites such as 2,4-diacetylphloroglucinol, phenazines, oomycin A, pyoluteorin, pyrrolnitrin, viscosinamide, pantocin A and B, butyrolactones etc. which showed antifungal activity (Raaijmakers et al., 2002). Although Pseudomonads produce ~50 phenazines with few strains producing more than one, 2-hydroxyphenazine-1-carboxylic acid, 1-hydroxyphenazine and PC are important as they were reported to have the maximum overall *in vitro* antifungal activity (Mavrodi et al., 2006). The isolated phenazines and the dipeptide along with two other natural products, namely, carvacrol and thymol were checked for antifungal activity against natural yeasts flora of grapes and wine spoilage yeasts.

3.10 Antifungal activity against grapes natural yeasts flora and wine spoilage yeasts

The cyclic dipeptide exhibited no antifungal activity against the grapes natural yeast flora. PC and PCME showed inhibitory effect on natural yeast flora of the grapes at both pH with more pronounced effect at pH 3.5. Whereas, potassium metabisulphite showed no effect on the tested strains at pH 6.5 due to no release of molecular SO_2. At pH 3.5, potassium metabisulphite exhibited antifungal activity against 6 tested strains from the natural flora with MIC in the range 64-256 μg/mL and no inhibition was observed for remaining 5 strains (MIC >256). In comparison, PC (32-128 µg/mL) and PCME (16-64 μg/mL) showed comparable or better antifungal activity at pH 3.5 against all the tested strains except for the *S. cerevisiae* strain used as inoculum for wine fermentation (MIC 256 μg/mL) (Table 3.10).

Carvacrol and thymol showed were also effective at both pH against natural yeast flora with more pronounced effect at pH 3.5 (Table 3.10). As compared to potassium metabisulphite, carvacrol and thymol showed comparable or better antifungal activity (16-128 μg/mL) at pH 3.5 against all the tested strains.

Table 3.10 Minimum inhibitory concentration (MIC in µg/mL) of natural products against natural yeast flora of grapes

Yeasts	$K_2S_2O_5$		PC		PCME		Carvacrol		Thymol	
	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)
C. azyma	>256	64	128	64	64	32	64	64	64	64
C. quercitrusa	>256	>256	128	64	64	32	128	128	128	128
D. hansenii	>256	256	128	64	64	32	128	128	128	64
H. guilliermondii	>256	64	64	32	32	16	64	16	64	32
H. uvarum	>256	64	128	64	64	32	64	64	64	64
I. orientalis	>256	>256	128	64	64	32	256	128	256	128
I. terricola	>256	256	64	32	32	16	64	32	64	32
P. membranifaciens	>256	128	256	128	128	64	256	128	128	128
S. cerevisiae	>256	>256	128	64	64	32	128	128	128	128
Z. steatolyticus	>256	>256	128	64	64	32	128	128	128	128
S. cerevisiae (inoculum strain)	>256	>256	>256	256	256	256	256	256	256	256

PC- Phenazine-1-carboxamide; PCME phenazine-1-caboxylic acid methyl ester

Table 3.11 Minimum inhibitory concentration (MIC in µg/mL) of natural products against wine spoilage yeasts

Yeasts	$K_2S_2O_5$		PC		PCME		Carvacrol		Thymol	
	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)	YPG (pH 6.5)	YPG (pH 3.5)
M. pulcherima NCIM 3109	>256	32	128	32	32	16	64	32	128	16
Z. rouxii NCIM 3460	>256	64	128	16	32	16	128	64	128	32
S. pombe NCIM 3457	>256	32	128	64	32	16	64	32	128	32
T. delbruekii NCIM 3295	>256	32	64	32	64	32	128	16	128	16
D. hansenii NCIM 3146	>256	32	128	32	64	32	64	16	128	16
D. bruxellensis NCIM 3534	>256	32	64	32	32	16	32	16	32	16

PC- Phenazine-1-carboxamide; PCME- phenazine-1-carboxylic acid methyl ester

For the seven wine spoilage yeasts viz. *Metchnikowia pulcherima*, *Zygosaccharomyces rouxii*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Debaryomyces* sp., *Kluveromyces marxianus* and *Dekkera bruxellensis*, similar pattern was observed with PC, PCME, Carvacrol and thymol i.e. comparable/better antifungal activity than potassium metabisulphite and at pH 3.5 than pH 6.5 (Table 3.11). PC and PCME exhibited MIC in the range of 16-64 µg/mL and 16-32 µg/mL against the tested strains at pH 3.5. Even at pH 6.5, maximum concentration of PC and PCME required for growth inhibition was 128 µg/mL and 64 µg/mL respectively. Whereas carvacrol and thymol exhibited MIC in the range of 16-64 µg/mL and 16-32 µg/mL against the tested strains at pH 3.5. Even at pH 6.5, maximum concentration of carvacrol or thymol required for growth inhibition was 256 µg/mL and 128 µg/mL respectively.

In wine the concentration of these compounds required to achieve desired control will be much less as their activity will be augmented by acidity, low pH, low temperature, low oxygen levels and presence of phenolic compounds. The yield of PC and PCME was significantly less (PC- 22 mg, PCME- 5 mg from 3 L fermentation broth). Large scale production of these compounds will not be cost effective; therefore, Carvacrol and thymol were chosen for further evaluation.

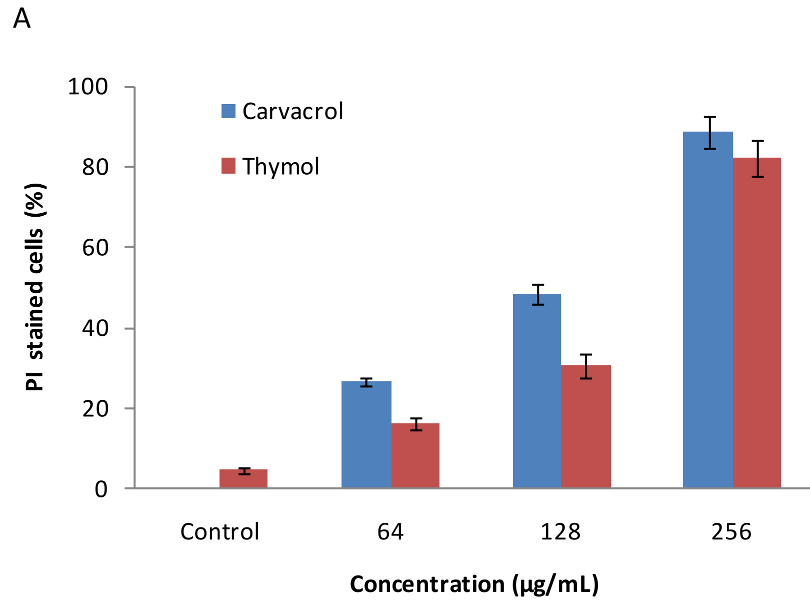
3.11 Mechanism of action of carvacrol and thymol in *Debaryomyces hansenii*

3.11.1 Propidium iodide staining

In present study, the mode of antifungal action of carvacrol and thymol was investigated in *D. hansenii.* Propidium iodide, a fluorescent membrane impermeable dye was used for staining of the cells after treatment with carvacrol and thymol. A dose dependent increase in the number of PI stained *D. hansenii* cells for both the compounds indicated damage to the cell membrane (Figure 3.12 A). Treatment with carvacrol and thymol at 256 µg/mL resulted in >80% PI positive cells (Figure 3.11C, D).

3.11.2 Release of cellular contents

Because of hydrophobicity, essential oil components including carvacrol and thymol partition in the lipids of the cell membrane, rendering the membrane permeable and leading to leakage of cell contents, thereby exerting their antibacterial action. Disruption of the membrane structure, formation of channels increases membrane permeability and leads to leakage of potassium ions and other cytoplasmic contents.



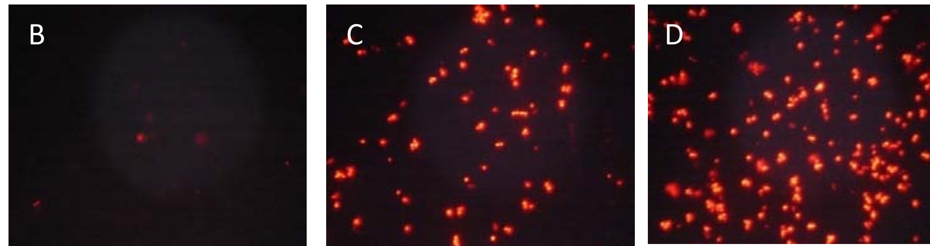


Figure 3.12 (A) Percentage of PI stained *Debaryomyces hansenii* cells after treatment with different concentrations of carvacrol and thymol, (B) control, (C) carvacrol 256 µg/mL and (D) thymol 256 µg/mL.

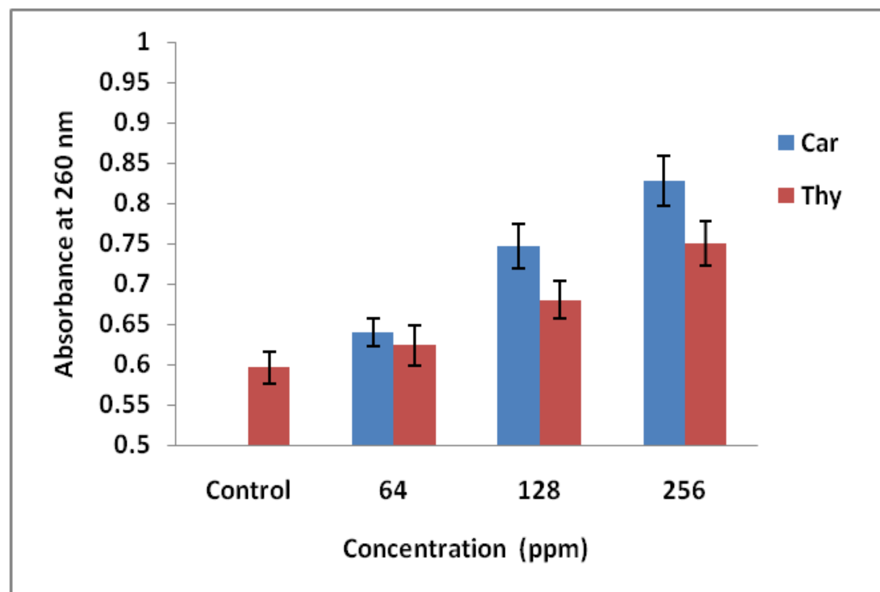


Figure 3.13 $A_{260\ nm}$ of the cell free supernatant after treatment of *D. hansenii* cells with different concentrations of carvacrol and thymol

This was evident from increase in the absorbance of the cell free supernatant after treatment with increasing concentrations of carvacrol or thymol (Figure 3.13).

