"YEAST DIVERSITY ASSOCIATED WITH DIFFERENT VARIETIES OF WINE GRAPES GROWN IN MAHARASHTRA (INDIA) AND ITS IMPORTANCE IN WINE MAKING"

A THESIS SUBMITTED TO

BHARATI VIDYAPEETH DEEMED UNIVERSITY, PUNE

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SCIENCE

SUBMITTED BY

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OCTOBER 2015

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled "**Yeast diversity associated with different varieties of wine grapes grown in Maharashtra (India) and its importance in winemaking**" for the degree of 'Doctor of Philosophy' in the subject of Biotechnology under the faculty of Science has been carried out by Ms. Sarika S. Mane in the Department of Biochemistry at Rajiv Gandhi Institute of IT and Biotechnology (RGITBT), Bharati Vidyapeeth Deemed University, Pune during the period from May 2008 to September 2015 under the guidance of Dr Shamim A. Shaikh.

Place: Pune
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CERTIFICATION OF GUIDE

This is to certify that the work incorporated in the thesis entitled "**Yeast diversity associated with different varieties of wine grapes grown in Maharashtra (India) and it's importance in winemaking**" submitted by Ms. Sarika S. Mane for the degree of 'Doctor of Philosophy' in the subject of Biotechnology under the faculty of Science has been carried out in the Department of Biochemistry, Rajiv Gandhi Institute of IT and Biotechnology (RGITBT), Bharati Vidyapeeth Deemed University, Pune during the period from May 2008 to September 2015, under my direct supervision/guidance.

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis entitled "**Yeast diversity associated with different varieties of wine grapes grown in Maharashtra (India) and its importance in winemaking**" submitted by me to the Bharati Vidyapeeth Deemed University, Pune for the Degree of **Doctor of Philosophy (Ph.D.)** in **Biotechnology** under the faculty of **Sciences** is original piece of work carried out by me under the supervision of Dr. Shamim A. Shaikh. I further declare that it has not been submitted to this or any other university or Institution for the award of any degree or diploma.

I also confirm that all the material which I have borrowed from other sources and incorporated in this thesis is duly acknowledged. If any material is not duly acknowledged and found incorporated in this thesis, it is entirely my responsibility. I am fully aware of the implications of any such act which might have been committed by me advertently or inadvertently.

Place: Name and signature of

Date: Research Student

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Sarika S. Mane

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Abbreviations

A_{600}	Absorbance at wavelength 600 nm
a.u.	Absorbance unit
BLAST	Basic local alignment search tool
bp	Base pair
CBS	Centraalbureau voor Schimmelcultures
CFU	Colony forming unit
CDE	Cuticle degrading enzymes
ºB	Degree Brix
DNase	Deoxyribo nuclease
dNTP	Deoxynucleotide triphosphate
DMAB	*p*-dimethyl amino benzaldehyde
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
GJ	Grape juice
GlcNAc	N-acetylglucosamine
HR-LCMS	High Resolution-Liquid Chromatography Mass spectrophotometry
h	Hour
ITS	Internal transcribed region
IC_{50}	Inhibitory concentration to kill 50% population
MGYP	Malt extract-glucose-yeast extract Peptone
NCBI	National Centre for Biotechnology Information
MIC	Minimum inhibitory concentration
ND	Not detected
OD	Optical density
RH	Relative humidity
PCR	Polymerase chain reaction
rpm	Revolutions per minute
rDNA	Ribosomal DNA
SSF	Soild state fermenation
SDS	Sodium dodecyl sulphate
sp.	Species
TCA	Trichloro scetic acid

TE	Tris EDTA
TPC	Total phenolic content
YP	Yeast extract-peptone
YPG	Yeast extract-peptone glucose
YNB	Yeast nitrogen base
U	Unit of enzyme activity
v/v	Volume by volume
w/v	Weight by volume

Chapter 1

Introduction and Review of Literature

"Wine is one of the most civilized things in the world and one of the most natural things of the world that has been brought to the greatest perfection, and it offers a greater range for enjoyment and appreciation than, possibly, any other purely sensory thing."

— Ernest Hemingway

The earliest known wine was made in Mesopotamia around 3500 BC (Robinson, 2006). However, it was reported that Chinese are deliberately fermenting grape juice (McGovern et al., 2004). The cultivation of grapes spread from Middle East to other regions in Europe, North Africa, and North America with major development in winemaking during Roman Empire.

Presently, France, Italy and Spain are the largest wine producing countries with total output of 84%, followed by Germany, Portugal, Greece, Romania and Austria. Italy tops the list with 4.49 billion liters of wine produced which is ~17% of the world market share (Bettini, 2014). According to the British Liv-ex Fine Wine Index, 84 of the world's 100 most famous wine brands are French. Given the rich diversity of its soil-types, climate and grapes, France is able to produce truly unique wines.

1.1 Current scenario of wine production in India

Wine production in India is ~17.3million litres/annum, which is negligible as compared to the rest of world production, 27100 million litres/annum. The wine industry in India is still in its nascent stage with a ~20% increase per annum for the last few years. Commercial wine grape production in India started since 1980s. Primarily, wine grapes are grown in states of Maharashtra and Karnataka. The Indian wine industry has seen rapid growth in the past decade, starting from a handful of wineries in Maharashtra in the eighties; to 93 wineries in the country in 2015. Maharashtra accounts for more than 84% of the country's total wine production. Within Maharashtra, most of the wine production takes place in the regions of Nashik and Sangli, followed by Pune district. Nashik is known as the wine capital of India, possessing the most beneficial climate for grape production, and thus is also the largest grape producer. Other main districts for grape growing in Maharashtra are:

Solapur, Ahmednagar, Latur, Osmanabad and Satara. In 2013, India exported almost 1.8 million liters of wine. Last year, the total wine production estimate for the states of Maharashtra and Karnataka was 14.2 million liters (1.58 million cases). Furthermore, production in Karnataka was predicted to increase to 5 million liters (555,000 cases), a rise of 1.3 million liters (145,000 cases) year-on-year. Thus, India is a rapidly making stride in wine production and consumption.

1.2 Wine as medicine

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) Moderate wine consumption (250-300 mL/day) has distinct health benefits (Doll et al., 2005; Thun et al., 1997). 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, 2008).

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 (Micallef et al., 2007; Pallas et al., 2010). Resveratrol is also known to protect the skin from ultraviolet radiation (Aziz et al., 2005).

1.3 Grape varieties used for winemaking

Different grape varieties are used for wine production. Wine is differentiated in to two types based on color as - red wine which is produced from grape varieties such as- Barbera, Cabernet Sauvignon, Carignan, Black Rieslin, Cabernet Franc, Cinsaut, Dornfelder, Gamay, Riesling, Sangiovese, Grenache, Malbec, Merlot, Shiraz, Syrah, Trollinger, Muscat, Montepulciano, Pinot Noir, Pinotage, Portugieser, Saperavi, and Zinfandel; and white wine made from grape varieties- Aligote, Sauvignon Blanc, Muscat, Mueller-Thurgau, Chardonnay, Feteasca Alba, Chenin Blanc, Clairette, Feteasca Regala, Prosecco, Ugni Blanc, Pinot Blanc, Pinot Grigio, Semillon, Silvaner Garganega, Viognier and Vermantino. White wines are made without must (Skin and seeds) and are much lower in phenolics as compared to red wines. Other regionally

important and aromatically distinctive varieties are Corvina, Dolcetto, Negro Amaro (red), Fiano, Garganega, and Torbato (white) from Italy; Malvasia, Parellada (white), and Graciano (red) from Spain; Arinto (white) and Ramisco (red) from Portugal and Rhoditis (white) from Greece; Furmint (white) from Hungary.

Grape variety used for wine making is an important factor determining wine quality as it imparts the "varietal character" to the wine. This in turn is due to 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 or 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.

The red grape varieties predominantly 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.

1.4 Red and white wine making process

Alcoholic fermentation is an anaerobic process carried out mainly by *S. cerevisiae* in which sugars, glucose and fructose are converted into ethanol and carbon dioxide. Yeasts present on grapes reach there by wind and insect dispersal, increasing in number from the onset of fruit ripening (Lafon-Lafourcade, 1983). After harvesting, the grapes are taken to winery, destemmed and crushed. In production of white wine, crushing is followed by limited maceration, pressing and extraction of juice for 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 adding starter culture *S. cerevisiae* to the must containing other non-*Saccharomyces* yeasts coming from the berries and which takes ~15 days. After the primary fermentation of red grapes the wine is pumped off into tanks and the skin is pressed to extract the wine. White wines are 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

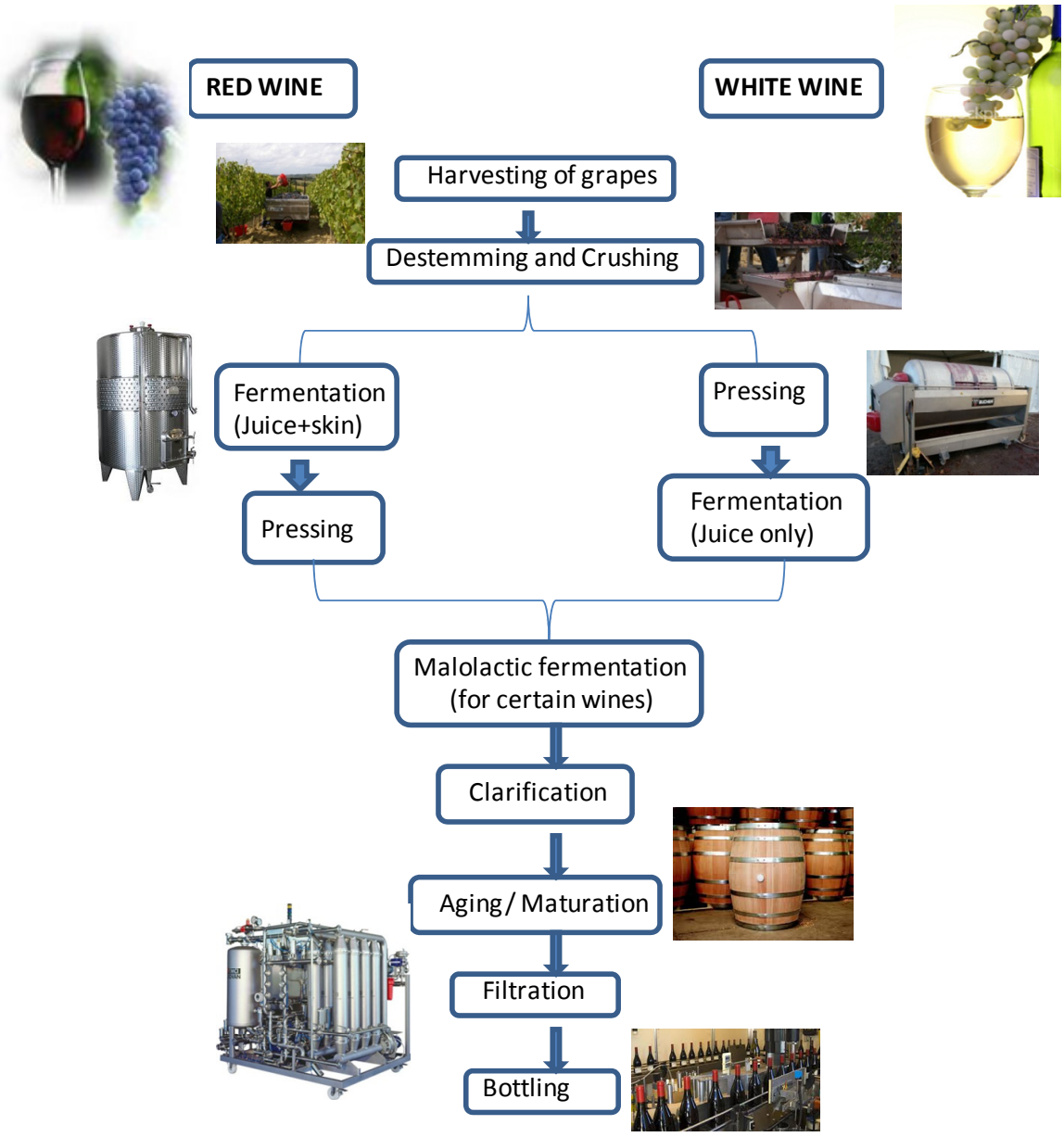

Fig. 1.1: Schematic outline of the main steps in red and white wine production.

extraction of phenolic compounds. An initial temperature of 20ºC is recommended for fermentation of 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 (for certain wines), filtered and bottled (Fig1.1).

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).

Along with the vine variety and fermentation process followed, the yeast diversity of the grapes and must is an important factor contributing to the quality of wine (Barata et al., 2012).