3.11.3 Quantitation of sterols

Fungicidal activity of carvacrol and thymol was shown to be due to inhibition of ergosterol biosynthesis and disruption of membrane integrity (Ahmad et al., 2011). Quantitation of sterols showed dose dependent decrease in ergosterol levels after treatment with 32 and 64 µg/mL carvacrol or thymol. However, the depletion in ergosterol was much higher for 32 µg/mL fluconazole, a known ergosterol biosynthesis inhibiting antifungal drug (Figure 3.14). Secondly, fluconazole and other azole class ergosterol biosynthesis inhibitors are fungistatic, whereas carvacrol and

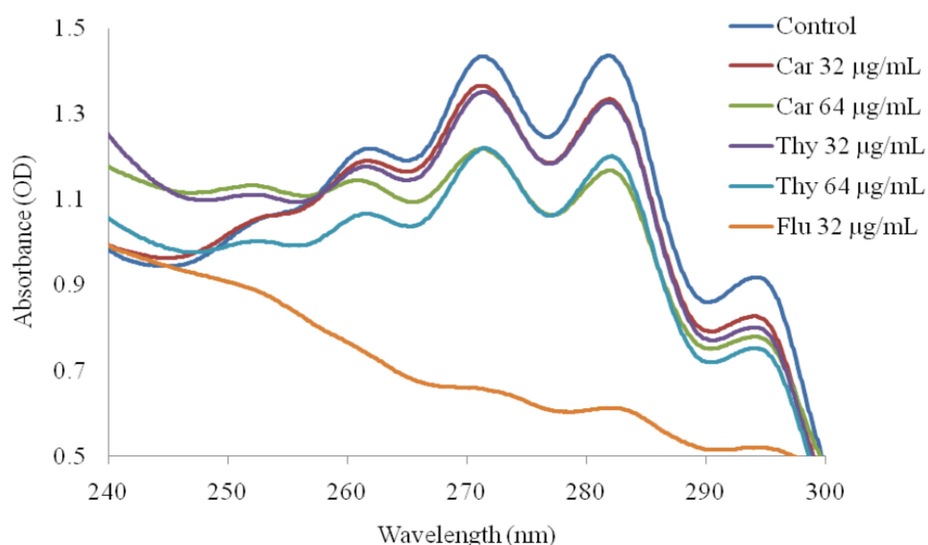


Figure 3.14 UV spectrophotometric sterol profiles of *D. hansenii* cells after treatment with 32 and 64 µg/mL of carvacrol (Car) and thymol (Thy). Fluconazole (Flu) at 32 µg/mL was used as positive control for ergosterol depletion.

thymol were fungicidal.

Thus, membrane damage and leakage of cytoplasmic content was found to be primary antifungal mechanism of carvacrol and thymol with ergosterol depletion as secondary effect.

3.12 Antimicrobial effect of carvacrol and thymol in wine

Effect of carvacrol and thymol against *M. pulcherima, T. delbrueckii, Z. rouxii, D. bruxellensis, S. pombe* and *D. hansenii* was also studied in red wine. Even after 16 d incubation, no visible growth of any of the yeasts was observed at 64 µg/mL

concentration of carvacrol or thymol. In absence of the compounds, increase in the absorbance and visible turbidity in the wine for all the cultures indicated survival and growth of the yeasts (Figure 3.15).

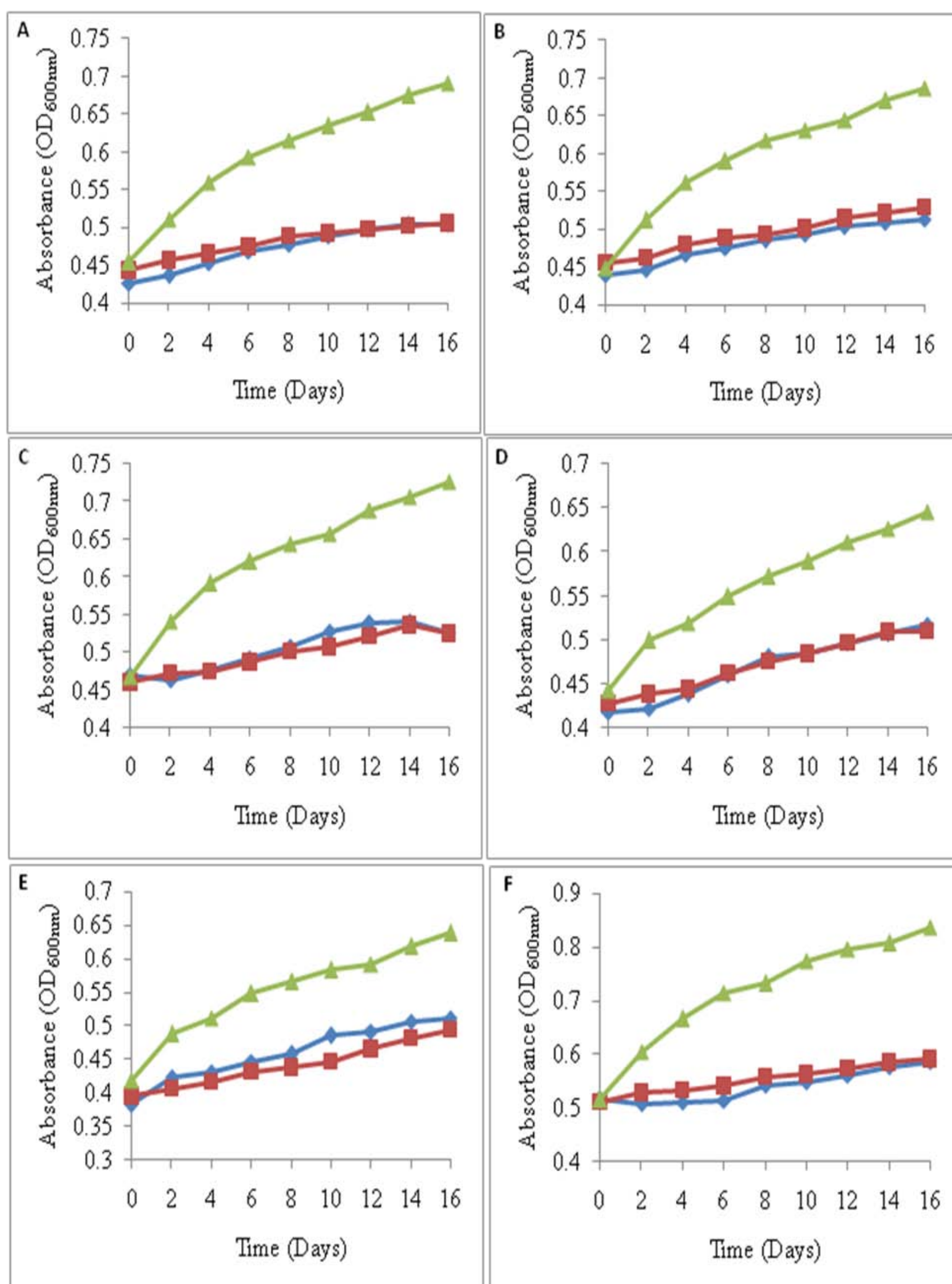


Figure 3.15 Inhibitory effect of carvacrol (—◆—) and thymol (—■—) at 64 µg/mL concentration on the growth of spoilage yeasts in wine (A) *M. pulcherima* NCIM 3109 (B) *T. delbrukii* NCIM 3295 (C) *D. hansenii* NCIM 3146 (D) *Z. rouxii* NCIM 3460 (E) *D. bruxellensis* NCIM 3534 and (F) *S. pombe* NCIM 3457. Growth without compounds is shown as (—▲—)

3.13 Toxicity testing

Hemolysis was not observed for carvacrol and thymol at concentrations ~256 μg/mL. Even at higher concentrations carvacrol and thymol (512 μg/mL) hemolysis was negligible (<5%) indicating that they are safe for use as preservative. Furthermore, carvacrol and thymol are USFDA approved food additives under flavoring agents and related substances category.

In conclusion, carvacrol and thymol showed promising *in vitro* and *in vivo* antifungal activity against wine spoilage yeasts. Their use in antimicrobial wine marinades at higher concentrations has been demonstrated previously. Oregano, main source of carvacrol and thymol, itself is being used to produce a oregano wine with many medicinal properties by Quezon Agricultural Experimental Station, Philippines. The antifungal concentrations of carvacrol and thymol demonstrated in present study are low and expected to have no effect on organoleptic properties of wine (accepted level of essential oil for food preservation is ~0.1%). Hence, carvacrol and thymol may have potential as preservative in wine and should be further explored as suitable natural alternative to chemical preservatives for the control of wine spoilage.

Chapter 4

Summary and conclusions

Detailed literature review presented in first chapter highlighted the important role of non-*Saccharomyces* yeast flora associated with grapes in determining the final quality of the wine. The diversity of non-*Saccharomyces* yeast flora associated with vineyards and wineries from different regions of wine producing countries have been widely documented. However, there are no such systematic studies for Indian vineyard and winery micro-flora. Therefore, present study was initiated with following objectives:

1) Isolation and identification of yeast flora of grapes from different varieties from different regions and its qualitative and quantitative changes during fermentation.
2) Profiling of enzymes, flavor and phenolic compounds produced by yeasts and its correlation with wine quality.
3) Effect of known preservatives, plant and microbial metabolites on the wine quality.

In India, Maharashtra is largest grape growing state accounts for most of the wineries with ~97% of India's wine production. Therefore, wine grapes of five varieties, namely, Cabernet, Bangalore Blue, Shiraz, Zinfandel and Sauvignon Blanc were collected from different vine yards located in Pune and Nashik regions of Maharashtra. The sugar content of grape juice from different varieties was between 163 g/L (Zinfandel) and 270 g/L (Sauvignon Blanc) and acidity was between 3.5 and 4.5. Total 78 yeasts were isolated and the numbers of yeasts obtained from different varieties were: Bangalore Blue, 24; Zinfandel, 19; Cabernet Sauvignon, 7; Shiraz, 17 and Sauvignon Blanc, 11.

Among the seventy eight isolates, 34 isolates had a creamy and glossy colony appearance with apiculate cell morphology typical of *Hanseniaspora* species. Creamy, smooth and butyrous colony type was displayed by 17 strains and their morphology was globose to ovoidal. Cells from 21 colonies were ovoidal to elongate with white dry appearance. Among these 21 strains five strains showed pseudomycelium formation. Two colonies were with yellowish mucoid morphology

and four pale brown colonies with pseudomycelium formation, probably *P. membrenifaciens,* were observed.

All seventy eight isolates fermented and assimilated glucose, while none of these isolates assimilated lactose. Nitrate and nitrite were not assimilated by the 78 isolates. Two clusters were generated, one with fifty one isolates from Pune region and second with twenty seven isolates from Nashik region. Cluster analysis of the isolates was carried out on the basis of comparison of results for 15 biochemical tests with 11 comparable standard strains reported in the literature. In cluster for Pune region, two major branches (I and II) with seven clusters were generated. Identification of *C. azyma, C. quercitrusa, C. diversa, P. manshurica* in the cluster 2, 3, 5 and 6, respectively was possible using the biochemical tests. In cluster for Nashik region, two major branches (I and II) with six clusters were generated. No clear grouping was observed in case of *Issatchenkia* sp. and *Hanseniaspora* sp. as they had several similar tests used for identification and strain dependant variations among the isolates.