1.5 Microbial diversity of grapes

Bacteria, unicellular and filamentous fungi with different physiological characteristics have been found on grapes. Some yeast species, lactic acid bacteria and acetic acid bacteria are unique to grapes which can survive and proliferate during fermentation, constituting the wine microbial consortium. The qualitative and quantitative differences of these microbes depend on the grape ripening stage and on the availability of nutrients. Furthermore, the microbial ecology is affected by grape health, abiotic and biotic factors which are involved in the primary damaging effect.

Different bacterial species found to be associated with grapes are *Bacillus* sp., *Enterobacter* sp., *Burkholderia* sp., *Serratia* sp., *Enterococcus* sp., and *Staphylococcus* sp. However, due to high acidity and ethanol concentration these bacterial species cannot grow in wine (Barata et al., 2012), where as lactic acid bacteria such as *Lactobacillus, Oenococcus, Leuconostoc,* and *Pediococcus* and acetic acid bacteria species of the genera *Acetobacter, Asaia, Acidomonas*, *Gluconobacter, Granulibacter, Neoasaia, Kozakia*, *Swaminathania, Saccharibacter* can grow and cause malolactic fermentation during wine making (Barata et al., 2011; Gonzalez et al., 2005; Lonvaud-Funel, 1999; Nisiotou et al., 2011; Osborne et al., 2005).

Valero et al. (2007) reported presence of filamentous fungi, like *Alternaria*, *Aspergillus, Cladosporium*, *Eurotium*, *Penicillium* and *Trichoderma* on grapes that are unable to grow in wine, similar to some bacterial genera. *Plamospara viticola, Erysiphe necator* and *Botrytis cinerea* are the main pathogens on grapes which cause downy mildew, powdery mildew and grey rot, respectively (Barata et al., 2012). Besides, *Erysiphe* and *Fusarium* were also observed on grapes (Diguta et al., 2011).

The yeast flora of five grape varieties, namely Chardonnay, Cabernet Franc, Cabernet Sauvignon, Marselan, and Merlot, on the basis of colony characteristics and sequencing of the 26S rDNA-D1/D2 domain revealed the identity of eight species of seven genera namely *Aureobasidium pullulans, Candida zemplinina, Hanseniaspora*

uvarum, Hanseniaspora occidentalis, Issatchenkia terricola, Metschnikowia pulcherrima, Pichia kluyveri, and *S. cerevisiae*. The predominantly isolated species were *H. uvarum* and *S. cerevisiae*. Presence of six different genotypes of *S. cerevisiae* at different time points of the fermentation (Marselan variety) was also observed (Sun et al., 2014).

Qualitative and quantitative profiling of yeasts during wine fermentation through colony morphology studied using WL nutrient medium, showed six different genera such as *Candida*, *Hanseniaspora*, *Issatchenkia, Pichia, Metschnikowia* and *Saccharomyces* and distinct colony subtypes were identified within a single species *M. pulcherrima* which produced antimicrobial pigment, the pulcherrimin (Pallmann et al., 2001). The use of local strains of yeast imparts unique regional qualities to the fermented wine. Natural yeast flora of the grape plays an important role in imparting varietal character to the wine and is discussed in detail in following section.

1.6 Natural yeast flora of grapes

1.6.1 Saccharomyces

Saccharomyces yeasts have a unicellular, globose, spheriodal shape. Multilateral (multipolar) budding is typical for vegetative reproduction (Vaughan-Matini and Martini, 1998) and is one of the most studied organisms at biochemical and molecular level.

Saccharomyces and 15 plus genera of non-*Saccharomyces* yeasts are associated some time or other with wine fermentation. *S. cerevisiae* is not a common phyllosphere isolate; in fact it is prevalent on the surface of winery equipment (Fleet et al., 2002; Von Wallbrunn, 2007). Earlier Mortimer and Polsinelli (1999) also reported the absence of *S. cerevisiae* on the grapes, in general. According to them only one in one-thousand grape berries carried *S. cerevisiae*. Furthermore damaged berries were rich depositories of microorganisms including *S. cerevisiae.*

S. cerevisiae has enormous capacity to ferment sugars to ethanol and carbon dioxide. As a result this organism is one of the key players in baking, wine making, brewing, and bioethanol industry. Additionally, *Saccharomyces* has also been used as a transformation host for protein production (Nevoigt, 2008). *S. cerevisiae* is relatively tolerant to low pH, high sugar and ethanol concentrations.

Capallo et al. (2004) isolated *S. cerevisiae* strains from 12 grape varieties grown in the experimental vineyard of Apulia, South Italy. One of the important

observations made was that these isolates were found to be well-adapted to the specific climatic conditions of the area and not the variety, *per se*. All these isolates were found to tolerate high ethanol concentration. Where as, Capace et al. (2010) reported that different *Saccharomyces* isolates from Nero d'Avola grapes collected from different areas of the Sicily showed similar physiological characteristics such as high ethanol and SO_2 tolerance. Chavan et al. (2009) have isolated *Saccharomyces* strains from different grape varieties grown in two different geographical areas, Pune (18° 31' N, 73° 55' E) and Sangli (16° 52' N, 74° 34' E), India. Out of four varieties grown in Pune region, namely Banglore Blue, Zinfandel, Shiraz and Cabernet, *Saccharomyces* strains were found only on Zinfandel variety. Where as, *Saccharomyces* strains were isolated from the berries of all four varieties grown in Sangli area namely Cabernet, Shiraz, Chenin Blanc and Sauvignon Blanc. These observations indeed suggest that no explicit role to either region (environmental factors) or variety could be assigned.

As the importance of role of *S. cerevisiae* in winemaking has long been established, the use of commercial strains of these yeast cultures in fermentation is a common practice in order to ensure a reproducible product and to reduce the risk of wine spoilage.

S. cerevisiae plays important role in wine fermentation mainly through metabolism of sugar to alcohol and CO_2 and it has an equally important role in the formation of secondary metabolites as well as in the conversion of grape aroma precursors to varietal aroma in wine. Molecular & biochemical studies have enabled researchers to develop sugar and alcohol tolerant, highly flocculent strains for wine production (Soares, 2010). Flocculation contributes significantly in the brewing industry, in the production of renewal fuels (bio-ethanol), in modern biotechnology (production of heterologous proteins) and in environmental applications (bioremediation of heavy metals), etc.

1.6.1.1 Status of *Saccharomyces* during wine fermentation

Various yeast species present on the berries and on winery equipments contribute significantly to wine fermentation. In the early stages of fermentation, genera like *Kloeckera, Hanseniaspora* and *Candida* were reported to be predominant followed by *Metschnikowia* and *Pichia*, when the ethanol concentration was to 3-4 %, while the later stages are dominated by alcohol tolerant strains of *Saccharomyces* species such as *S. cerevisiae, S. bayanus, S. paradoxus* and *S. pastorians* (Pretorius et al., 1999).

For the production of sherry wine, two successive processes, alcoholic fermentation of the must by yeast and biological aging are crucial. Species like *Candida stellata, Dekkera anomala, Hanseniaspora guilliermondii, Hanseniaspora uvarum, Issatchenkia terricola* and *S. Cerevisiae* were observed at higher frequencies than other species like *Candida incommunis, Candida sorbosa*, and *Zygosaccharomyces cidri* or *Z. Fermentati* during alcoholic fermentation, while *S. cerevisiae, Pichia membranaefaciens*, *Pichia anomala* were found during biological aging. The *S. cerevisiae* strains involved in fermentation (*S. cerevisiae, S. bayanus, S. paradoxus* and *S. Pastorians*) are different from the strains responsible for biological aging (flor yeast, *S. cerevisiae* races *beticus, cheresiensis, montuliensis*, and *rouxii*) has been demonstrated by studying the *Saccharomyces* diversity using mtDNA restriction analysis and karyotyping of strains during sherry wine production (Esteve-Zarzoso et al., 2001).

1.6.2 Non-*Saccharomyces* yeasts

Grape berry surface provide physical environment suitable for the growth of microorganisms. *Rhodotorula*, *Cryptococcus* and *Candida* are the predominant candidates on unripe-grapes. With an increase in sugar concentration and decrease in acidity during maturation of berries, *Kloeckera/Hanseniaspora* become dominant, accounting for more than 50% of the total yeast flora., 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.

1.6.2.1 Non-*Saccharomyces* yeasts associated with fermenting must

The non-*Saccharomyces* yeast population changes during cold maceration and alcoholic fermentation which can be attributed to the changes in micro-environment. Availability of the oxygen, and /or ethanol concentration also affects the predominance of different species of yeasts in the fermenting must. During

fermentation, due to low oxygen and increasing level of ethanol most of the non-*Saccharomyces* yeasts cannot survive (Combina et al., 2005; Fleet et al., 1984; Hansen et al., 2001; Henick-Kling, 1998; Jackson, 1994). A ubiquitous presence of *Kloeckera apiculata, Candida stellata* and *Metschnikowia pulcherrima* was observed in the must of Malbec variety of grapes during the spontaneous fermentation (Combina et al. 2005) and *H. osmophila*, *C. tropicalis* and *Z. bisporus* species were predominantly found during cold maceration (Hierro et al. 2006). The clarification of white must (centrifugation, enzyme treatments, cold settling) also reduces the initial population of yeasts (Fleet, 1990; Lonvaud-Funel, 1999; Pretorius, 2000).

Predominance of non-*Saccharomyces* yeasts in fermenting must at the later stages is influenced by barrels and post-fermentation spoilage (Loureiro and Malfeito-Ferreira, 2003). *Brettanomyces* sp. and *Zygosaccharomyces* sp. are ethanol tolerant like *S. cerevisiae* and can 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). 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.

Grape variety, physical damage of the grapes, weather conditions and chemical composition of the must influenced *Sacharomyces* and non-*Saccharomyces* yeast diversity. The ascomycetes yeasts (*Aureobasidium, Candida, Hanseniaspora, Metschnikowia, Pichia, Saccharomyces* and *Saccharomycopsis*) and basidiomycetous yeasts (*Cryptococcus, Dioszegia, Filobasidium, Rhodotorula* and *Sporidiobolus)* were reported to be associated with fermenting must of three grape varieties namely Blue Frankish, Green Veltliner and Sauvignon Blanc, while *Hanseniaspora uvarum, Metschnikowia pulcherrima, Pichia kluyveri, Pichia kudriavzevii* and *Sporidiobolus pararoseus* were observed on the berries. However, damaged berries were found to support the growth of *P. kluyveri* and *P. kudriavzevii* (Nemcova et al., 2015). Yeast flora of Chenin Blanc variety cultivated in the "Sao Francisco Valley" region of Brazil observed that *Hanseniaspora opuntiae* and mixed cultures of *H. opuntiae* and *Saccharomyces cerevisiae* that could influence the wine quality (Assis et al., 2014).

1.7 Region specific non-*Saccharomyces* yeasts

The diversity of natural yeast flora of grapes changes significantly with geographical locations or regions and influenced by the grape varieties, and level of maritime (closeness of sea), temperature and rainfall. The vineyards from Italy, Spain and China show higher diversity of yeast flora followed by France, India, Argentina and Portugal, while relatively low species diversity was observed in vineyards of Australia, Brazil, Canada, Greece and Japan (Table 1.1).

Studies on diversity of yeast in two wine regions of northwest Spain has shown that four species, *C. albidus, C. stellata, H. anomala*, and *H. silvicola* were predominant in the Atlantic region (near sea) where climate is moderate, while six species, *C. vini, H. canadensis, H. jadinii, P. carsoni, D. intermedia*, and *Sp. roseus*, were exclusive to the interior region (arid lifted plains with low lying river valleys). This geographic variation perhaps is also responsible for the oxidative behaviour of the yeast (Longo et al., 1991).

Assessment of the long term relationship (about 15 years) between yeast quantity and composition has demonstrated that weather conditions, particularly rainfall and relative humidity, 25 to 30 days before harvesting can be correlated with total yeasts, and *Kloeckera apiculata* and *Candida zemplinina* represent almost the entire non-*Saccharomyces* yeasts in grape and fresh musts. Along with climate, management strategies of harvest can also affect the microbial communities. For eg. *K. apiculata* and *C. zemplinina* were found to be correlated with temperature 10 days before grape harvest at the same time as leaf pulling (Brilli et al., 2014). A significant change in the yeast diversity, species heterogeneity was observed in presence of *Botrytis cinerea* infection, with *Hanseniaspora opuntiae* being encountered as an inhabitant of the grape ecosystem (Longo et al., 1991; Nisiotou et al., 2007).

Various methods like restriction fragment length polymorphism, sequence analyses of the 5.8S internal transcribed spacer and the D1/D2 ribosomal DNA (rDNA) regions, PCR-RFLP have been used to study and establish yeast species diversity. During initial phase of fermentation *Botrytis*-affected grape juice showed more biodiversity than grape juice without infection. The species such as *Zygosaccharomyces bailii* and *Issatchenkia* spp. or *Kluyveromyces dobzhanskii* and *Kazachstania* sp. were predominant. Li et al. (2010) evaluated the yeast diversity and its quantitative changes in three grape varieties cultivated in four different regions of

Table 1.1. Diversity of yeasts associated with grapes from different countries

Country	Grape variety (red/white)	Associated yeast genera	References
Argentina	Malbec (red)	*Pichia, Kloeckera, Saccharomyces, Zygosaccharomyces, Rhodotorula, Metschnicowia, Issatchenkia, Kluyveromyces*	(Combina et al., 2005)
Australia	Cabernet Sauvignon (red)	*Cryptococcus, Rhodotorula, Sporobolomyces, Hanseniaspora, Metschnikowia, Kluyveromyces, Torulaspora, Saccharomyces*	(Prakitchaiwattana, 2004)
Brazil	Isabeal (red)	*Hanseniaspora, Saccharomyces, Issatchenkia, Sporidiobolus, Candida, Cryptococcus*	(Baffi et al., 2011)
Canada	Icewine (red)	*Sporobolomyces, Cryptococcus, Rhodotorula, Hanseniaspora*	(Subden 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*	
France	Chardonnay (white)	*Candida, Rhodotorula, Pichia, Sporidiobolus, Cryptococcus, Hanseniaspora, Rhodosporidium*	(Renouf et al., 2005)
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*	

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*	
Japan	Niagara (white)	*Kloeckera, Candida*	(Yanagida et al., 1992)
	Chardonnay (white)	*Cryptococcus, Rhodotorula*	
	Zenkoji (white)	*Cryptococcus, Rhodotorula, Candida*	
	Koshu (white)	*Kloeckera, Cryptococcus*	
Portugal	Periquita (red)	*Metschnikowia, Kluyveromyces, Candida, Pichia, Hanseniaspora, Saccharomyces, Issatchenkia, Zygosaccharomyces, Zygoascus, Torulaspora*	(Barata et al., 2008)
Slovenia	Žametovka, Modra Frankinja (red) and Kraljevina (white)	*Cryptococcus, Debaryomyces, Hanseniaspora, Metschnikowia, Pichia, Rhodotorula, Sporobolomyces*	(Raspor et al., 2006)
Spain	Shiraz, Grenache, Barbera (red)	*Metschnikowia, Kluyveromyces, Candida, Pichia, Hanseniaspora, Torulaspora, Saccharomyces*	(Cordero-Bueso et al., 2011)
	Abarino, Godello (white) and Mencia (red)	*Rhodotorula mucilaginosa*	(Longo et al., 1991)
Spain (North)	Folle Blanche and Hondarrabi Zuri (white)	*Candida, Cryptococcus, Kloeckera, Rhodotorula, Saccharomyces*	(Rementeria et al., 2003)
South Africa	Chardonnay (white)	*Kluyveromyces, Debaryomyces Candida, Pichia, Kloeckera, Saccharomyces, Zygosaccharomyces, Rhodotorula,*	(Jolly et al., 2003)
Southern Slovakia	Frankovka (red)	*Pichia, Candida, Metschnikowia, Hanseniaspora, Issatchenkia, Saccharomyces*	(Brezna et al., 2010)
	Veltlin (white)	*Saccharomyces, Metschnikowia, Hanseniaspora*	

China that revealed presence of seventeen different yeast species belonging to eight genera on the grape berries.These include: *Hanseniaspora uvarum*, *Cryptococcus flavescens*, *Pichia fermentans*, *Candida zemplinina*, *Cryptococcus carnescens*, *Candida inconpicua, Zygosaccharomyces fermentati*, *Issatchenkia terricola*, *Candida quercitrusa*, *Hanseniaspora guilliermondii*, *Candida bombi*, *Zygosaccharomyces bailii*, *Sporidiobolus pararoseus, Cryptococcus magnus, Metschnikowia pulcherrima*, *Issatchenkia orientalis* and *Pichia guilliermondii*. Among these *H. uvarum* and *C. flavescens* were the dominant species with *Sporidiobolus pararoseus* being found for the first time.

1.8 Factors affecting yeast diversity

Yeast diversity of grapes and must is quite important in wine production because of its influence on fermentation speed, wine flavour and wine quality. The density and diversity of the yeast population on grape berries is affected by numerous factors such as, grape variety (Cordero-Bueso et al., 2011), grape health (Barata et al., 2008; Loureiro and Malfeito-Ferreira, 2003), grape ripeness (Martins et al., 2012), climatic condition and geographic location (Bezerra-Bussoli et al., 2013; Nicholas et al., 2013), application of different chemicals (Milanovic et al., 2013), use of different oenological practices (Andorra et al., 2008; Andorra et al., 2011) as well as application of different farming systems (Cordero-Bueso et al., 2011; Martins et al., 2012). 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 determines the yeast diversity in must (Martini et al., 1996). At harvest, grape temperature, method of harvest (manual vs. mechanical), method of transport to the cellar (picking crates / baskets, tipsters), time of transport to the cellar, time lapse before crushing, and sulphite and enzyme addition can all affect yeast populations (Lambrechts and Pretorius, 2000; Pretorius et al., 1999). Despite all the variables in grape harvest and wine production, the yeast species generally found on grapes and in wines are similar throughout the world (Amerine and Kunkee, 1968). However, the proportion (or population profile) of yeasts in different regions shows distinct differences (Longo et al., 1991). Cordero-Bueso et al. (2011) studied the biodiversity of yeasts in the conventional and organic viticulture in Spain. *K.*

thermotolerans, C. stellata, T. delbrueckii and *P. anomala* were reported from the vineyard with both farming systems. However, the organic viticulture supported diversity of yeast species significantly more than conventional agriculture practices. For instance, in organic vineyard, in a must of a Shiraz variety*, K. thermotolerans* was the most abundant, while *S. cerevisiae, C. stellata, M. pulcherrima* and *H. guilliermondii* were also significant. While in Grenache must *H. guilliermondii* was more abundant than *K. thermotolerans, P. anomala, S. cerevisiae* and *C. stellata*. *S. cerevisiae* strains were reported to be in high number in Barbera must. Under conventional viticulture in the Barbera must *C. stellata* was in the highest proportion, followed by *T. delbrueckii* and *K. thermotolerans.* However, in Grenache must only two species, *K. thermotolerans* and *H. guilliermondii* were in significant number. *P. toletana, C. sorbosa* and *T. delbrueckii* were isolated from Shiraz variety from Spain (Cordero-Bueso et al., 2011).

1.9 Importance of yeast diversity in wine making

1.9.1 Profiling of yeasts during wine fermentation

The qualitative and quantitative changes in *Saccharomyces* and non-*Sacharomyces* yeast strains during wine fermentation influence the wine quality. Traditionally the samples at different time intervals are analyzed using microbiological techniques of enrichment, isolation and identification. Combina et al. (2005) used the conventional microbiological techniques and showed the significant participation of non-*Saccharomyces* yeasts during spontaneous fermentation of Malbec musts, with the ubiquitous presence of three main species: *K. apiculata, C. stellata* and *M. Pulcherrima*. In view of the advances in molecular techniques, denaturing gradient gel electrophoresis of PCR- amplified 26 rRNA genes was reported to be useful to analyze mixed yeast community during wine fermentation (Cocolin et al., 2000)

1.9.1.1 Succession of yeast flora

It was observed that the early stage of fermentation was always dominated by non-*Saccharomyces* yeast flora of grapes (Fleet, 1990). For instance, *Candida* sp., *Hanseniaspora* sp., *Pichia* sp., *Rhodotorula* sp. and *Kluyveromyces* sp. were dominant in grape must during the early stages due to their low fermentative activity. Subsequently, as the ethanol level (5-7%) increased, most of the non-*Saccharomyces* yeasts did not

survive and finally *S. cerevisiae* proliferated, became dominant and completed the wine fermentation (Fleet, 2003; Fleet and Heard, 1993; Gao et al., 2002; Heard and Fleet, 1988). Hansen et al. (2001) reported that two wine related yeasts, *Kluyveromyces thermotolerans* and *Torulaspora delbrueckii* could not survive in the later stages due to the presence of ethanol, lack of oxygen, nutrient depletion or the presence of toxic compounds and cell-to-cell contact mechanism. Moreover, *S. cerevisiae* strains were reported to secrete peptides that inhibited the growth of some non-*Saccharomyces* yeast (Albergaria et al., 2010; Nissen and Arneborg, 2003). However, some non-*Saccharomyces* yeast could survive till later stage of fermentation (up to 12 days) (Fleet, 1990; Fleet et al., 1984). Heard and Fleet (1988) studied the effect of temperature and pH on the growth of the non-*Saccharomyces* yeasts during fermentation in mixed culture. It was observed that at low temperature (15-20°C) the ethanol tolerance of *Candida* and *Hanseniaspora* was more and thus has more impact on the wine flavor at the end. On the other hand, species like *Schizosaccharomyces pombe, Zygosaccharomyces bailii* and *Zygosaccharomyces fermentati* were reported to survive in presence of high ethanol concentrations (>10%) (Fleet, 2000; Romano and Suzzi, 1993).

Furthermore, the ability of the yeasts to utilize malic acid was a positive attribute in many wine-making processes (Volschenkla et al., 2006). Usually commercially available *Saccharomyces* strains cannot degrade malic acid effectively during alcoholic fermentation. The expression of the malolactic pathway genes, i.e. the malate transporter *(mael)* of *S. pombe* and the malolactic enzyme *(mleA)* from *Oenococcus oeni* in *Saccharomyces*, can improve the malate- utilization and thus improve the quality of wine. However, Volschenkla et al. (2006) suggested that the improper strain selection may give an off-flavor to the wine.

Jolly et al. (2013) have extensively reviewed the contributions and successions of non-*Saccharomyces* yeasts in wine fermentation. Ocon et al. (2010) analyzed the quantitative and qualitative changes of non-*Saccharomyces* yeasts present in spontaneous alcoholic fermentations of a tempranillo grape variety. Though qualitatively 17 different yeast species were reported, quantitatively *Candida stellata, Kloeckera apiculate* and *Saccharomyces cerevisiae*, appeared in large numbers.

Clemente-Jimenez et al. (2004) reported that in the initial phase of the natural fermentation in Macabeo grape varieties, *Kloeckera* and *Candida* genera appeared prominantly, followed by *Metschnikowia*, *Pichia* and sometimes, *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula* and *Zygosaccharomyces.* They further reported that the best profile of higher alcohols was given by *Saccharomyces cerevisiae* followed by *Hanseniaspora uvarum*, *Issatchenkia orientalis* and *Candida stellata*. While due to the presence of *Metschnikowia pulcherrima* and *Pichia fermentans* highest production of ethyl caprilate and 2-phenyl ethanol, compounds associated with pleasant aromas was seen.

1.9.1.2 Factors affecting succession of yeast flora

The succession of yeast during fermentation is affected directly or indirectly, by a number of factors including grape variety, ripening stage, physical damage to berries, if any, climatic conditions, viticulture practices, etc. Renouf et al. (2005) observed qualitative and quantitative differences in yeast populations isolated from Merlot, Cabernet Sauvignon and Cabernet Franc varieties according to berry development stages, namely berry set, varaison and harvest. For instance, at berry set, *A. pullulans* was predominant which was never detected at harvest, while *Saccharomyces* was detected at harvest and not in the first stage of grape growth. The specific condition of the must with respect to the osmotic pressure, presence of SO_2 and temperature play a role in determining species which can survive and grow (Bisson and Kunkee, 1991). The species of *Cryptococcus, Rhodotorula, Sporobolomyces, Candida* and *Hanseniaspora* which were low in number at the initial stage were seen in other two stages also, which can be attributed to their adaptive nature under environmental perturbations such as anaerobic condition, increased alcohol level etc. Excessive rainfall or even pesticide sprays especially during ripening stages affect the number of non-*Saccharomyces* yeasts in the initial stages and later in the fermentation (Guerra et al., 1999; Querol et al., 1990). *Botrytis cinerea* infection to grapes was found to increase the population of *C. krusei* and *K. apiculata* while decrease in *R. glutinis* (Le Roux et al., 1973). In fact, the methods of isolation and enumeration, type of growth media used are also important for the quantitative estimation. For example, on a medium containing lysine as a sole carbon source *S. cerevisiae* could not grow luxuriantly (Heard and Fleet, 1986).