Species level identification was done by ITS1-5.8S-ITS2 sequencing. Total sixty four isolates showed 99% similarity with type strain and fourteen isolates showed less than 98% similarity. Therefore, further identification of fourteen isolates was done by 26S rDNA sequencing. Based on the sequence analysis from both methods, the isolates identified were from, two isolates as *C. azyma* and three as *C. quercitrusa*. Twenty isolates were identified as *H. guilliermondii*, nine as *H. uvarum,* and five as *H. opuntiae.* Six isolates identified as *I. orientalis*, ten isolates were *I. terricola.* From *Pichia* two isolates were *P. membrenifaciens* and two isolates were *P. manshurica.* Thirteen isolates were identified as *S. cerevisiae* and two isolates were identified as *Z. steatolyticus*. Single isolate of each were identified *as C. diversa, T. delbrueckii, P. kluyveri* and *P. fermentans.*

Species such as *C. diversa*, *I. orientalis, I. terricola, H. guilliermondii, H. opuntiae, H. uvarum, P. membranifaciens, P. manshurica, P. fermentans, P. kluyveri, T. delbrueckii, Z. steatolyticus* and *S. cerevisiae* found in the present study are similar to those species studied on grapes and wines in other parts of the world. *Hanseniaspora* sp. was predominantly present among the isolates obtained from the five vine varieties. Among the 78 identified indigenous grape yeast isolates, the largest diversity of yeast species (eight) was found in the Bangalore Blue and Shiraz vine variety followed by Zinfandel (four), Sauvignon Blanc (seven), and Cabernet

Sauvignon (Two). Species such as *P. fermentans, P. kluyveri,* and *T. delbrueckii* were specifically associated with Nashik region whereas *C. azyma, C. diversa, P. membranifaciens* and *Z. steatolyticus* were only found from Pune region. For the first time, *C. azyma* isolated from Bangalore Blue variety of Pune region was reported as grape yeast flora. This association may be attributed to the change in cropping pattern from sugarcane to viticulture in the vine growing regions and the known association of *C. azyma* with sugarcane phylloplane.

Non-*Saccharomyces* yeasts are known to produce different enzymes responsible for extraction of flavor and phenolics in the wine. Therefore, all the seventy eight yeast strains were screened for the extracellular hydrolytic production of enzyme of enological interest. From the four enzymes evaluated, pectinase activity was present in the all isolates. Among the 78 isolates, highest glucosidase (1010 nmol/mL/h), pectinase (27458 nmol/mL/h), glucanase (27279 nmol/mL/h) and protease (10810 nmole/mL/h) activity were observed for isolates *I. terricola* I68, *H. guilliermondii* I5, *I. terricola* I57, and *H. guilliermondii* I56 respectively. *Candida* and *Hanseniaspora* species were major contributors for protease activity. Almost all isolates were capable of β-1, 3 glucanase productions, but *H. uvarum* (14271 nmol/mL/h) and *I. terricola* (27353 nmol/mL/h) were significant β-1, 3 glucanase producers. Maximum glucosidase activities were detected in *I. terricola* (624-904 nmol/mL/h) strains. *Saccharomyces* and non-*Saccharomyces* isolates studied in this work produced a broad range of enzyme activities of enological interest especially those related to hydrolysis of polymers present in grape juice. This could contribute to hydrolysis of natural precursors and consequently affect the aromatic characteristics and quality of the wine.

I. orientalis, I. terricola, D. hansenii and *P. membranifaciens* were significant producers of β-glucosidase. Importance of β-glucosidase in the wine industry is to release flavor compound i.e. terpenes from glycosidically bound precursors. Significant protease activity was observed in *Candia* (*C. azyma* and *C. quercitrusa*) species. Few strains of *Hanseniaspora* sp. and few *Issatchenkia* sp. also showed good protease activity. These strains if present may be helpful in clarification of wine.

Several studies have evaluated the use of controlled mixed fermentations using *Saccharomyces* and different non-*Saccharomyces* yeast species that are part of the winemaking environment. Multi starter non-*Saccharomyces*/*Saccharomyces* fermentations with *Hanseniaspora*, *Issatchenkia* and *Candida* sp. may enhance wine

quality. The trend in wine industry is to develop new fermentation techniques and to produce alternative alcoholic beverages. In such settings, the selection of non-*Saccharomyces* yeasts could be an important aspect for their potential application in the wine industry.

Profiling of microflora during fermentation was carried out to understand how it changes during fermentation. *Hanseniaspora* sp., *Issatchenkia* sp., *Pichia* sp. and *Torulaspora* sp. were found to be present on 0 d. The count of *Hansenispora* sp. was 1.6×10^4 cells/mL in grape juice and it reached to 2×10^6 on 3rd d and further 10^7 cells/mL on 6th d of fermentation. Afterwards, the cell concentration decreased with increase in ethanol concentration. From the five species present on 0 d, *Torulaspora* sp. could not be detected on 3rd d, *Pichia* sp. disappeared on 6th d, whereas only *S. cerevisiae* was present in the sample from 9th d of fermentation. *Issatchenkia* sp. and *Pichia* sp. were present in grape juice at 3×10^4 and 4×10^4 cells/mL, respectively. On 3rd d of fermentation, *Issatchenkia* sp. and *Pichia* sp. count reached to 7×10^6 and 5.2×10^5 cells/mL, respectively. Inoculum of *S. cerevisiae* was 1×10^6 cells/mL on 0 d which first increased and then decreased to 8.1×10^6, 4×10^8, 1.6×10^8, 1.6×10^7 and 8×10^5 cells/mL on 3rd, 6th, 9th, 12th, and 15th d, respectively.

It was essential to study the enzymatic activities during winemaking, since their levels may vary throughout the fermentation. In present study, fluctuations in the enzyme levels were detected during alcoholic fermentation but all four activities were detected during early days of fermentation. The levels of protease, pectinase and glucosidase decreased in late stage of fermention. Pectinase levels were high (>4500 nmol/mL/h) and constant till 6th d, which then dropped by >60% from 9th d. Glucanase activity was not detected at 0 d, which then increased gradually and was maximum, i.e. 122 nmol/mL/h on 6th d. Glucanase activity was not detected in samples from 9, 12 and 15th d. Protease level was also maximum (568 nmol/mL/h) on 6th d and decreased in late stage of fermentation. Maximum level of β-glucosidase (36 nmol/mL/h) was on 0 d. Monitoring of these enzymes during fermentation could be useful in deciding strategies to prevent stuck fermentation.

Phenolics analysis showed that tartaric and malic acid concentration in grape juice were 13310 mg/L and 10140 mg/L, respectively, which reduced to 1620 mg/L and 2340 mg/L, respectively in test wine. Acidity of wine is important for the quality and taste; organic acids like tartaric acid and malic acid impart sour taste to the wine. Gallic acid, succinic acid, fragments of piceatannol and syringic acid found in test

wine were not detected in grape juice because they may be bound to skin or present in seeds. These compounds may have got extracted in wine during fermentation by microbial action. Catechin (5.97 mg/L), p-coumaric acid (8.23 mg/L), and resveratrol (18.28 mg/L) were also detected in the test wine. Catechin reacts with tannin and is important for thae primary flavor whereas resveratrol has antimicrobial, antioxidant and anticancer properties. The levels of tartaric acid, gallic acid, catechin and p-coumaric acid were comparable and malic acid, resveratrol concentrations were higher than that reported by Ciu et al. (2012).

In wine making non-*Saccharomyces* yeasts are important in clarification, filtration and to enhance flavor of wine, but there are certain yeasts which affect the wine by producing compounds beyond the threshold value which leads to off taste, odor and flavor. Along with *Dekkera bruxellensis,* few other species are involved in wine spoilage such as *Candida, Debaryomyces, Hansenula, Kluveromyces, Pichia, Metschnikowia, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces.*

Control of wine spoilage is one of the challenges in wine industry. Different chemical preservatives are used to control these yeasts. In our study, two microbial metabolites, phenazine-1-carboxamide (PCN) and phenazine -1-carboxymethyl ester (PCME) were produced, extracted and purified from *Pseudomonas* strain MCC 2142. Antimicrobial activity of PCN, PCME and plant essential oils (carvacrol and thymol) was checked against natural yeasts flora and seven wine spoilage yeasts, namely, *Metchnikowia pulcherima*, *Zygosaccharomyces rouxii*, *Schizosaccharomyces pombe*, *Torulaspora delbrueckii*, *Debaryomyces* sp., *Kluveromyces marxianus* and *Dekkera bruxellensis*. PC and PCME showed potent inhibitory effect on natural yeast flora of the grapes at both pH with more pronounced effect at pH 3.5 than 6.5. As expected, potassium metabisulphite showed no effect on the tested strains at pH 6.5 due to no release of molecular SO_2. At pH 3.5, potassium metabisulphite exhibited antifungal activity against six tested strains from the natural flora with MIC in the range 64-256 μg/mL and no inhibition was observed for remaining 5 strains (MIC >256 μg/mL).In comparison, PC (32-128 μg/mL) and PCME (16-64 μg/mL) showed comparable or better antifungal activity at pH 3.5 against all the tested strains except for the *S. cerevisiae* strain used as inoculum for wine fermentation (MIC 256 μg/mL).

Carvacrol and thymol also showed potent inhibitory effect on natural yeast flora of the grapes at both pH with more pronounced effect at pH 3.5. In comparison to potassium metabisulphite, carvacrol and thymol showed comparable or better

antifungal activity (16-128 μg/mL) at pH 3.5 against all the tested strains. MIC was higher only for the *S. cerevisiae* strain used as inoculum for wine fermentation (MIC 256 μg/mL).

In case of spoilage yeasts, similar pattern was observed with carvacrol, thymol exhibiting comparable/better antifungal activity than potassium metabisulphite and at pH 3.5 than pH 6.5. Carvacrol and thymol exhibited MIC in the range of 16-64 μg/mL and 16-32 μg/mL, respectively, against the tested strains at pH 3.5. The potential of carvacrol and thymol as a natural preservative for the control of spoilage yeasts was evaluated in wine. For both the compounds all spoilage yeasts were inhibited at 64 μg/mL. The growth was not observed even after 16 days of incubation.

The mode of antifungal action of carvacrol and thymol was investigated in *D. hansenii.* After treatment with carvacrol and thymol, a dose dependent increase in the number of PI stained *D. hansenii* cells indicated damage to the cell membrane. Treatment with carvacrol and thymol at 256 μg/mL resulted in >80% PI positive cells. Disruption of the membrane structure, formation of channels increases membrane permeability and leads to leakage of potassium ions and other cytoplasmic contents. This was evident from increase in the absorbance of the cell supernatant after treatment with increasing concentrations of carvacrol or thymol. Quantitation of sterols showed dose dependent decrease in ergosterol levels after treatment with 32 and 64 μg/mL carvacrol or thymol. Hemolysis was not observed for carvacrol and thymol at concentrations upto 256 μg/mL. Even at higher concentrations of carvacrol and thymol (512 μg/mL) hemolysis was negligible (<5%). Thus, carvacrol and thymol holds promise as natural preservatives for wine.

Thus, the salient findings of the thesis can be summarized as follows:

- Systematic study of yeast diversity from the different wine grape varieties commonly used in India was carried out. Total 78 natural yeasts associated with five grape varieties (Bangalore Blue, 24; Zinfandel, 19; Cabernet Sauvignon, 7; Shiraz, 17 and Sauvignon Blanc, 11) were isolated and identified from Pune and Nashik region.
- Higher levels of in vitro enzymes production by strains *I. terricola* I68; glucosidase, *H. guilliermondii* I5; pectinase, *I. terricola* I57; glucanase, and *H. guilliermondii* I56; protease indicated towards their potential use in mixed culture fermentation along with starter strain to develop good quality wine with regards to flavor, aroma and taste.

- Profiling of non-*Saccharomyces* yeasts flora and its relation to enzyme activities during fermentation and final wine quality were demonstrated in Shiraz variety. This study will also be useful to control the fermentation process and avoid wine spoilage.
- Carvacrol and thymol (USFDA approved flavoring agent) may have potential as alternative to the currently used chemical preservatives in wine industry. Considering that *Debaromyces, Pichia* and *Dekkera/Brettanomyces* yeasts cause heavy economic losses in winemaking; these natural antimicrobial compounds appear to be apt tools for the control of wine spoilage.