1.9.2 Profiling of enzyme activities during fermentation

The quality of wine is mainly determined by aroma which is due to terpenes. The pivotal role of endogenous enzymes from grapes and also from natural flora in the wine making has been well emphasized Van Rensburg and Pretorius (2000). The enzymes like pectinases, glucanases, xylanases and proteases are involved in the clarification and processing of wine and glucosidase plays a major role in release of aroma compounds (Pombo et al., 2011). The indigenous enzymes from grapes are not adequate in developing specific aroma by hydrolyzing non-volatile glycosidic precursors present in the grapes (Fia et al., 2005). The glycosidases from grapes have narrow substrate specificity, are inhibited by low pH (i.e. from 3 to 4) and glucose at concentrations >1%. Enzymes such as pectinases and glucanase increase juice extraction from grapes improve wine clarification and facilitate wine filtrations (Canal-Llauberes, 1998; Canal-Llauberes, 1993; Villettaz and Duboudieu, 1991), which however, are inactivated due to low pH and SO_2 conditions prevalent during wine fermentation. *S. cerevisiae* does not produce significant quantities of extracellular proteases, lipases or pectinolytic enzymes, while the non-*Saccharomyces* yeasts contribute significantly to a variety of enzyme reactions involved in aroma production during wine fermentation. Yet, as the indigeneous enzymes are not sufficient and efficient under the prevailing conditions of wine making, commercially produced enzymes are often employed for achieving desired quality of wine. Haze formation from proteins in the finished wine may be decreased by the use of proteolytic enzymes (Waters et al., 2005). The reduction in ethyl carbamate as well as alcohol levels is catalysed by urease and glucose oxidase, respectively (Van Rensburg and Pretorius, 2000).

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-glucosidases (Strauss et al., 2001). Esteve-Zarzoso et al. (1998) reported that non-*Saccharomyces* yeast species are important contributors to the final taste and flavor of wines due to their capacitiy to produce different enzymes such as protease, β-glucosidase, esterase, pectinase and lipase. Enzymes of enological interest found in different non-*Saccharomyces* wine yeasts are presented in Table 1.2.

Table 1.2 Enologically important enzymes 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. membranefaciens	+	+	+	+	-	-
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., 2006; Jolly et al., 2006; Lagace and Bisson, 1990; Otero et al., 2003; Rosi et al., 1987; Rosi et al., 1994; Strauss et al., 2001)

The predominant genera which produce these enzymes are *Brettanomyces, Candida, Cryptococcus, Debaryomyces, Hanseniaspora, Hansenula*, *Kloeckera, Kluyveromyces*, *Metschnikowia, Pichia, Rhodotorula, Saccharomycodes, Schizosaccharomyces* and *Zygosaccharomyces.* Maturano et al. (2012) studied the enzymes from *Saccharomyces* and non-*Saccharomyces* species in pure and mixed culture during the fermentation. Pure cultures of *S. cerevisiae, H. vinae*, *T. delbrueckii* and mixed cultures of *Saccharomyces* with *H. vinae* or *T. delbrueckii* were used for fermentation of sterilized grape juice. In mixed cultures, *H. vinae and T. delbruckii* were detected in the initial half of the fermentation. Nevertheless β-glucosidase, protease and pectinase secreted by *H. vinae* and *T. delbruckii* in mixed culture could be detected up to the end of fermentation.

The commercial wine yeast *S. cerevisiae* is not attributed with production of extracellular proteases, β-glucosidase, or glucanases (Hernandez et al., 2003). The commercial β-glucanase preparations used in winemaking for clarification, filtration and maturation of wines were produced by *Trichoderma* species (Canal-Llauberes, 1993). Mojsov et al. (2011) studied the effect of three commercial pectolytic enzyme preparations on the wine fermentation of white grape cultivar, Smederevka. These pectolytic preparations were found to be important in improving filtration rates, lees settling rates and clarity of wines. It was further suggested that such preparations can be used to increase sensory quality in a shorter time with cost effectiveness. However the activity of such exogenously added enzymes are compromised due the conditions prevailing during fermentation. Therefore, non-*Saccharomyces* yeasts as sources of these enzymes are important during wine fermentation.

1.9.3 Profiling of flavor and aroma compounds during fermentation

It was earlier thought that the flavor of alcoholic beverages was due to a small number of volatile compounds. In 1985, the number of volatile compounds estimated was 1300 plus (Nykanen, 1986). Many precursors of volatiles are present in grapes, which are processed more due to activities of non-*Saccharomyces* yeasts than *S. cerevisiae* (Cordente et al., 2012; Nykanen, 1986). Non-*Saccharomyces* yeasts positively contribute to the analytical and sensorial composition of wine with production of hundreds of flavor active secondary metabolites such as acids, alcohols, esters, terpenoids, phenolic compounds, aldehydes, ketones, higher alcohols, glycerol, acetaldehyde, acetic acid and succinic acid volatile sulphur compounds (Jolly et al., 2013; Lambrechts and Pretorius, 2000). Interestingly, though not much studied, the

role of non-*Saccharomyces* yeasts such as *Schizosaccharomyces pombe, P. guilliermondii* and *H. uvarum* in enriching wine color is also documented (Benito et al., 2013; Morata et al., 2012). For example, *P. guilliermondii* and *S. cerevisiae* were shown to increase the formation of vinylphenolic pyranoanthocyanins molecules which show greater color stability (Benito et al., 2013). *S. pombe* which can ferment grape must with high sugar contents also produced high levels of vitisin A type pigments-a natural phenol, while some strains with the help of hydroxycinnamate decarboxylase activity favored the formation of vinylphenolic pyranoanthocyanins (Morata et al., 2012).

Lambrechts and Pretorius (2000) extensively reviewed the significance of both *Saccharomyces* and non-*Saccharomyces* yeasts in developing specific flavor to wine. The variety of grapes, conditioning of must, activities of microbial flora and aging contribute in to the flavor production. Further, the combination of yeast starter cultures can be used to have more or less predictable wine flavor. However, though it appears easy to have desired flavor, the understanding of physiological characteristics of each strain present and it's qualitative and quantitative interactions with other factors are important for avoiding undesirable flavor. The common flavor compounds produced due to yeast enzymes are esters, fatty acids, fatty acid esters and higher alcohols. Usually saturated, straight chain fatty acids along with one unsaturated fatty acid (palmitoleic acid) are produced during wine fermentation. The volatile fatty acid contents of wine are 10-15% of the total acid content which is mainly acetic acid. The predominant non-volatile organic acids are tartaric acid and malic acid in grape juice. Citric acid and lactic acid also add to acidity of the juice. The succinic and keto acids are present initially in traces but increase quantitatively during fermentation. These acids can influence wine flavor depending on concentration and type of wine (Swiegers and Bartowskya, 2005).

The primary flavor of wine is derived from the grapes. However, secondary flavors are derived from ester formation by yeasts during 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). Phenolic compounds contribute to the color, flavor, bitterness and astringency of wine. 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).

Yeast strains in the *Hanseniaspora* genus produce high levels of phenylethyl acetate and phenyl ethanol that contribute to the complexity of wine aroma (Rojas et al., 2001; Viana et al., 2008; Moreira et al., 2005). Glycerol produced by *Candida, Hanseniaspora, Pichia* sp., *L. thermotolerans* and *C. zemplinina* contributes to smoothness (mouth-feel), sweetness and complexity in wines (Comitini et al., 2011; Toro and Vazquez, 2002; Soden et al., 2000). Acetic acid produced by *Hanseniaspora* spp., *Zygosaccharomyces* spp. and succinic acid producers such as *Candida stellata, Saccharomyces ludwigii* and *T. delbrueckii* contribute to the total acidity of the wine (Ciani and Maccarelli, 1998). Different secondary metabolites produced by non-*Saccharomyces* yeasts that contribute to wine quality are depicted in Fig. 1.2.

The multi-starter fermentation with selected non-*Saccharomyces* yeasts and *S. cerevisiae* was found to be useful to avoid problems of natural fermentation, if any (Sadoudi et al., 2012). However, the interactions among the cultures used were complex and majority of the interactions were unpredictable.

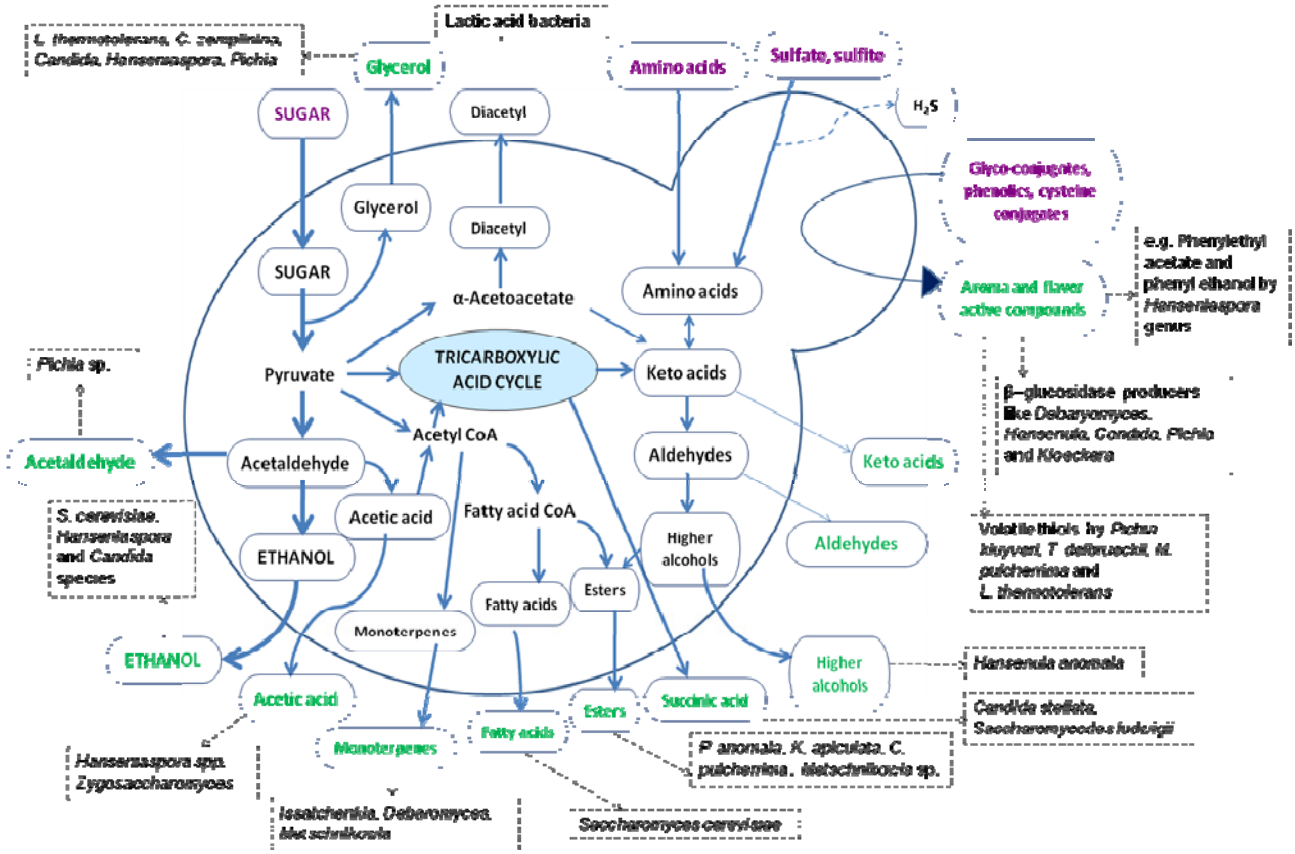

Fig. 1.2 The metabolites produced by *Saccharomyces* and non-*Saccharomyces* yeasts that contribute to wine quality (Modified from Swiegers et al, 2005).

To understand these interactions the volatile profiles of *Candida zemplinina*, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* either in mono-culture or in co-culture with *S. cerevisiae* were studied. It was reported that *C. zemplinina* produced high levels of terpenes and lactones which were decreased in co-culture with *S. cerevisiae*. On the other hand a synergistic effect on aromatic compound production by *M. pulcherrima* was reported in co-culture with *S. cerevisiae* (Sadoudi et al., 2012). No effect was seen in aromatic profiles of *T. delbrueckii* and *S. cerevisiae* in mono-culture and in co-culture. This study can be used to design a specific microbial profile for defined wine quality.