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Annexure I - List of accession numbers

Region	Variety	Isolate	Name of organism	Accession No
Pune	Bangalore blue	I1	*Candida azyma*	FJ231458
		I2	*Hanseniaspora guilliermondii*	FJ231450
		I3	*Issatchenkia terricola*	FJ231425
		I4	*Issatchenkia terricola*	KJ729273
		I5	*Hanseniaspora guilliermondii*	FJ231448
		I6	*Candida quercitrusa*	FJ231428
		I7	*Candida quercitrusa*	KJ729274
		I8	*Hanseniaspora guilliermondii*	KJ729335
		I9	*Hanseniaspora guilliermondii*	FJ231442
		I10	*Hanseniaspora guilliermondii*	KJ729275
		I11	*Issatchenkia orientalis*	FJ231418
		I12	*Pichia membranifaciens*	FJ231459
		I13	*Pichia membranifaciens*	KJ729276
		I14	*Pichia manshurica*	KJ729277
		I15	*Issatchenkia terricola*	KJ729278
		I16	*Issatchenkia terricola*	FJ231426
		I17	*Issatchenkia terricola*	FJ231427
		I18	*Hanseniaspora uvarum*	FJ231455
		I19	*Issatchenkia orientalis*	FJ231421
		I20	*Issatchenkia orientalis*	FJ231422
		I21	*Hanseniaspora guilliermondii*	KJ729279
		I22	*Hanseniaspora guilliermondii*	KJ729336
		I23	*Hanseniaspora guilliermondii*	FJ231444
		I24	*Candida azyma*	FJ231456
	Zinfandel	I25	*Saccharomyces cerevisiae*	KJ729280
		I26	*Hanseniaspora guilliermondii*	FJ231466
		I27	*Issatchenkia orientalis*	FJ231419
		I28	*Hanseniaspora guilliermondii*	KJ729281
		I29	*Saccharomyces cerevisiae*	FJ231434
		I30	*Hanseniaspora guilliermondii*	KJ729282
		I31	*Saccharomyces cerevisiae*	KJ729283

		I32	*Saccharomyces cerevisiae*	KJ729284
		I33	*Saccharomyces cerevisiae*	KJ729285
		I34	*Saccharomyces cerevisiae*	FJ231432
		I35	*Hanseniaspora guilliermondii*	KJ729286
		I36	*Zygoascus steatolyticus*	FJ231438
		I37	*Zygoascus steatolyticus*	FJ231439
		I38	*Hanseniaspora guilliermondii*	KJ729287
		I39	*Hanseniaspora guilliermondii*	KJ729288
		I40	*Issatchenkia orientalis*	FJ231420
		I41	*Hanseniaspora guilliermondii*	FJ231443
		I42	*Saccharomyces cerevisiae*	FJ231429
		I43	*Saccharomyces cerevisiae*	KJ729289
	Shiraz	I44	*Hanseniaspora uvarum*	KJ810829
		I45	*Candida diversa*	KJ810830
		I46	*Hanseniaspora opuntiae*	KJ810831
		I47	*Hanseniaspora opuntiae*	KJ810832
		I48	*Hanseniaspora uvarum*	KJ810833
	Cabernet	I49	*Hanseniaspora opuntiae*	KJ810834
		I50	*Hanseniaspora uvarum*	KJ810835
		I51	*Hanseniaspora uvarum*	KJ810836
Nashik	Shiraz	I52	*Torulaspora delbrueckii*	KJ810802
		I53	*Hanseniaspora guilliermondii*	KJ810803
		I54	*Hanseniaspora uvarum*	KJ810804
		I55	*Issatchenkia terricola*	KJ810805
		I56	*Hanseniaspora guilliermondii*	KJ810806
		I57	*Issatchenkia terricola*	KJ810807
		I58	*Issatchenkia terricola*	KJ810808
		I59	*Pichia kluyveri*	KJ810809
		I60	*Hanseniaspora opuntiae*	KJ810810
		I61	*Hanseniaspora guilliermondii*	KJ810811
		I62	*Saccharomyces cerevisiae*	KJ810812
		I63	*Saccharomyces cerevisiae*	KJ810813
	Cabernet	I64	*Hanseniaspora uvarum*	KJ810814

	I65	*Hanseniaspora uvarum*	KJ810815
	I66	*Hanseniaspora uvarum*	KJ810816
	I67	*Hanseniaspora opuntiae*	KJ810817
Sauvignon	I68	*Issatchenkia terricola*	KJ810818
Blanc	I69	*Hanseniaspora guilliermondii*	KJ810819
	I70	*Issatchenkia terricola*	KJ810820
	I71	*Pichia fermentans*	KJ810821
	I72	*Saccharomyces cerevisiae*	KJ810822
	I73	*Saccharomyces cerevisiae*	KJ810823
	I74	*Candida quercitrusa*	KJ810824
	I75	*Pichia manshurica*	KJ810825
	I76	*Hanseniaspora guilliermondii*	KJ810826
	I77	*Saccharomyces cerevisiae*	KJ810827
	I78	*Issatchenkia orientalis*	KJ810828

Annexure II - High Resolution-Liquid Chromatography Mass Spectrometry chromatograms

Standards

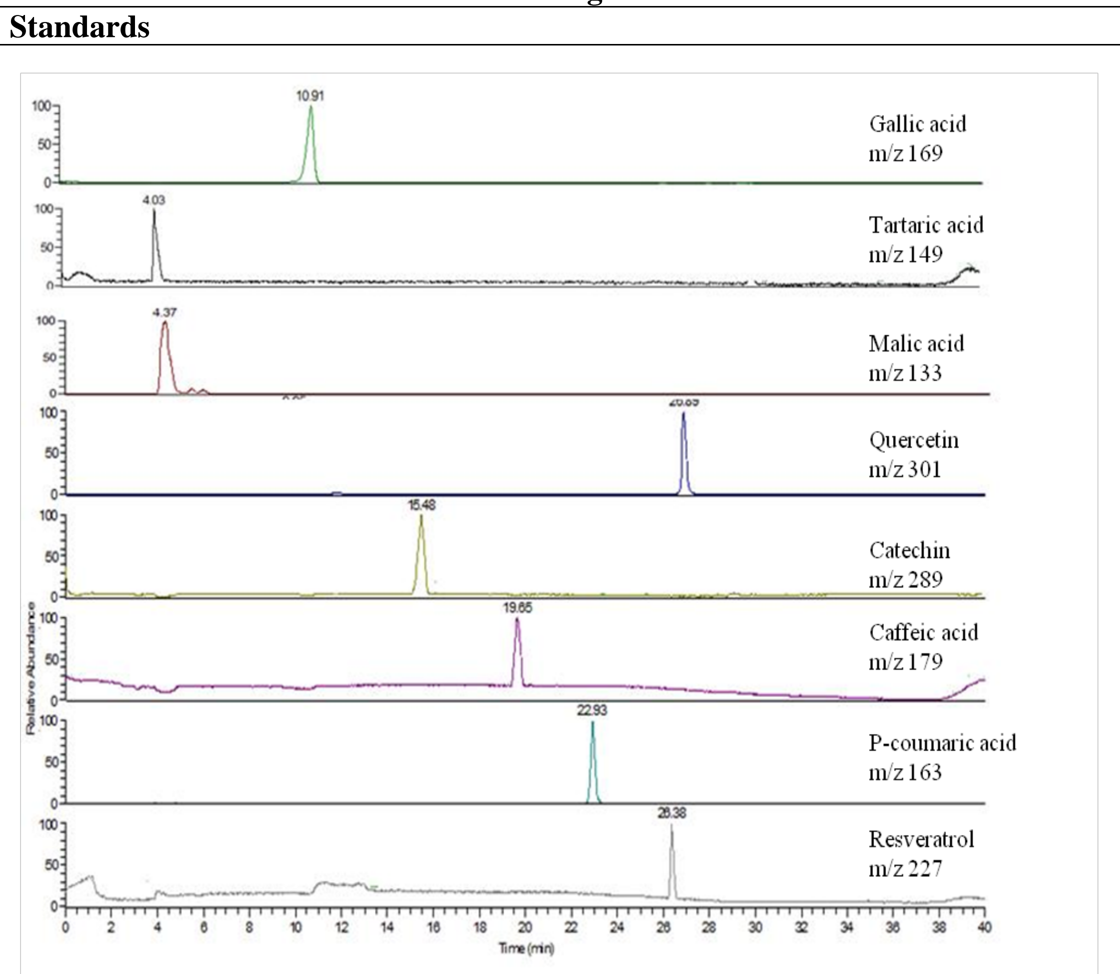


Figure II.1 Chromatogram showing peaks of standard compounds

A. Shiraz Grape Juice

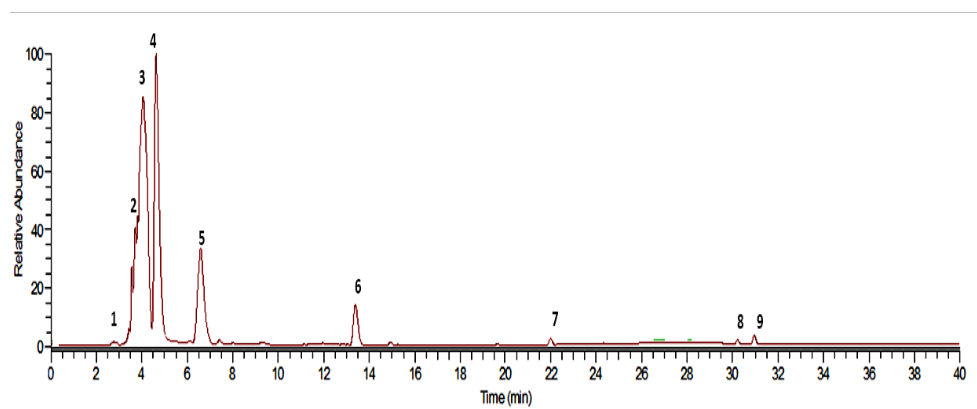


Figure II.2 LC/MS chromatogram of Shiraz

Table II.1 Retention Times (RT), molecular mass and compounds in Shiraz grape juice

Peak No	RT (min)	Mass m/z	Compound Name
A1	2.84	272	NI
A2	3.76	195	NI
A3	4.05	149	Tartaric acid
A4	4.74	133	Malic acid
A5	6.51	191	Citric acid
A6	13.39	616	Glutationyl caffeoyl tartarate
A7	22.0	366	NI
A8	30.24	329	Vanilic acid β d glucopyranoside
A9	30.95	207	NI

RT; Retention time, m/z; mass:charge, ND; Not identified

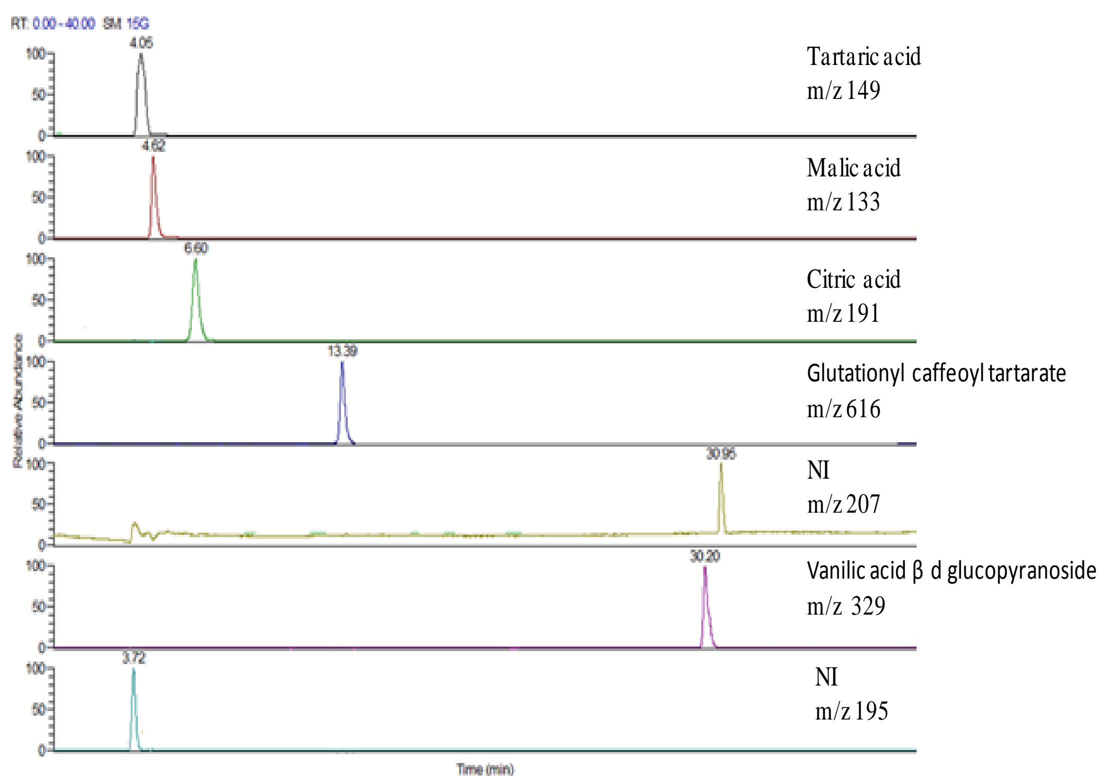


Figure II.3 LC/MS chromatogram showing base peaks of Shiraz grape juice

B. Shiraz Laboratory wine

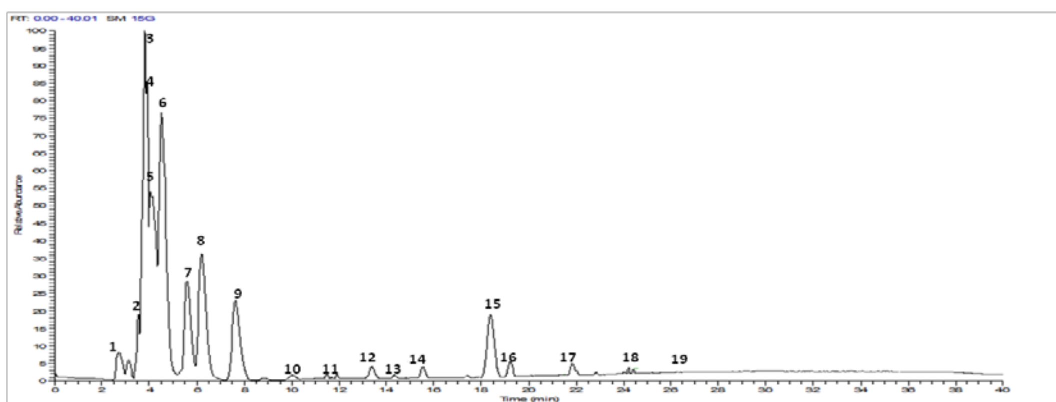


Figure II.4 LC/MS chromatogram of Shiraz test wine

Table II.2 Retention Times (RT), molecular mass and compounds in Shiraz test wine

Peak	RT(min)	m/z	Compounds
C1	3.09	273	NI
C2	3.54	225	NI
C3	3.80	369	NI
C4	3.89	545	NI
C5	4.00	149	Tartaric acid
C6	4.50	133	Malic acid
C7	5.60	147	NI
C8	6.22	191	Citric acid
C9	7.63	117	Succinic acid
C10	10.04	169	Gallic acid
C11	11.91	174	NI
C12	13.37	616	Glutationyl caffeoyl tartarate
C13	14.34	311	NI
C14	15.53	289	Catechin
C15	18.38	175	Fragment of piceatannol
C16	19.19	289	Catechin
C17	21.84	153	Syringic acid
C18	24.20	463	Quercetin 3 glucoside
C19	26.24	227	Resveratrol

RT; Retention time, m/z; mass:charge, ND; Not identified

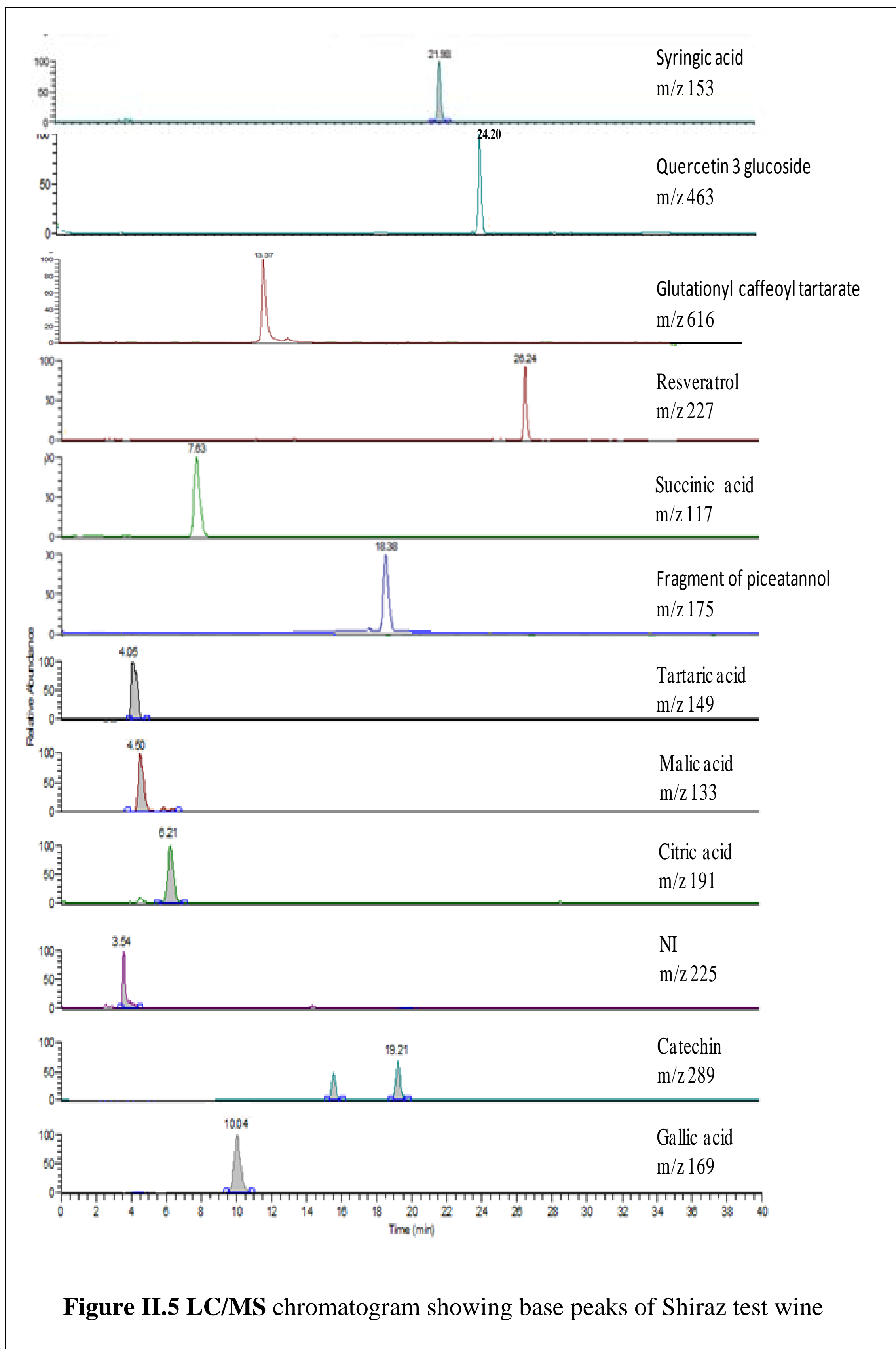
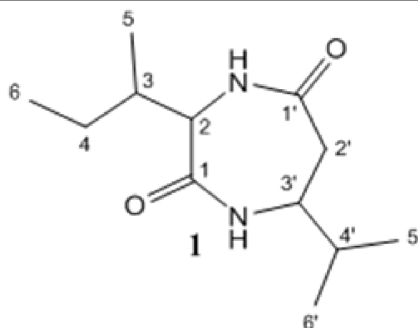


Figure II.5 LC/MS chromatogram showing base peaks of Shiraz test wine

Annexure III - Structure and spectra of *Pseudomonas* sp. MCC 2142 metabolites

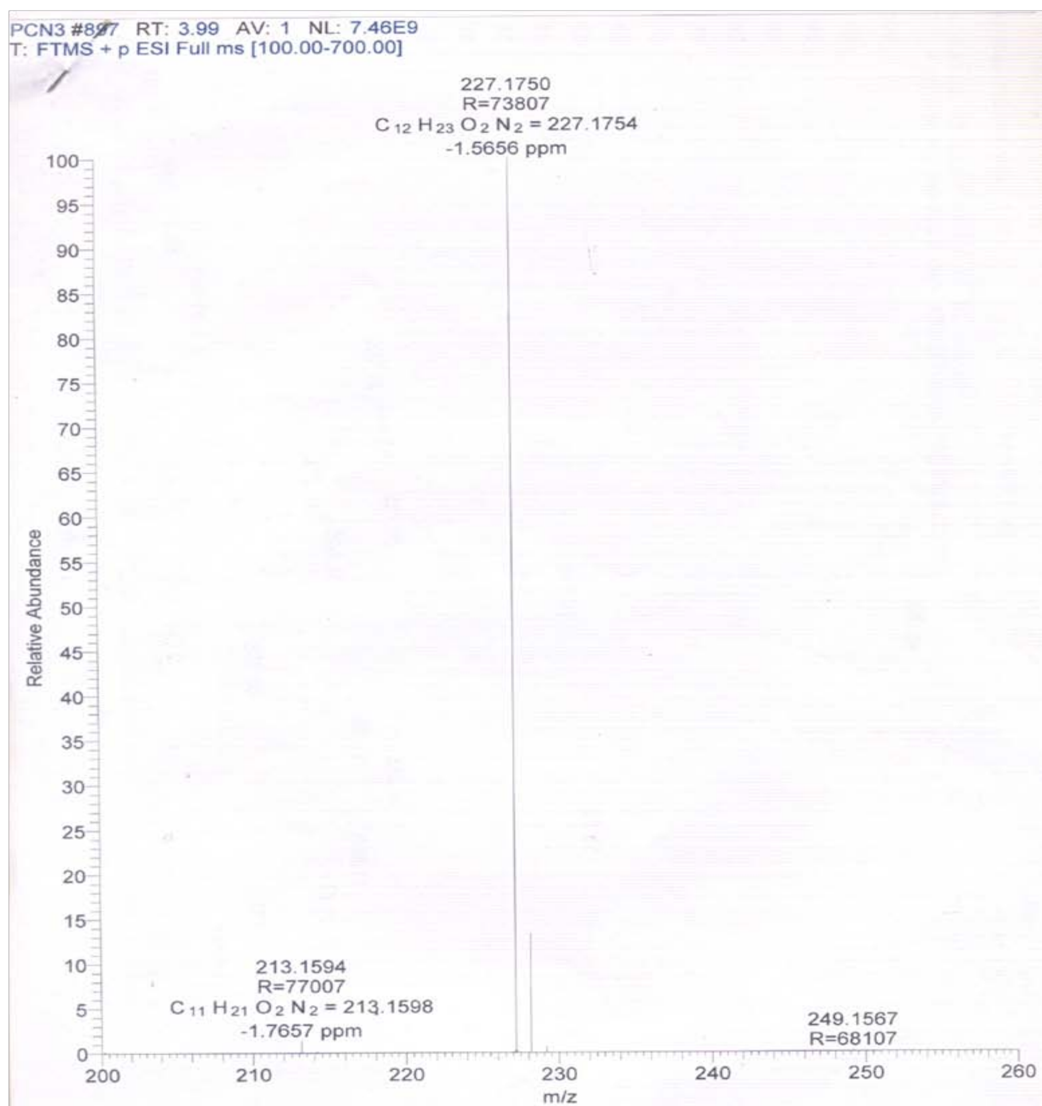
Compound 1- dipeptide cyclo (-Ile-homoVal)