In addition to the role in the production of flavor compounds some of the non-*Saccharomyces* yeasts were also useful to reduce the alcohol levels indirectly enhancing the perception of wine aroma (Quiras et al., 2014). Contreras et al. (2013) earlier evaluated number of non-*Saccharomyces* strains for their capabilities to ferment sugar to ethanol. Although number of yeasts such as *Zygosaccharomyces bailii, Kluveromyces marxianus*, *Hanseniaspora uvarum, Pichia kluyveri, Issatchenkia terricola, Candida sake* and others, produce more ethanol (>0.45g) per g of sugar under anaerobic condition, the % utilization of sugar for ethanol production in most of the strains was far lesser (< 16%) as compared to *S. cerevisiae.* In case of *S. cerevisiae* 98.5% sugar was consumed for ethanol production (0.44g/g sugar) from an initial concentration of 150 g/L sugar under anaerobic condition (Contreras et al., 2013). Therefore, the regulation of level of non-*Saccharomyces* inoculum along with *S. ceervisiae* can be a key feature to avoid masking of wine aroma due to ethanol.

1.10 Wine quality

The term "wine quality" is a subjective term, and has many different meanings, depending upon the context in which the term is used. Perceived quality is the reflection of the chemical composition of the wine at the time at which it is being consumed. What one person perceives as quality may not be so thought of by another. This is in part due to physiological differences in the detection of compounds. There are also differences in the relative concentration or threshold at which a given compound is detected. Wine quality is significantly influenced by the grape composition and yeasts that participate and interact during the fermentation: body, viscosity, colour, flavour and aroma of wines are strongly determined by these yeasts (Sturm et al., 2006).

Many quality components are carried over from grape juice into wine and a few undergo reactions to form compounds that are distinctive to wines. Alcohol content in wine is directly dependant on the sugar content of berries. Vitamins, minerals and nitrogenous compounds are essential for yeast growth and fermentation and basic flavour of a wine is due to the relative contents of sugars, acids, phenolics, and ethanol. The color of wine is an indicator of its condition, quality, age, and even style. Anthocyanins are the main pigments contributing to red color of wine. Phenolics contribute to the astringency/bitterness of wine, and are also responsible for the color. Six types of phenolics are found in grapes, namely, catechins, procyanidins, anthocyanins, flavonols, hydroxycinnamates and hydroxybenzoates. The difference between red and white grapes (wines) is due to the presence of different types of phenolics. More complex phenolics collectively known as "flavonoids" occur in the skin and seeds; hence are more in red wines.

Flavor compounds in combination with specific volatile aroma compounds present in different grapes will give distinct aroma to the wines (Bouquet-as quite often called). More than 100 volatile compounds have been identified in different wines. The most important volatiles in grape are the monoterpenes present in minute quantities (<4 ppm). These give a range of odors in different classes, viz; floral, spicy or fruity. The monoterpenes exist in both free volatile form and as bound glycosides. The glycosides are slowly hydrolysed due to acid conditions of the wine and contribute to aroma as the wine ages. In addition to terpenes, other volatiles also contribute to aroma, such as methoxy pyrazines contributing to distinct 'Sauvignon" aroma in Cabernet Sauvignon and Sauvignon Blanc. It is therefore extremely important to control the acid fraction (depends on many wine properties and phenomena that take place inside), density and color (appearance, astringency and structure). Increased production of glycerol is usually associated with increased acetic acid production (Prior et al., 2000), which can be detrimental to wine quality.

1.11 Winery waste utilization

During wine production huge amounts of by-products mainly consisting of organic wastes- pomace and yeast lees, wastewater, emission of greenhouse gases, and inorganic residues are generated. After grape juice extraction the remaining pomace and stems are sent for composting, land-filling or discarded in open areas potentially causing environmental problems. For instance, degradation products of pomace can

inhibit root growth. Development of innovative procedures to recycle/reuse these residues and recover green materials, nutrients and bioactive compounds for the feed/food, pharmaceutical, and cosmetic sectors is warranted for value addition. In this sense, the valorization of these wastes will provide alternatives to reduce the environmental impact of winery activity.

Winery waste and by-products as percent of grapes include grape stalks (2.5−7.5 %), grape pomace (~15 % dry wt.; wet wt. up to 25−45 %), grape seeds (3−6 %) and yeast lees (3.5−8.5 %). Grape pomace contains up to 15 % sugars, 0.9 % phenolics/pigments (in red grape pomace), 0.05−0.08 % tartarate and 30−40 % fibre. Grape seeds contain 4−6 % phenolics and 12−17 % oil very rich in linoleic acid-omega-6 fatty acid (~76 %). The yeast lees contain 0.012 % pigments, 0.1−0.15 % tartrate and 6−12 % β-1, 3-glucans (Wadhwa and Bakshi, 2013). Grape pomace bioactives have many potential applications in development of functional foods, cosmetics, pharmaceutical/biomedical, for food processing, and supplements. Grape pomace, seeds and stalks are known sources of antioxidants (phenolic acids, quercetin, flavonoids, phytoalexins and pterostylbenes, resveratrol, etc.). These substances also have anti-inflammatory, anti-carcinogenic and anti-mutagenic effects (Shrikhande, 2000). Grape pomace, seeds, skin and stems extracts have exhibited potent anti-bacterial activity against different food spoilage bacteria and may find application in food preservation (Ozkan et al., 2004).

Seeds, pulp and skin waste due to their high fiber content are usually used as feed for animals. However these by-products are a good and cheap source of high quality polyphenolic compounds which have different therapeutic uses (Lafka et al., 2007). Polyphenols represent the third most abundant constituent in grapes and wines after carbohydrates and fruit acids. The most common polyphenolic compounds in grapes and pomace include - cinnamic acids (coumaric, caffeic, ferulic, chlorogenic and neochlorogenic acid), stilbenes (the most common being resveratrol and its derivatives) and flavonoids such as catechin, epicatechin, glycosides of flavonols and phenolic acids (Iriti et al., 2006). Pomace may also prove to be good source for other value added products such as biosurfactants, grapeseed oil and pullulan. In recent years, the use of grape seed extract (GSE) with strong antioxidant activity has begun to become popular as a nutritional supplement. These extracts contain a heterogeneous mixture of monomers, oligomers and polymers formed by subunit of flanan-3-ol. These phenols have demonstrated anti-carcinogenic activities as well as

in-vivo activity against the oxidation of the low-density lipoproteins (Meyer et al., 1998).

After polyphenol extraction, grape pomace can be used as a medium for solid state fermentation (SSF) to produce single cell protein (Lo Curto and Tripodo, 2001), bacterial cellulose (Tataridis et al., 2006) and other valuable metabolites. There is a significant interest in use of pomace as substrate for SSF to produce various enzymes mainly from fungi (Botella et al., 2005). Several bioprocesses using grape pomace as substrate have been developed for the production of various chemicals including ethanol, gluconic acid, carotenoids, xanthan and citric acid (Botella et al., 2007).

During wine fermentation yeast flora, natural as well as added inoculum flourishes to an extent to be a component for value addition to winery. Klis et al. (2006) extensively reviewed the cell wall architecture of *S. cerevisiae* revealing that mannoproteins were 30-50%, 1-6-β-glucan 5-10%, 1,3-β-glucan 30-45 % while chitin 1.5-6% of the cell wall mass. The yeast lees obtained may be useful as source material for the isolation of these cell wall polymers having diverse applications.

Aims and Objectives

Study of yeast diversity to gain more information about yeast communities present on the grape berries and their influence on the wine making process is a major challenge in wine microbiology. The vine micro-flora may be useful or detrimental affecting the quality of wine (Fleet, 2003).Various environmental factors such as geographic location, climatic conditions, agricultural practices have an impact on the population of yeast on grape surfaces (Raspor et al., 2006). Certain non *Saccharomyce*s yeasts produce extracellular enzymes and metabolites that contribute to the quality of wine (Esteve-Zarzoso et al., 1998).

Quantification of *Saccharomyces* and non-S*accharomyces* yeasts from grapes berries and during fermentation along with enzyme and metabolites produced can help to improve the knowledge and understanding of the role of these yeasts in wine making.

Literature review presented in first chapter 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. The commercial wine grape production areas of Maharashtra and Karnataka in India has increased in the past decade, and the Indian wine industry expanded from a handful of wineries to 93 wineries (in Maharashtra) in 2015. There is a need to study and understand the yeast diversity associated with the different wine variety grapes commonly used in different locations for wine production 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 producing good quality Indian wine. Thus 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, huge amount of waste such as pomace and yeast lees is generated as by-product of wine industry. Since grape pomace finds limited use in animal feed or as manure, and its use as land-fill causes environmental pollution, therefore, management of this waste is remains a major challenge to the wine industry.

Based on the background presented in literature review, the present investigation is aimed toward understanding the role of *Saccharomyces* and non-*Saccharomyces* yeasts in wine making and valorization of winery waste with following specific objectives -

- Isolation and identification of natural *Saccharomyces* and non *Saccharomyces* yeasts from different varieties of wine grapes grown in different regions of Maharashtra (India).
- Role of *Saccharomyces* and non-*Saccharomyces* yeasts in wine fermentation
- Uses of winery waste for possible value addition to the wine Industry

Chapter 2

Materials and Methods

2.1 Chemicals

All the bulk chemicals, solvents used in the study were of analytical reagent (AR) grade and procured from suppliers including SD fine-chemicals, Loba Chemie, Sisco Research Laboratories and Qualigenes, India.

The microbiological media components were purchased from Hi-Media Corporation, Mumbai, India. Fine chemicals like caffeic acid, coumaric acid, D-galacturonic acid, ethidium bromide, laminarin, N-acetylglucosamine polygalacturonic acid, quercetin, syringic acid, trans-resveratrol, tyrosin, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenylthiazolium bromide (MTT) were obtained from Sigma–Aldrich, Germany.

DNA ladder mix, RNAase was procured from Promega, UK; dNTP, HF PCR buffer, and Phusion HF DNA polymerase was procured from Finnzyme, Finland; PCR primers were provided by IDT, USA and PCR purification kit was purchased from Quiagen, India.

2.2 Grape sample collection

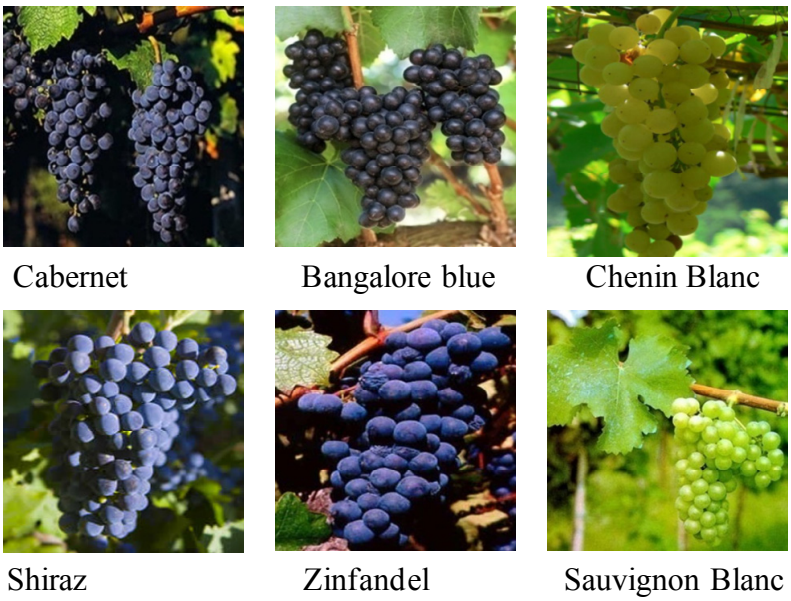

Fig. 2.1 Grape varieties used for isolation of yeasts

Red Wine grapes varieties i.e. Shiraz and Cabernet; and white wine grape varieties Chenin Blanc and Sauvignon Blanc were collected from vineyards located in Sangli district of Maharashtra (16^0 52'N, 74^0 34'E). Samples of red grape varieties Shiraz, Cabernet and white variety Sauvignon Blanc were collected From Nashik district (19º 99´ N, 73º 78´ E),. Four red grape varieties Bangalore Blue, Cabernet, Shiraz and Zinfandel were collected from Pune district (18º 31´ N, 73º 55´ E) (Fig. 2.1).

Mature grapes (2 Kg) with 19-27º Brix level and without any visible damage or infection were collected for each variety.

2.3 Isolation of the natural *Saccharomyces* and non-*Saccharomyces* yeasts

Sample of each grape variety was processed separately. After crushing, serial dilutions of the grape juices was prepared and 0.1 mL from each dilution was spread plated 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 for 48 h in an incubator at 28ºC. After incubation, single colonies having different morphologies were selected and transferred to new MGYP plates to obtain pure yeasts.

2.4 Maintenance of microorganisms

Pure yeasts isolates obtained were maintained on MGYP agar slats at 4ºC and sub-cultured on fresh slants before use and every 30 days. Wine spoilage yeasts namely *Schizosaccharomyces pombe* NCIM 3457, *Dekkera bruxellensis* NCIM 3534, *Zygosaccharomyces rouxii* NCIM 3460, *Torulaspora delbrueckii* NCIM 3295, *Metchnikowia pulcherima* NCIM 3109, and pathogenic yeast namely *Candida albicans* NCIM 3557*, Cryptococcus neoformans* NCIM 3542 were obtained from National Collection of Industrial Microorganisms (NCIM), CSIR-National Chemical Laboratory, Pune, India and were grown on MGYP agar slants.