1A. Structure

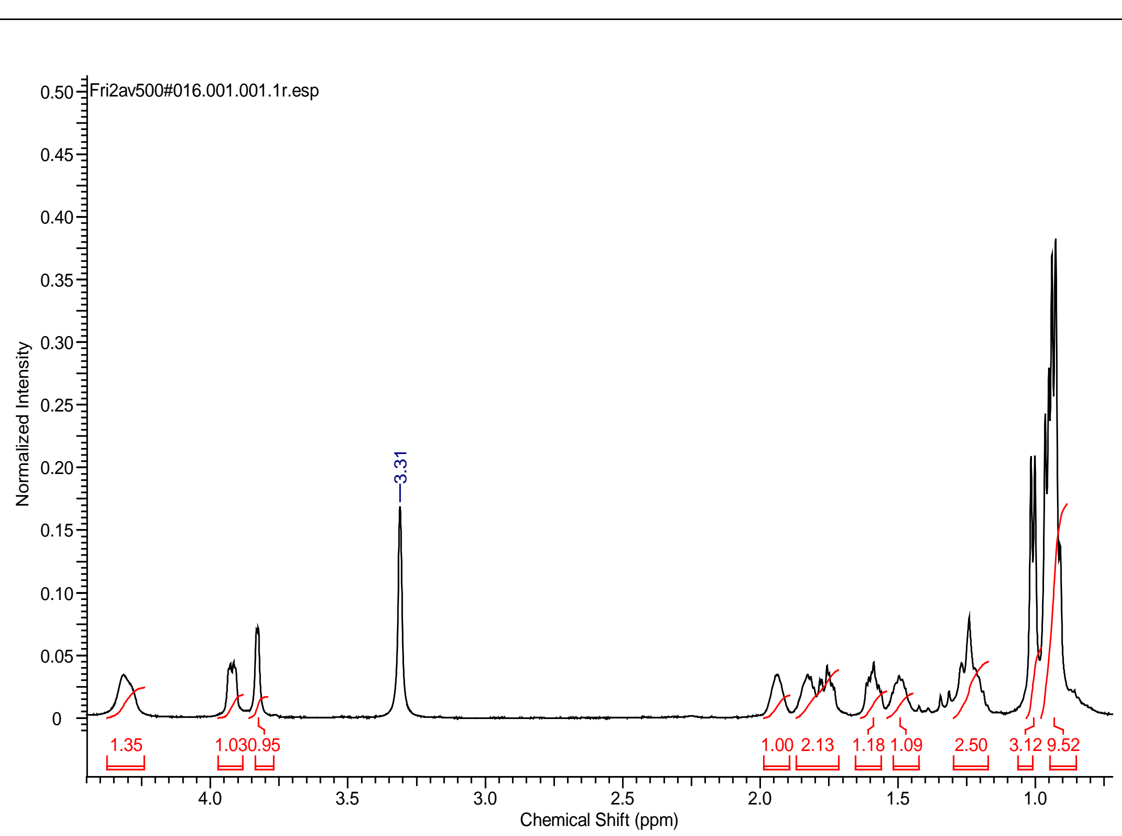


dipeptide cyclo (-Ile-homoVal)
($C_{12}H_{22}N_2O_2$ M^+ 227)

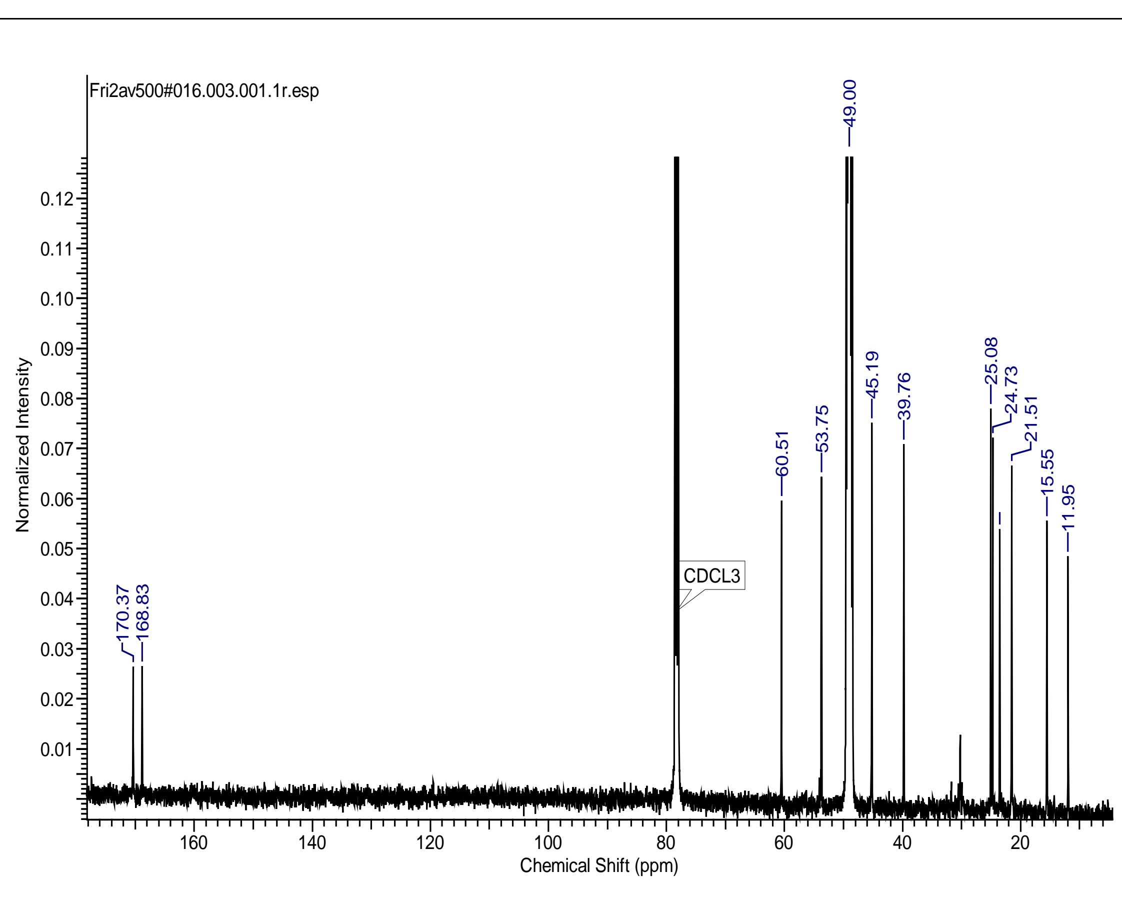
1B. HR-ESI-MS of compound **1**



1C. ^{1}H NMR spectrum of compound **1**



1D. ^{13}C NMR spectrum of compound **1**



1E. DEPT NMR spectrum of **1** (125 MHz, $CDCl_3$: CD_3OD)