Potato dextrose agar (PDA powder, 39 g/L; agar, 5g/L; pH, 5.5-6.0) was used as a medium for growth and maintenance of *Myrothecium verrucaria* MTCC 5191. Bacterial cultures *Mycobacterium tuberculosis* H37Ra, *Mycobacterium bovis* BCG, *Staphylococcus aureus, Bacillus subtillus* from NCIM, CSIR-NCL, Pune were used in antibacterial testing.

2.5 Identification of the yeast isolates

2.5.1 Colony characteristics and morphology

The yeast isolates were identified based on the colony characteristics, morphology and different biochemical tests. Individual colonies of the isolates grown on MGYP agar were examined for different characteristics, namely, size, shape, color, margin, consistency, opacity, elevation and appearance. The yeast cells were observed under light microscope (40 x) for size and shape.

2.5.2 Biochemical tests

In biochemical characteristics, assimilation and fermentation of different sugars as sole carbon source was checked as described by Kurtzman and Fell (1998).

2.5.2.1 Sugar assimilation tests

Different sugars namely, glucose, galactose, sucrose, maltose, lactose, cellobiose and salicin were added (0.5 %) separately in 5 mL of 0.6% yeast nitrogen base (YNB). The yeast cells ($1x10^6$ CFU/mL) were inoculated and incubation in a rotary shaker (180 rpm) at 28ºC for 48 h. Tubes with glucose and without any carbon source were also kept as positive and negative controls. The growth in liquid medium was measured after 28°C for 24–48 h at 600 nm. Absorbance was compared with the controls and recorded as + or - (positive/negative).

2.5.2.1 Sugar fermentation tests

Sterile basal medium YP broth (4.5 g/L yeast extract, 7.5 g/L peptone and bromothymol blue indicator) supplemented separately with 2% sugars, namely, glucose, galactose, sucrose, maltose, lactose, raffinose were added in test tubes. Inverted Durham's tubes were put in the tubes in order to to collect the gas formed during fermentation. After inoculation with the yeast cultures, the tubes were incubated at 28ºC for 48 h.

After incubation, results were recorded for all the sugars as positive (+), weak positive (w) and negative (-) based on the amount of acid (change in the color of bromothymol blue from dark green to yellow) and gas produced (which is accumulated in Durham's tube).

2.5.3 Cluster analysis

Results of the biochemical tests for all the 152 isolates recorded as + (positive), w (weak) and – (negative) were given an ordinal scale of 1, 2 and 3 and used as raw data along with comparable standard strains from Centraalbureau voor Schimmelcultures CBS fungal biodiversity center and Kurtzman and Fell (1998) was used for cluster

analysis. Identification of the yeasts was done by carrying out cluster analysis using SPSS 11 software.

2.6 Molecular analysis for identification of the yeasts

Confirmation of cultures identified by morphological and biochemical methods were further identified till species level by amplification and sequencing of ITS1, 5.8S, ITS2 and 26S ribosomal DNA region.

2.6.1 DNA isolation

For the isolation of DNA, yeast cells were grown in MGYP broth. DNA was extracted using standard procedures according to Chavan et al. (2009). MGYP broth (5 mL) containing 1 x 10^8 cells/mL was centrifuged at 16,000 x g at 4°C for 10 min and the cell pellet (approximately 100 µL volume) was resuspended in 200 µL of breaking buffer (2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8). The cells were homogenized by vortexing with 0.3 g of glass beads (0.5 mm diameter; BioSpec Products, Bartlesville, USA) in the presence of 200 µL phenol/chloroform/isoamyl alcohol (50:48:2). TE buffer 200 µL (10 mM Tris, 1 mM EDTA, pH 7.6) was added and the bead/cell mixture was centrifuged at 16,000 x g at 4°C for 10 min. After this the aqueous phase was collected. The DNA was precipitated with 2.5 volumes of 100% ethanol and centrifuged at 16,000 x g at 4°C for 10 min and the pellet was washed with 70% ethanol, dried and resuspended in 50 µL of sterile distilled water containing 2 IU RNase (Sigma, USA). The sample was incubated at 37ºC for 30 min and stored at -20°C until further use. Purity and quantity of the DNA in the samples was determined using the A260/A280 ratio measured on Nanodrop 1000 UV-visible spectrometer. A260/A280 ratio ≥ 1.8 was considered as pure DNA.

2.6.2 Amplification of ITS region

The ITS1, 5.8S and ITS2 regions of rDNA gene were amplified by PCR using the

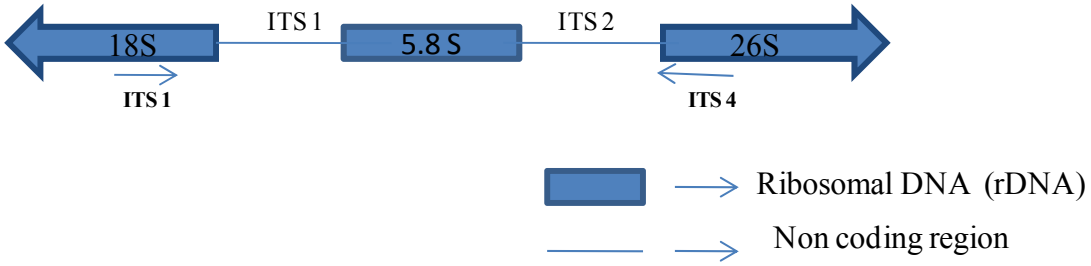

Fig. 2.2 ITS region of ribosomal DNA

primer ITS1 (19-mer 5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (21-mer 5'-TCCTCCGCTTTATTGATATGC-3') according to White et al. (1990) (Fig 2.2).

PCR was performed in a final volume of 20 µL containing 1X water, 1X HF PCR buffer, 0.2 mM dNTP, 0.5 mM of the each reverse and forward primer, 0.4 U Phusion polymerase (Finnzyme, Finland) and 1 µL of the extracted DNA (~ 15 ng). After an initial 30 s denaturation at 98°C the reactions were run for 32 cycles: denaturation was at 98°C for 10 s, annealing at 5 °C for 20 s and extension at 72°C for 90 s followed by final 7 min extension at 72°C.

2.6.3 Amplification of 26S rDNA

Amplification of the 26S rDNA (Fig. 2.3) was carried out using the primer pair forward - NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3") and reverse - NL4 (3'-GGTCCGTGTTTCAAGACGG-5') (O'Donnell, 1993). Programme conditions for PCR were same as described above in section 2.6.2.

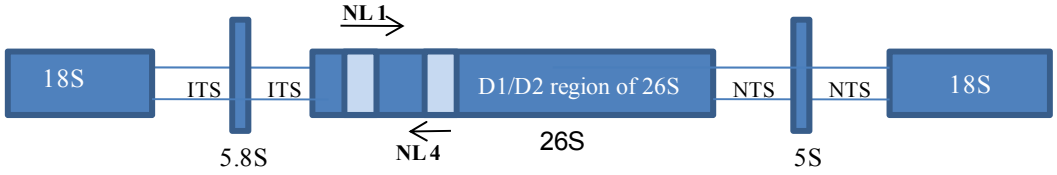

Fig. 2.3 D1/D2 region 26S rDNA

2.6.4 Purification of PCR product

PCR products were purified with QIAquick PCR purification kit (Qiagen, Germany). Briefly, PCR product (one volume) was mixed with phosphate buffer (five volumes) and transferred to the QIAquick column placed in a provided 2 mL collection tube. The column was centrifuged (60 sec) and flow-through was discarded. Column was then washed with 750 µL PE Buffer by centrifugation for 60 sec. Flow-through was discarded and the residual wash buffer was removed by additional 2 min centrifugation. The column was then placed in a clean 1.5 mL micro-centrifuge tube. Elution Buffer (50 µL; 10 mM Tris·Cl, pH 8.5) was added and the column was centrifuged for 1 min to elute the PCR product. Products were analyzed on 1.5% agarose gel containing 0.7 µg/mL of ethidium bromide and visualized under UV light. Approximate size of amplicons was determined using standard molecular weight markers (range 100 bp-10 kb gene ruler DNA ladder mix, Promega, Madison, USA).

2.6.5 Sequencing and data analysis

Purified PCR products were directly sequenced by using the ABI prism 3730 DNA analyzer (Applied Biosystems, USA). Chromas 2.1 software was used for reading and editing the DNA sequences. Blast searches of sequences were performed at the 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). Evolutionary analyses were conducted in MEGA6. The phylogeny was estimated using the Maximum Parsimony method for 152 grape isolates and their closest related species. The analysis involved 170 nucleotide sequences. Bootstrap was performed for 100 replicates. All the sequences were deposited in NCBI GenBank for accession number.

2.7 Spot assay for pesticide susceptibility

Commonly used commercially available pesticide formulations were used for the pesticide susceptibility assay. All the pesticide stocks were prepared in DMSO as per the recommended dose. YPG agar plates containing recommended field concentration of test pesticide were prepared. Cells were grown at 28 °C for 24 h, collected by centrifugation and washed with sterile distilled water. The cell suspensions were adjusted to 10^8 cells/mL (counted with a Neubauer chamber), ten-fold serial dilutions were prepared and 10 µl from each dilution were spotted on agar plates containing different pesticides. The plates were incubated at 28°C for up to 120 h. After incubation sensitivity/resistance of yeast isolates were determined on the basis of presence or absence of growth.

2.8 Enzyme assays

Yeast isolates grown for 24 hrs were inoculated ($1x10^6$ cells/mL) separately in YPG broth (5 mL) and incubated in a rotary shaker (180 rpm) at 28°C for 48 h. The medium was centrifuged at 16,000 x g for 10 min at 4°C. The supernatant was used for the determination of different enzyme activities, viz., pectinase, β-1, 3 glucanase, β-glucosidase and protease. For all assays mentioned below, suitable blanks were made as required.

2.8.1 Pectinase assay

Pectinase activity was determined according to Akhter et al (2011). The reaction mixture contained substrate 0.9 mL; 0.5% pectin in 0.05 M sodium acetate buffer, pH

5 and enzyme sample 0.1 ml. Reaction mixture was incubated for 30 min at 50°C. DNSA reagent (1 mL) was added to stop the reaction. Tubes were kept in boiling water bath for 5 min, cooled under running water and 8 mL distilled water was added. Absorbance of the mixture was measured at 540 nm. D-galacturonic acid (10 µg/mL - 100 µg/mL) was used as a standard to calculate the amount of D-galacturonic acid released. One unit of pectinase activity was defined as one nmole of D-galacturonic acid released/mL/h under the given assay conditions.

To prepare DNSA reagent, 10.66% NaOH (150 mL) was added drop-wise to stirring solution of 3, 5 dinitrosalicyclic acid (10 g 3, 5-dinitrosalicyclic acid in 200 mL water). Potassium sodium tartarate (300 g) was added in it and volume was made up to 1 L with distilled water. Solution was filtered with sintered glass filter.

2.8.2 β-1, 3 glucanase assay

The β-1, 3 glucanase activity was estimated by following method of Vyas and Deshpande (1989). Laminarin (0.5 mL; 1% in 0.05 M sodium acetate buffer, pH 4.5) was mixed with the supernatant (0.1 mL) and incubated at 37°C for 30 min. After incubation, 1 mL DNSA reagent was added. The mixture was boiled for 5 min, cooled and 8 mL distilled water was added to it. Absorbance of the sample was measured at 540 nm. Amount of reducing sugars released was calculated using D-glucose (100 µg/mL-1000 µg/mL) as standard. One unit of β-1, 3 glucanase activity was defined as the amount of enzyme required to liberate one nmole of glucose/mL/h from the substrate laminarin under the given assay conditions.

2.8.3 β-glucosidase assay

The β-glucosidase assay was performed according to Sadana et al. (1980) with modification. Para-nitrophenyl-β-D-glucanopyroside (0.1 %) in 0.1 M acetate buffer (pH 4.5) was used as substrate. Supernatant (0.2 mL) was added to 1.8 mL substrate and incubated at 37°C for 15 min. Reaction was stopped with 2 mL 2% Na_2CO_3. *P*-nitrophenol released was estimated by reading absorbance at 405 nm. One unit of enzyme activity was defined as the amount of enzyme required to liberate one nmole of paranitrophenyl/mL/h under the given assay conditions.

2.8.4 Acid protease assay

For estimation of acid proteases activity, method reported by Nelson and young (1986) was used. Haemoglobin (1% in 0.1M citrate phosphate buffer, pH 3.2) was used as a substrate. Briefly, reaction mixture (2 mL substrate and 0.5 mL of the

supernatant) was incubated in a temperature controlled water bath at 37°C for 30 min. Reaction was stopped by adding 3 mL trichloroacetic acid (5% TCA in 3.3 N HCl). Reaction mixture was filtered using Whatmann filter paper No. 1 and absorbance of the sample was measured at 280 nm. Tyrosine (50 - 500 µg/mL) was used as standard. One unit of acid protease activity was defined as the amount of enzyme required to liberate one nmole of tyrosine/mL/h under the given assay conditions.

2.9 Chemical analysis of grape juice

2.9.1 Color and phenolic measures

Diluted grape juice (1:100), 2 mL was added in three set of tubes, and 100 µL was added in 4th set of tubes. In 2nd set, 20 µL acetaldehyde solution (10% w/v) was added, mixed and incubated for 45 min. In 3rd set, 30 µL sodium metabisulfite (25% w/v) was added; mixed gently and spectral readings were taken. In the 4th set, HCL solution (10 mL, 1 M) was added, mixed thoroughly by inverting many times and incubated for 3 h. For samples from set 1, 2 and 3, absorbances were measured both at 520 and 420 nm, whereas for 4th set, readings were taken at 520 and 280 nm. Following formulas were used to calculate different wine color parameters (Iland et al., 2004).

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

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

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

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

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

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

2.9.2 Reducing sugars

DNSA method was used for estimation of reducing sugars (Miller, 1959). Diluted grape juice sample (1:100), 1 mL, was mixed with DNSA reagent (1 mL) and boiled for 5 min in water bath. After cooling, 8 mL distilled water was added to the mixture. Absorbance of the solution was measured at 540 nm. D-glucose (100 µg/mL -1000 µg/mL) was used as a standard to calculate the amount of released reducing sugars.

2.9.3 Total phenolics content (TPC)

Grape juice obtained from Shiraz variety was diluted (1:50) with distilled water and used for estimation of TPC. The sample (1 mL) was mixed with 2% sodium carbonate (2.5 mL) and 10 % Folin-Ciocalteu reagent (2.5 mL). After incubation for 30 min at room temperature (RT), absorbance was measured at 765 nm (Khatoon et al., 2013). Gallic acid was used as standard. Gallic acid stock solution (1 mg/mL of methanol) was diluted with distilled water to prepare working standards (10 µg/mL-100 µg/mL). TPC was expressed as milligrams of gallic acid equivalent (GAE)/L.

2.9.4 Flavonoids content

Flavonoid contents were determined by method of Feliciano et al. (2009). Grape juice (1 mL, 1:50 diluted), 5% sodium nitrite (150 µL) and distilled water (2 mL) were mixed. The mixture was incubated for 5 min at RT, 10% aluminium chloride (150 µL) was added and incubated further for 6 min. Sodium hydroxide (1 mL, 1 M) was added and total volume was made upto 5 mL with distilled water. Absorbance was measured at 510 nm. Catechin (10-100 µg/mL) served as the standard and total flavonoids content was expressed as mg catechin equivalents (CE)/L.

2.9.5 Tannin content

Hagerman and Butler‘s (1978) method of protein tannin precipitation was used for estimation of total tannins. Tannic acid (1mg/ml) was used as a standard. Reaction mixture containing sample (1 mL) and bovine serum albumin (BSA), (2 mL; 1 mg/mL in 0.2 M acetate buffer, pH 5.0 containing 0.17 M sodium chloride) was mixed by vortexing and allowed to stand at room temperature for 15 min. Centrifugation was done at 5000 x g for 15 min. Pellet was separated, washed with 0.2 M acetate buffer and dissolved in 4 mL SDS- triethanolamine mixture (1% & 5% w/v respectively). Ferric chloride reagent (1 mL; 0.01 M $FeCl_3$ in 0.01 N HCl) was added, reaction mixture incubated for 30 min and the absorbance was measured at 510 nm.

2.10 Ethanol tolerance test

The experiment was carried out in 96 well microtiter plates. YPG (Yeast extract, peptone and glucose) 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

Shiraz variety grapes were crushed and 1.5 L of the juice containing seeds and skin was added in 2 L fermenter bottles. Home-brewing fermentation locks filled with 25 mL of sterile water were used to seal the fermenter. Commercially available yeast (25 mg/L; Lalvin EC 1118, Zytex, Mumbai) and 75 mg $K_2S_2O_5$ were added to the must. Fermentation was carried out for 15 d at 20ºC. After every three days, samples were collected. Serial dilutions of the samples were done and plated on MGYP agar plates. Colonies were counted and cells were observed microscopically after 48 h incubation.

For estimation of enzyme activities, the samples (50 mL) were centrifuged at 5000 x g for 20 min at 4ºC. Supernatant was separated, mixed with same volume of acetone: ethanol (1:1), and kept overnight at -20ºC for precipitation. The solution was centrifuged at 5000 x g for 15 min at 4ºC. Pellet obtained was dissolved in sodium acetate buffer (5 mL; 0.05 M, pH 5) and used as enzyme source for the estimation of different enzyme activities described in section 2.8.

2.12 Wine analysis

Parameters namely, residual sugars, color and phenolic measures, TPC, flavoinoid and total tannin contents for the wine sample were estimated by following procedures used for grape juice as described in section 2.9.

2.12.1 Ethanol concentration

Ethanol concentration in the final wine (15^{th} d sample) was determined using Ebulliometer (Dujardin-Salleron, Paris, France). It involves estimation of boiling point of a sample relative to the boiling point of distilled water. Condenser of the Ebulliometer was filled with cold water, whereas boiling chamber was filled with distilled water/ wine sample. The chamber was heated with spirit lamp and after the thermometer reached a constant temperature, it was recored as boiling point of the sample. Ethanol concentration (%; v/v) of the sample was estimated using the calculating dial.

2.12.2 Analysis of glycerol

Glycerol concentration in the wine sample was determined by HPLC (Waters) with Sugar Pak-1 column (300 mm x 6.5 mm). Ca-EDTA (0.01 mM) at a flow rate of 0.4 mL/min was used as the mobile phase. Column oven temperature was maintained at

80°C and sample size was 30 µL samples /standard. Peak area of 150 µg/mL glycerol was used to calculate glycerol concentration in the samples.

2.13 High Resolution-Liquid Chromatography Mass spectrophotometry (HR-LCMS) analysis

HR-LCMS was used for qualitative and quantitative determination of phenolic compounds and organic acids present in the grape juice, fermented wine sample and wine purchased from market. Samples were centrifuged at 5000 x g for 5 min and filtered through 0.45 µm membrane. Polyphenols standards (1 µg/L) were prepared in methanol.

Analysis was performed on Q-Exactive hybrid quadropoule orbitrap mass spectrometer (Thermo scientific, Germany). Injection volume was 5 µL. Thermo hypersil gold C-18 (150 mm × 4.6 mm, 8 µm) reverse phase column was used for liquid chromatographic separation. Column and auto sampler tray temperature were kept constant at 25ºC and 8ºC, respectively. Methanol (A) and 0.1% formic acid aqueous (B) was used as mobile phase with flow rate of 0.5 mL/min and gradient program mentioned in Table 2.1.

Table 2.1 Mobile phase gradient program for HR-LCMS

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

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.

2.14 Separation of seeds and skin/pulp from pomace

Grape pomace of four different variety grapes namely, Shiraz, Cabernet, Sauvignon Blanc and Chenin Blanc was collected from a winery located in Nashik district. The pomace was shade dried and powered in a grinder.

2.15 Physico-chemical analysis of grape pomace

Pomace sample (1 g) was dissolved in distilled water and reducing sugars, TPC were determined as described in section 2.9. pH was recorded using pH meter. Percentage of C, H and N were estimated by Flash EA1112 Series (C, H, N, and S) analyzer. Protein content was calculated by using formula Nitrogen percentage x 6.25 (Llobera and Canellas, 2007)

For moisture content, pomace (10 g) was weighed in glass petri plate, placed in a desiccator and dried in an oven at 100°C till a constant weight is achieved. Moisture content was calculated using this formula

Moisture content (*%*) = (*Initial weight-Final weight/Weight of the sample*) *X 100*

2.16 Extraction of polyphenolic enriched fraction

Seeds were separated manually from skin/pulp and processed separately. Dried seeds and skin/pulp (50 g) were extracted separately with 100 mL methanol and 200 mL petroleum ether with mechanical stirring for 30 min. Solubles were filtered and the residual powder was extracted two more times with fresh methanol 100 mL) and petroleum ether (200 mL) with stirring. Methanol and petroleum layers were separated and the solvents were evaporated under reduced pressure to yield methanolic extract and petroleum extract (fat).The methanolic extract was partitioned in n-butanol and water (50 mL each), n-butanol layer was separated and dried using rotary evaporator to yield polyphenolic enriched fractions.

2.17 Biological activities of polyphenolic enriched fractions

2.17.1 Antioxidant assay

Total antioxidant capacity was measured spectrophotometrically as described by Prieto et al. (1999). Samples (with different dilutions) were prepared in methanol and

mixed with 1 mL of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, 4 mM ammonium molybdate mixture) in microcentrifuge tubes. The tubes were kept in a water bath at 95º C for 90 min. The mixture was cooled to room temperature and the absorbance was read at 695 nm against blank. Ascorbic acid equivalents were calculated from standard graph of ascorbic acid (10-200 µg/mL). Results were expressed as equivalents of ascorbic acid in µg/mg of extract.

2.17.2 Antiglycation assay

BSA (50 mg/mL) and 0.5 M glucose in 0.1 M phosphate buffer, pH 7.4 were incubated in presence or absence of test sample under aseptic conditions at 37ºC for seven days. The samples were checked for glycation specific fluorescence (excitation, 370 nm; emission, 440 nm) using a Varian Spectrofluorometer. The inhibition of the glycation reaction was calculated by (C-T)/Cx100 for each test sample, where C is the fluorescence of glycated BSA in the absence of sample and T is the fluorescence of glycated BSA in the presence of sample. Aminoguanidine was used as a positive control for glycation inhibition.

2.17.3 Antibacterial assay

Antibacterial activity of the polyphenolic fractions was checked against *Staphylococcus aureus* and *Bacillus subtillus.* The fractions (100 µg/mL) were added in tubes with Mueller-Hinton broth (1 mL) and the cultures were inoculated (100 µL of $1x10^6$ cells/mL). All the tubes were incubated at 35ºC for 24 h. Optical density of the broth was measured at 450 nm (AL-Janabi, 2009).

2.17.4 Antitubercular assay

Briefly, *M. tuberculosis* H37Ra (ATCC 25177) and *Mycobacterium bovis* BCG cells were grown to logarithmic phase (OD 0.595−1.0) in a defined medium (M. pheli medium) under aerobic conditions in a shaker incubator (37°C, 150 rpm). After growth, the culture was sonicated for 2 min in sonicator. Sonicated cells were used for the inoculation (250 µL of $\sim 10^5$ cells/mL) in microtiter plate wells. Test samples dissolved in DMSO were added to the wells to achieve a concentration of 100 µg/mL for the preliminary screening. A dose response curve of the active compounds was carried out by serial dilutions of the test samples. Isoniazid was used as a positive control. The plate was incubated in a CO_2 incubator at 37 °C. The plate was taken out on the eighth day of incubation to measure the viable cell counts. The optical density of the culture was measured at 470 nm before the addition of XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], which served as a

blank. XTT (200 μM) was added, the mixture was shaken for 1 min and incubated for 20 min at 37°C. Subsequently, menadione (60 μM) was added, the mixture was shaken for 1 min and incubated further at 37°C for 40 min. Finally, the optical density of the suspension was measured at 470 nm using a microplate reader.

2.17.5 Anticancer assay

Human cancer cell line, SiHa (squamous cell carcinoma; Cervix) was obtained from National Animal Cell Repository at National Center for Cell Science, Pune. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM L-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin and maintained at 37ºC in humidified 5% CO_2 atmosphere. For cytotoxicity evaluation, cells were trypsinized and the cell suspension containing 1 x 10^4 cells were seeded into each well of 96 well microtiter plates. The plates were incubated at 37°C in humidified 5% CO_2 atmosphere for 24 h to allow the adherence of cells prior to addition of various test samples for testing. After 24 h of incubation, cells were treated with 0.39 μg/mL-25 μg/mL concentrations of test sample in multiple wells of microtiter plates. To evaluate possible effect of DMSO on cell viability, cells were also treated with similar concentrations of DMSO. Equal amount of PBS was added to wells which served as control. After addition of all test samples, plates were incubated in 5% CO_2 atmosphere for 48 h. The cytotoxic effect was analyzed by MTT assay.