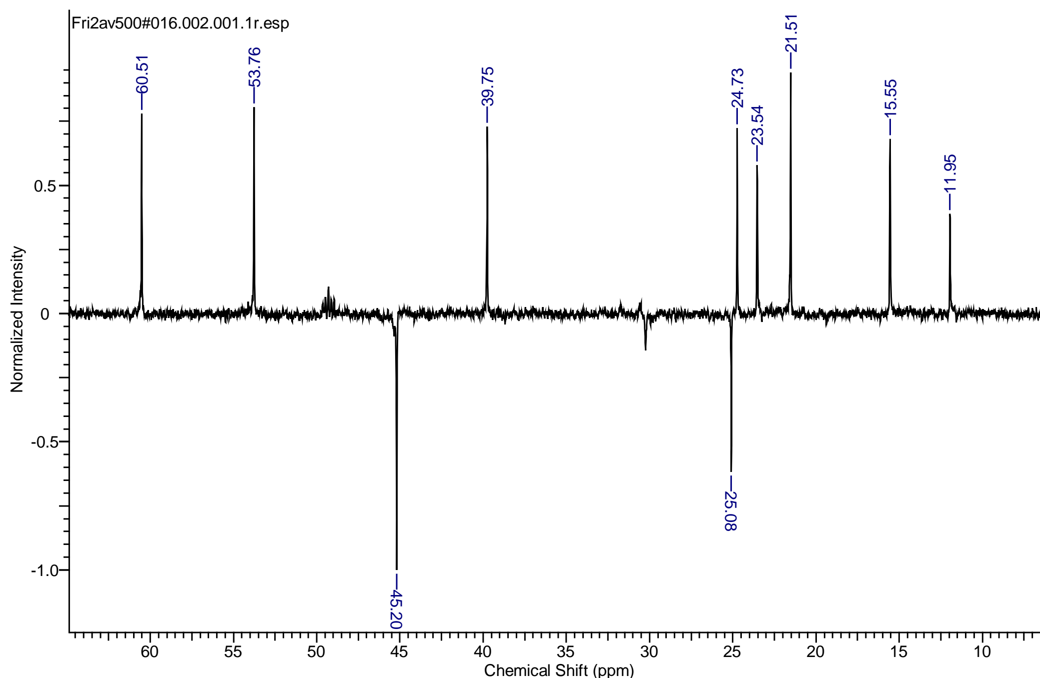
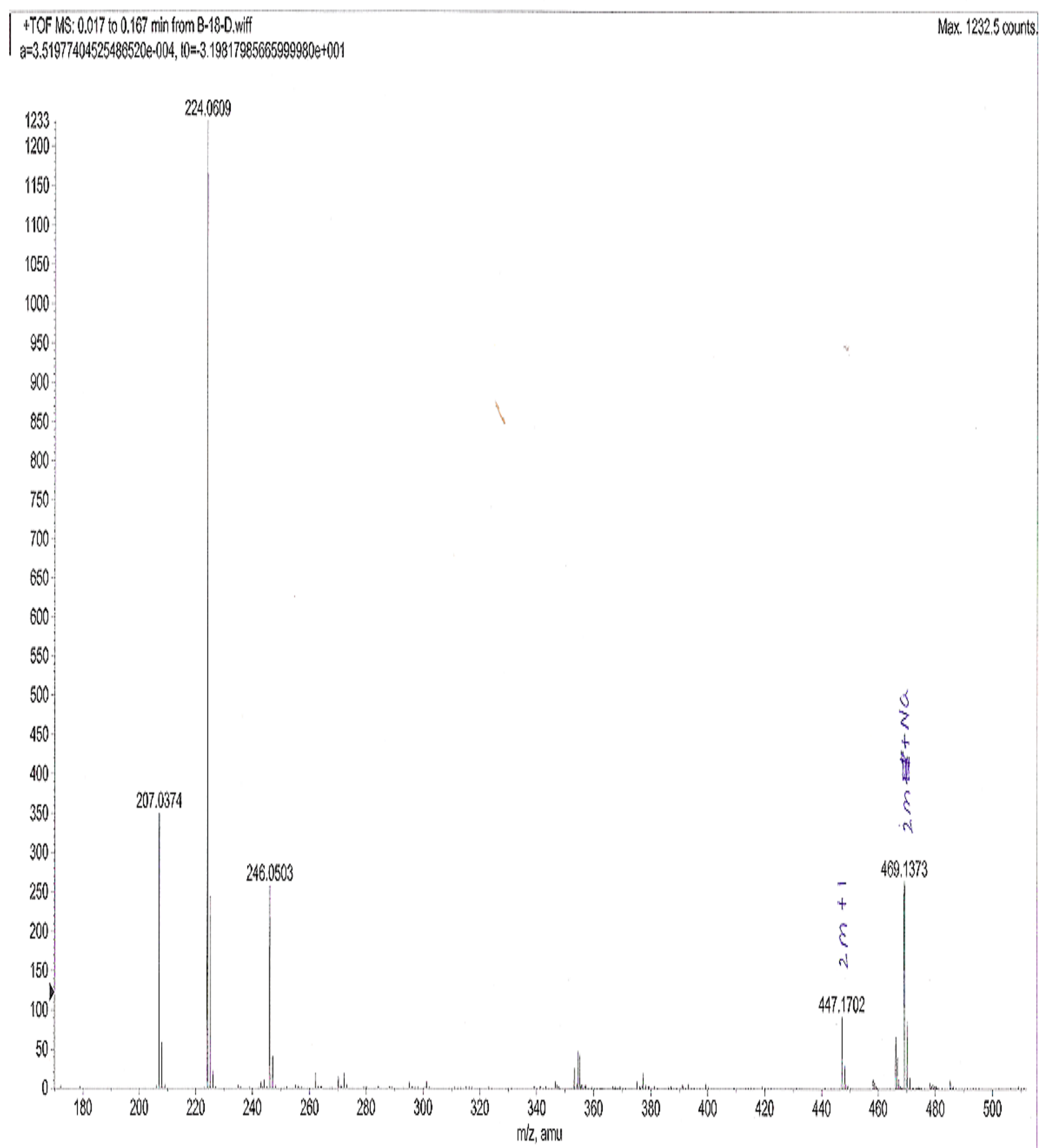
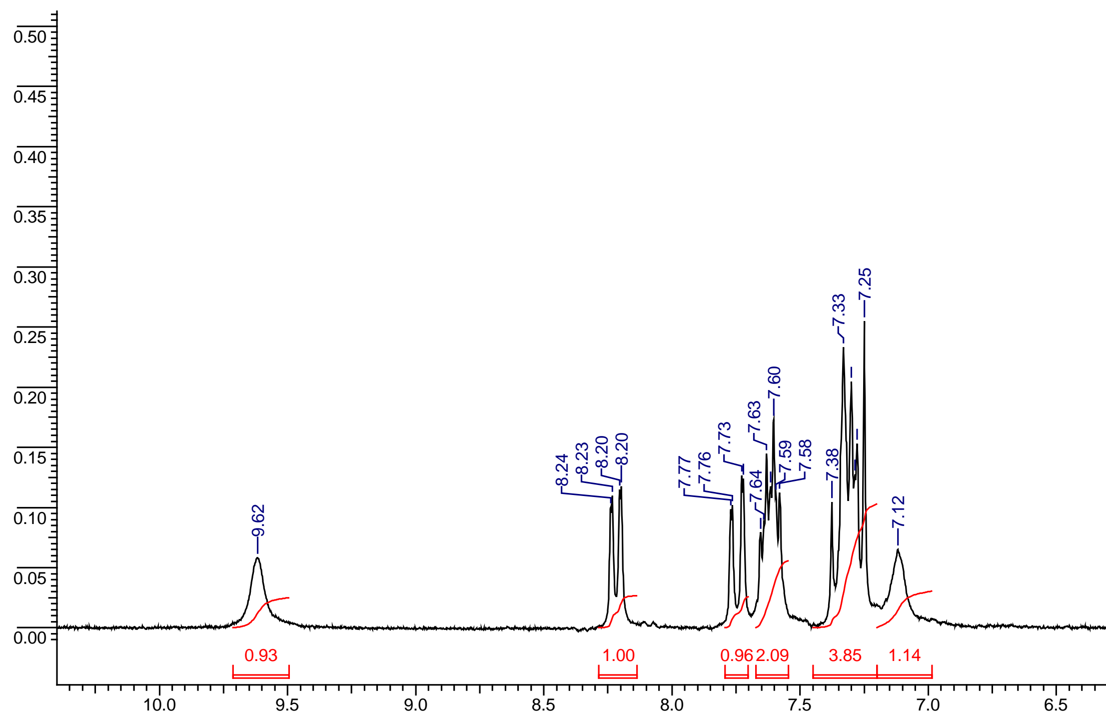


Table 1 The HSQC and HMBC correlations of compound **1**

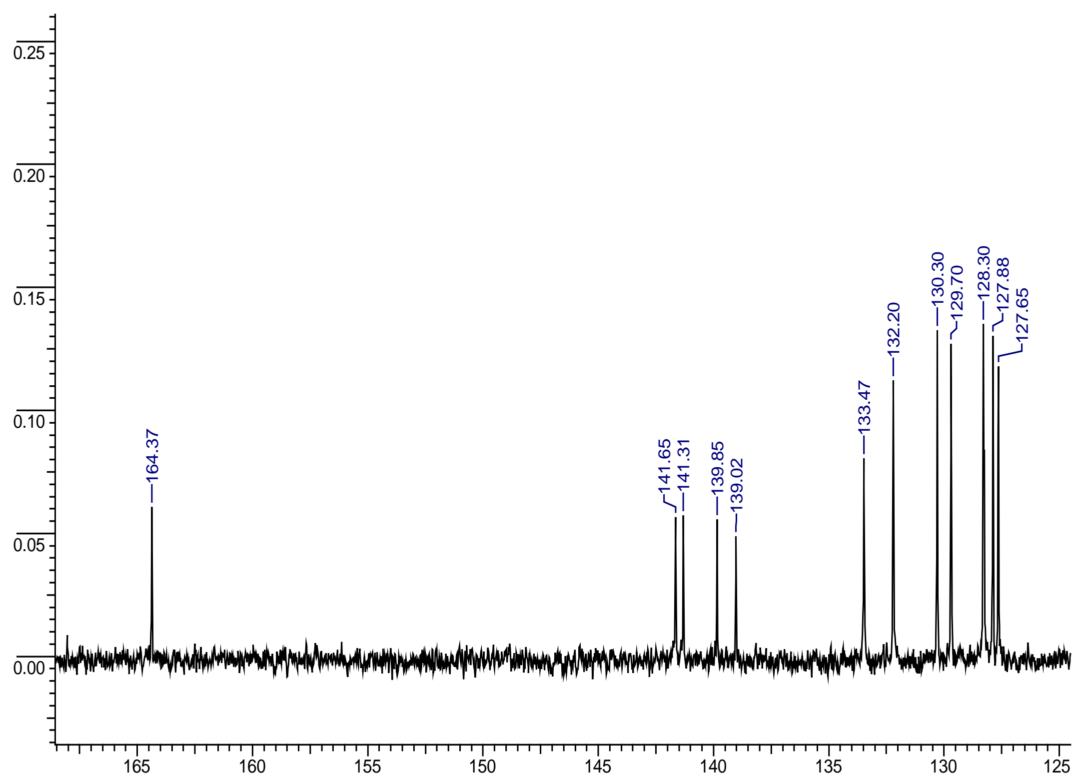
No.	δ_C	δ_H (J, Hz)	HMBC H→C
Isoleucine			
1	168.8	-	
2	60.5	3.83 bd (3.1)	C-1, **C-1'**, C-3, C-4, C-5
3	39.8	1.94 bs	
4	25.1	1.48 m	C-2, C-3, C-5, C-6
5	15.6	1.01 d (7.0)	C-2, C-3, C-4
6	12.0	0.93 t (6.7)	C-3, C-4
Homovaline			
1'	170.4	-	-
2'	45.2	1.58 m, 1.74 m	C-1', C-3', C-4', C-5', C-6'
3'	53.8	3.93 dd (3.4, 8.5)	C-1', **C-1**, C-2', C-4'
4'	24.7	1.81 bs	C-5', C-6'
5'	21.5	0.93 d (6.4)	C-2', C-6'
6'	23.5	0.95 d (6.4)	C-5'

Compound 2-Phenazine -1-carboxamide
2A. Structure **Phenazine-1-carboxamide (PCN)** **($C_{13}H_9N_3O$; M^+ 224)**
2B. EI mass spectrum of Compound 2


2C. ^{1}H-NMR of compound 2



2D. ^{13}C-NMR signals of compound 2



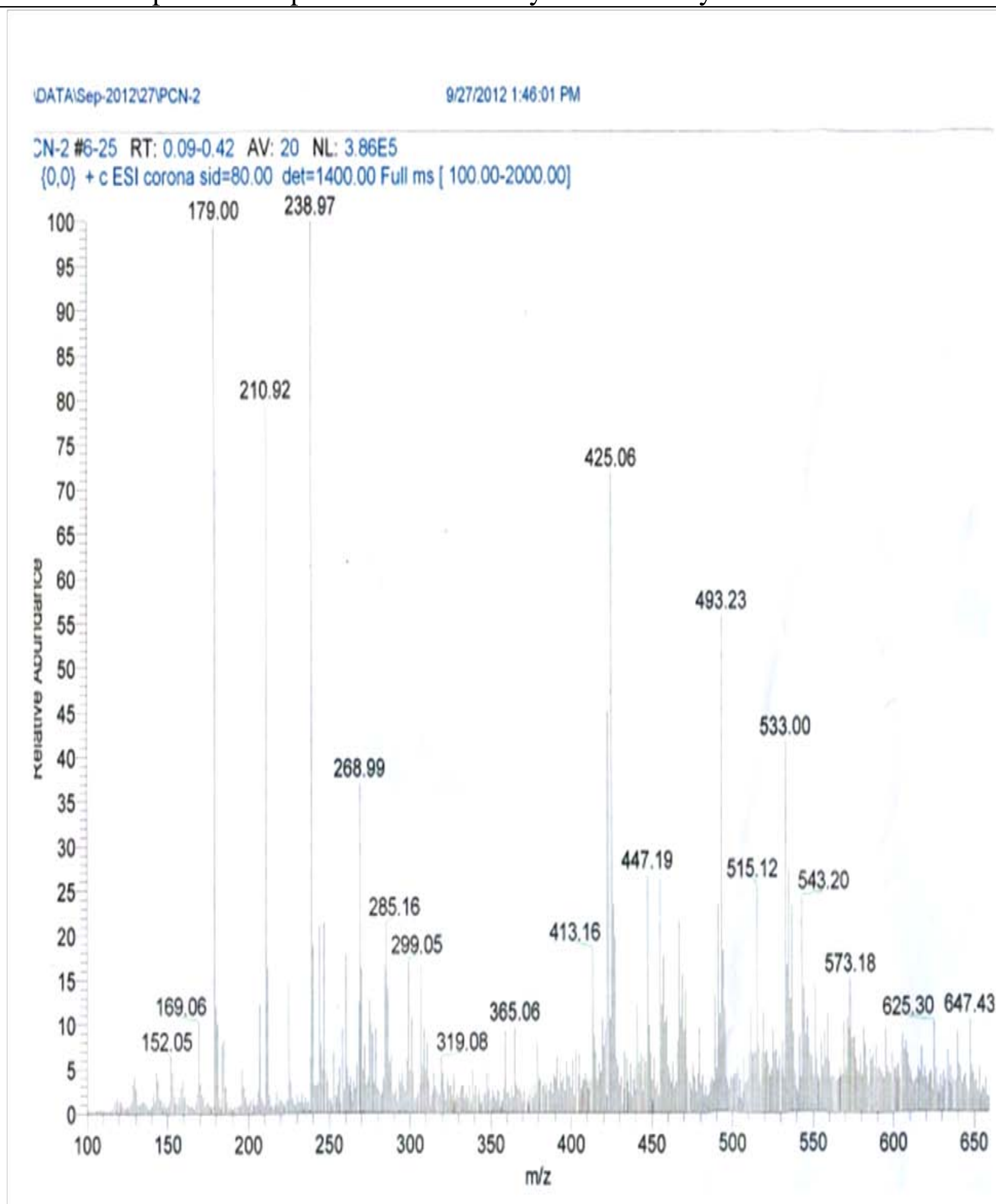
Nuclear magnetic resonance (NMR) signals from phenazine-1-carboxamide (A) ^{1}H-NMR (B) ^{13}C-NMR

Table2 A. ^{1}H-NMR

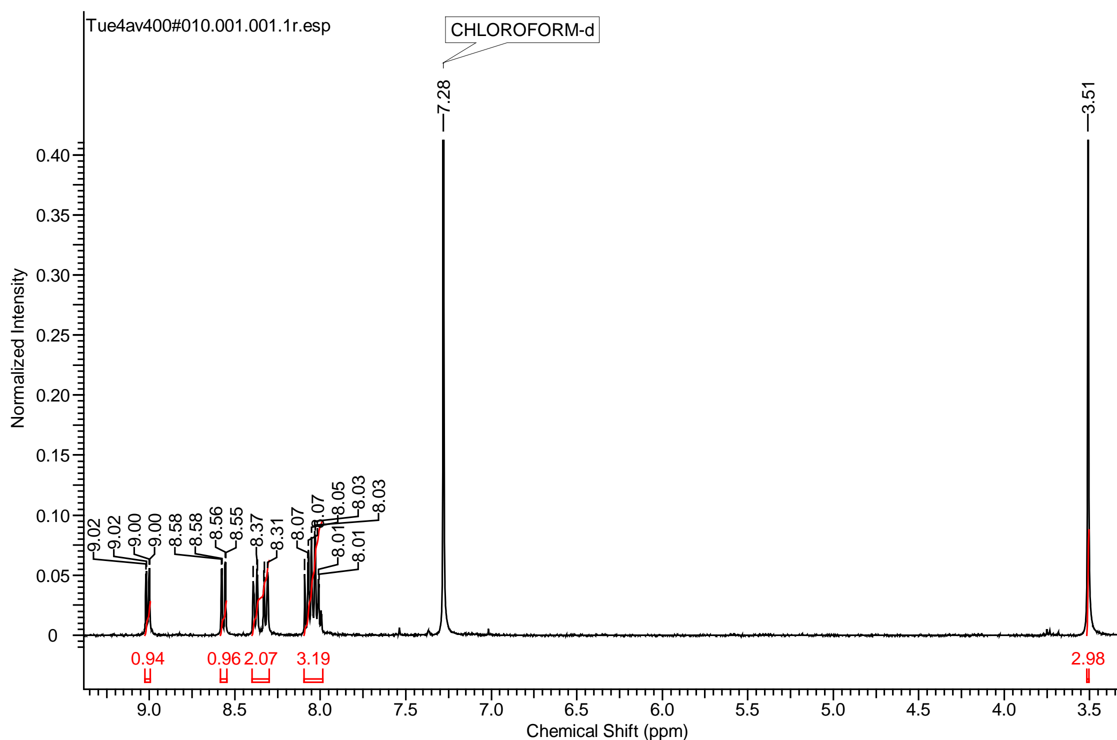
Proton	Chemical shift δ (ppm)	Splitting pattern [coupling constants (*Hz*)]
NH2	10.72	bs
	6.32	bs
H-2	8.54	dd (8.7; 1.3)
H-3	8.015	dd (8.7; 7.0)
H-4	8.97	dd (7.0; 1.3)
H-6	8.29	ddd (8.0; 1.5; 0.5)
H-7	7.97	ddd (8.0; 7.5; 1.3)
H-8	8.04	ddd (7.5; 7.5; 1.5)
H-9	8.34	ddd (7.5; 1.3; 0.5)

Table 2B. ^{13}C-NMR

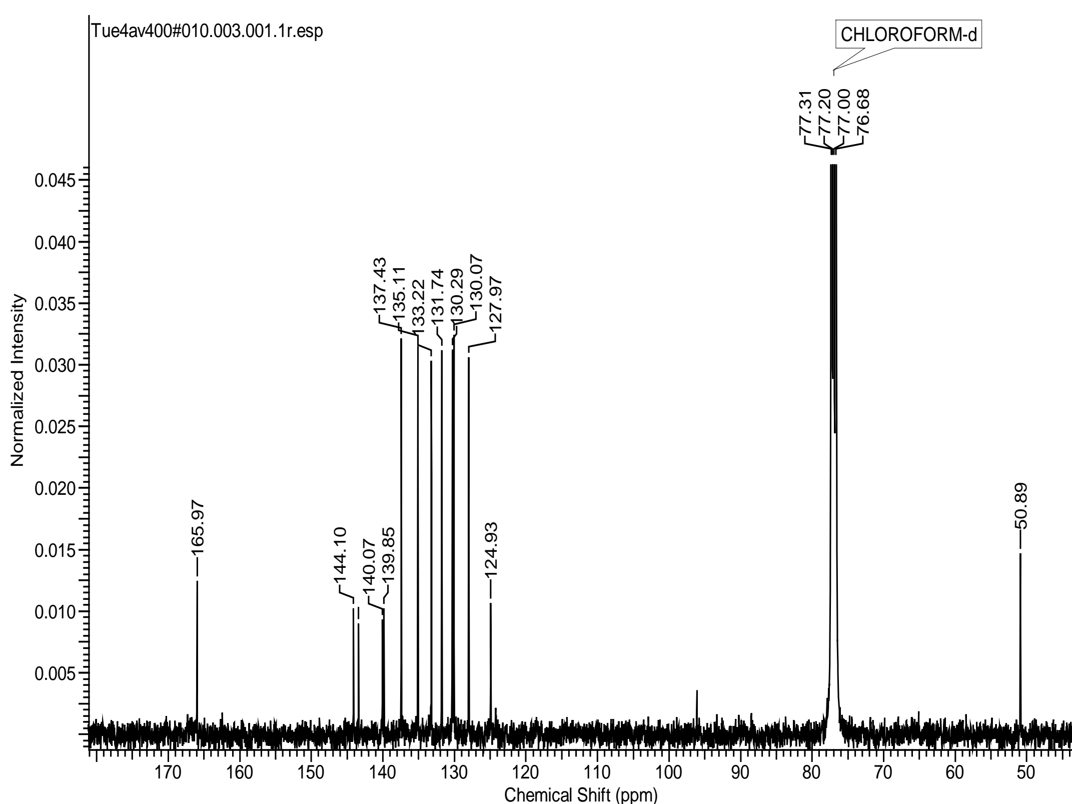
Carbon	Chemical shift δ (ppm)
CONH2	166.54
C-1	128.93
C-2	134.35
C-3	129.12
C-4	136.00
C-4a	141.55a
C-5a	143.56a
C-6	131.75b
C-7	129.81c
C-8	129.90c
C-9	131.07b
C-9a	143.20a
C-10a	140.85a

Compound 3 - Phenazine-1-carboxylic acid methyl ester
3A. Structure **Phenazine-1-carboxylic acid methyl ester** **($C_{14}H_{10}N_2O_2$; M^+ 238)**
3B. EI-MS spectrum of phenazine-1-carboxylic acid methyl ester


3C. ¹H NMR of phenazine-1-carboxylic acid methyl ester



3D. ¹³C NMR of phenazine-1-carboxylic acid methyl ester



Nuclear magnetic resonance (NMR) signals from phenazine-1-carboxylic acid methyl

ester(A) ^{1}H-NMR (B) ^{13}C-NMR

Table3 A. ^{1}H-NMR

Proton	Chemical shift δ (ppm)	Splitting pattern (Coupling constant (Hz))
O-CH_3	3.51	S
H-2	8.55	dd (8.7; 1.25)
H-3	8.01	dd (8.7; 7.0)
H-4	9.00	dd (7.0; 1.25)
H-6	8.33	ddd (8.0; 1.5;0.5)
H-7	7.99	ddd (8.0; 7.5;1.3)
H-8	8.04	ddd (7.5; 7.5;1.5)
H-9	8.37	ddd (7.5;1.3;0.5)

Table 3 B. ^{13}C-NMR

Carbon	Chemical shift δ (ppm)
$COOCH_3$	165.97
O-CH_3	50.89
C-1	124.93
C-2	133.22
C-3	127.97
C-4	135.11
C-4a	139.85
C-6a	140.07
C-6	137.43
C-7	130.07
C-8	130.29
C-9	131.74
C-9a	143.39
C-10a	144.10

List of Publications

1. **Pradnya Chavan** and Santosh Tupe (2014) Antifungal activity and mechanism of action of carvacrol and thymol against vineyard and wine spoilage yeasts. *Food Control* 46:115-120.
2. **Pradnya Chavan**, Sarika Mane, Girish Kulkarni, Shamim Shaikh, Vandana Ghormade, Devidas P. Nerkar, Yogesh Shouche and Mukund V. Deshpande (2009) Natural yeast flora from different grape varieties used for wine making in India. *Food Microbiology* 26:801-808.
3. Namdev S. Vatmurge, Braja G. Hazra, Vandana S. Pore, Fazal Shirazi, **Pradnya Chavan** and Mukund V. Deshpande (2008) Synthesis and antimicrobial activity of β-lactam-bile acid conjugates linked *via* triazole. *Bioorganic and medicinal chemistry Letters* 18: 2043-2047.

Papers/posters presented in Symposia/Conferences/Meetings, etc.

1. **P**. **Chavan**, S. Mane, S. Tupe, V. Ghormade and M.V. Deshpande (2012) Biochemical and microbial profiling of yeasts to identify their role in wine making. Presented at research student meet (RSM) at NCL, Pune.
2. **P. Chavan**, V. Ghormade, R. Mirlekar, S. Shaikh, D.P. Nerkar, G. Kulkarni Y. Shouche and M. V. Deshpande (2007) Biochemical and molecular profiling of wine spoilage yeast isolated in wine spoilage. Association of Microbiologist of India at IIT, Chennai.
3. **P. Chavan**, A. Dahiya, F. Shirazi, S.P.Sawargave, U. Phalgune, S.P. Joshi and M.V. Deshpande (2007) Biocontrol of plant pathogenic fungi using Phenazine-1-carboxamide from *Pseudomonas fluorescens* strain B-18. Presented in the International Conference on the Biology of Yeasts and Filamentous Fungi at NCL**,** Pune.
4. F. Shirazi, **P. Chavan**, A. Dahiya, G. J. Kaur, V. Panchanadikar and M. V. Deshpande (2007) *Benjaminiella poitrasii*: A model system to study differentiation and its linkage to programmed cell death. The International Conference on the Biology of Yeasts and Filamentous Fungi at NCL**,** Pune.