After 48 h incubation, MTT (10 μL, 5 μg/mL) was added to all the wells of the test plates and the plates were incubated in dark for 5 - 6 h. DMSO (100 μL) and glycine buffer (25 μL) were added to dissolve the formazan crystals resulting from the reduction of the tetrazolium salt by metabolically active cells. The absorbance was measured at 540 nm using a microtiter plate reader (Spectramax plus 384 plate reader, Molecular Devices Inc). Since the absorbance directly correlates with the number of viable cells, cell survival was measured as absorbance (OD) of the mean of the replicate wells compared to that of control. IC_{50} values, defined as the concentration of the drug/compound that killed 50% of cells in comparison with the untreated cultures, were estimated by plotting OD readings versus the drug concentrations.

2.17.6 Antifungal assay

Evaluation for antifungal susceptibility was carried out using microbroth dilution method according to the recommendations of the Clinical Laboratory Standards Institute (CLSI, 2008). Appropriate amount of test samples were dissolved in

dimethyl sulfoxide (DMSO) to get 100 X final strength. The samples were serially diluted two fold in successive wells to get a range of 4-512 µg/mL and 500-2000 µg/mL. Yeast cells (~$1x10^3$ CFU/mL), freshly grown (24 h) in YPG broth in logarithmic phase were suspended in fresh YPG medium and 200 µL was inoculated in the wells of the plate. The microtitre plate was incubated for 48 h, and the absorbance was measured at 600 nm by using microtiter plate reader to assess cell growth. The Minimal Inhibitory Concentration (MIC) was defined as the concentration exhibiting > 99 % inhibition of growth as compared to the growth of the control.

2.18 Solid state fermentation for Cuticle degrading enzymes (CDE) production

Powdered grape pomace of Shiraz variety and crude chitin (3:1) mixture was used as substrate for solid state fermentation. Chitin medium with following composition (g/L) was prepared - KH_2PO_4, 3.0; K_2HPO_4, 1.0; $MgSO_4$, 0.7; $(NH_4)_2SO_4$, 1.4; NaCl, 0.5; $CaCl_2$, 0.5; yeast extract; 0.5; bacto-peptone, 0.5; chitin, 5.0; 1 mL trace metal solution (mg/mL) $FeSO_4$, 5.0; $MnSO_4$, 1.56; $ZnSO_4$, 3.34; $CoCl_2.2H_2O$, 2.0; pH, 6.0. Ten grams of substrate (pomace: chitin; 3:1), was soaked separately in different volumes of chitin medium (6 mL, 7 mL and 8 mL). The media was sterilized by autoclaving at 15 psi for 45 min. After cooling, the substrate was inoculated with *Myrothecium verrucaria* mycelial inoculum (1 mL and 3 mL) grown in chitin medium. The fermentation was carried out at 28°C for 7 d in humidity chamber (RH 80%). After fermentation, CDE complex was extracted using 50 mL 1% NaCl by shaking at 120 rpm for 30 min at room temperature. After extraction, the CDE complex was separated by centrifugation at 10,000 × g, 10 min. Supernatant was used as enzyme source for the assays.

2.18.1 Chitinase assay

Total chitinase activity was estimated using acid-swollen chitin as a substrate. To prepare acid-swollen chitin, 10 g chitin (purified powder from crab shells, Sigma) was suspended in 300 mL O-phosphoric acid (88% w/v) and kept at 4ºC for 1 h with occasional stirring. The mixture was poured into ice-cold distilled water (4 L) and left for 30 min. The swollen chitin was repeatedly washed with ice-cold distilled water, followed by a wash with 1% (w/v) $NaHCO_3$ solution to adjust pH 7. The swollen chitin was then dialyzed at 4ºC against distilled water. After homogenization in a

waring blender for 1 min, the concentration of acid swollen chitin was adjusted to 7 mg/mL using 50 mM acetate buffer, pH 5.

The reaction mixture containing 1 mL 0.7% swollen chitin, 1 mL of acetate buffer (pH 5.0, 50 mM) and 1 mL of suitably diluted enzyme solution was incubated at 50ºC for 1 h. After 1 h, p-dimethyl amino benzaldehyde (DMAB, 10% in acetic acid), 3 mL, was added and the mixture was incubated for 20 min at 37°C. The absorbance was measured at 585 nm (Reissig et al., 1955). N-Acetylglucosamine 10-100 µg/mL was used as standard. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol N-Acetylglucosamine (GlcNAc) per ml per min under given assay condition.

2.18.2 N-Acetylglucosaminidase assay

The N-acetylglucosaminidase activity was determined as described by Nahar et al. (2008). Reaction mixture containing 0.9 mL of p-nitrophenyl-N-acetyl-β-D-glucosaminide (1 mg/mL in of 50 mM acetate buffer, pH 5.0) and 0.1 mL of enzyme was incubated at 50°C for 30 min. The reaction was terminated by adding 2 mL of 0.2 M Na_2CO_3. The amount of p-nitrophenol released was measured at 410 nm. One unit of enzyme activity was defined as amount of enzyme that produced 1 µmol of p-nitrophenol per mL per min under given assay condition.

2.18.3 Protease assay

Protease activity was measured using Hammerstein casein as a substrate (Kulkarni et al., 2008). The reaction mixture contained 100 µL of suitably diluted enzyme solution, 1 mL casein (1%) and 0.9 mL carbonate-bicarbonate buffer, pH 9.7. The reaction mixture was incubated at 37ºC for 20 min and terminated by the addition of 3 mL TCA (2.6 mL of 5% trichloroacitic acid + 0.4 mL of 3.3N HCl). The precipitate was filtered (Whatman filter paper 1) and absorbance of supernatant (TCA soluble fraction) was measured at 280 nm. One unit of activity was defined as amount of enzyme that produced 1 µmol of tyrosine per mL per min under given assay condition.

2.19 Solid state fermentation for pectinase enzyme production

Powdered grape pomace (10 g) was soaked with 6 mL of YPG medium in 250 mL flasks. After sterilization, 24 h grown yeast isolates, viz. *P. membrenifaciens*, *S.*

cerevisiae, Z. steatolyticus, D. hansenii and *H. guillermondii* (1 mL, ~10^6 cells/mL), were inoculated. The fermentation was carried out at 28°C for 7 d in humidity chamber (RH 80%). Enzyme was extracted in NaCl as described in section 2.18 and supernatant was used for determination of pectinase activity (section 2.8.1).

2.20 Isolation of cell wall polymers from yeast lees

Chitosan was extracted from yeast lees as described by Cardoso et al. (2012). Yeast lees was deproteinized by adding 30 mL of 1 M sodium hydroxide solution and autoclaving at 121°C for 15 min. Alkali insoluble fraction was separated by centrifugation (4,000 rpm for 15 min). Pellet was washed twice with saline (0.85% NaCl) followed by washing with cold distilled water, 4-5 times to attain pH 7.0. To this residue 30 mL 2% acetic acid was added and the solution was heated at 100°C for 15 min, insolubles removed by filtering, to get chitin fraction. This filtrate was precipitated by adding 1 M NaOH (till pH 9.0) and stored in a refrigerator for 24 h. The contents were centrifuged (4000 rpm for 15 min) and the pellet was considered as chitosan fraction. The chitosan was washed several times with saline and cold distilled water till pH 7.0. Samples were placed in petri dishes for drying (24–48 h at 30ºC) to get dry chitin and chitosan fraction.

2.20.1 FT-IR analysis

The Fourier transform infrared (FT-IR) spectra were taken on a Bruker Optics ALPHA-E spectrometer with a universal Zn-Se ATR (attenuated total reflection) accessory in the 600-4000 cm^{-1} region. The degree of deacetylation (DDA) for fungal chitosan sample was determined using absorbance ratio A1655/A3450. The following formula was used for the % degree of acetylation (% DA) calculation (Van de Velde and Kiekens, 2004).

$$\% \text{ DA} = 31.918 (A1320/A1420) - 12.20$$

The degree of deacetylation (% DDA) was calculated by using formula:

$$\%\text{DDA} = 100 - \%\text{DA}$$

Chapter 3

Results and Discussion

A. Isolation and identification of *Saccharomyces* and non-*Saccharomyces* yeasts from different varieties of grapes grown in Maharashtra, India

Microbial ecology of grape berries is complex and includes various filamentous fungi, bacteria and yeasts with different physiological properties. Some species are only found on grape, such as parasitic fungi, while others, more specifically few types of yeast have the ability to survive and grow in grape must and during fermentation, constituting the wine microbial consortium. The natural *Saccharomyces* and non-*Saccharomyces* yeast flora present on the grape berries is one of the important factors responsible for wine quality (Clemente-Jimenez et al., 2005; Fleet, 2003; Sabate et al., 2002). Various factors such as rainfall, temperature, soil type, berry maturity, damage due to birds, insects and fungi, mechanical damage, application of fungicides, insecticides and geographic location affect the yeast flora present on grapes (Chavan et al., 2009; Combina et al., 2005; Guerra et al., 1999; Mortimer and Polsinelli, 1999). Besides our report, so far there are no reports on the yeast flora associated with vineyards and winerys from India (Chavan et al., 2009). Therefore, the present investigation was undertaken to further study the yeast diversity of different grape varieties commonly used for wine making in India.

3.1 Grape varieties used for isolation of Yeasts

Six grape varieties, viz. Bangalore Blue, Cabernet, Shiraz, Zinfandel, Chenin Blanc and Sauvignon Blanc were collected from Pune, Sangli and Nashik districts of Maharashtra, India. The sugar concentration of the grape juice from different varieties was between 161 g/L (Zinfandel) and 270 g/L (Sauvignon Blanc), whereas acidity was between 3.5 and 4.5. The sugar content (ºBrix) and pH for the collected samples are given the Table 3.1.

Grape variety plays an important role in determining the wine quality. Varietal flavor and aroma of wine is determined by volatile compounds such as monoterpenes, norisoprenoids, benzene derivatives which are naturally present in grapes (Fia et al.,

2005). Grape juice and must overwhelmingly constitute the key raw materials in winemaking and are, therefore, the principal contributors to the varietal flavours and characteristic aroma of the end product (Pretorius and HoJ, 2005).

Table 3.1. Sugar content (ºBrix) and pH for different grape samples used for yeast isolation

Region	Type of grapes	Grape variety	Sugar (Brix °)	pH
Sangli	Red	Shiraz	19.7	3.9
		Cabernet	20.2	3.5
	White	Chenin Blanc	23.4	4.5
		Sauvignon Blanc	27.0	4.5
Nashik	Red	Shiraz	26.1	3.4
		Cabernet	25.0	3.7
	White	Sauvignon Blanc	23.8	2.9
Pune	Red	Bangalore blue	20.4	3.6
		Zinfandel	16.1	4.1
		Shiraz	26.0	3.6
		Cabernet	25.0	3.7

3.2 Isolation of the yeasts

Totally 152 different yeast were isolated from six different varieties of grapes. The number of isolates and their nomenclature from each variety and region is listed in Table 3.2. The number of isolates from each variety was: 24, Bangalore blue; 19, Zinfandel; 25, Cabernet; 48, Shiraz; 24, Sauvignon Blanc and 12, Chenin Blanc.

Table 3.2 Nomenclature of yeast isolates

Type	Grape variety	Region		
		Sangli	Nashik	Pune
White	Sauvignon Blanc	I_105 –I_117	I_134 –I_144	-
	Chenin Blanc	I_62 –I_73	-	-
Red	Bangalore Blue	-	-	I_1 –I_24
	Zinfandel	-	-	I_25 –I_43
	Shiraz	I_74 –I_104	I_118 –I_129	I_145 –I_149
	Cabernet	I_44 –I_61	I_130 –I_133	I_150–I_152
Total isolates		74	27	51

3.3 Identification of the yeast isolates

Preliminary identification of the yeast isolates was done on the basis of morphology, colony characteristics and biochemical tests. Further identification and confirmation was done by sequence analysis.

3.3.1 Morphology and colony characteristics

Six types of colony morphologies were observed for the 152 isolates. Creamy and glossy colony with apiculate cell morphology is typical of *Hanseniaspora* species which was observed for 74 isolates (Fig. 3.1 A). Twenty isolates exhibited white dry colonies (Fig. 3.1 B). Sixteen isolates showed pale brown and dry colony morphology also produced pseudomycelia typical of *Pichia* sp (Fig. 3.1 C). Thirty two isolates showed creamy, smooth and buytrous colonies (Fig. 3.1 D). Eight isolates had yellowish mucoid colonies (Fig. 3.1 E). Two isolates exhibited white, smooth butyrous colonies (Fig 3.1 F).

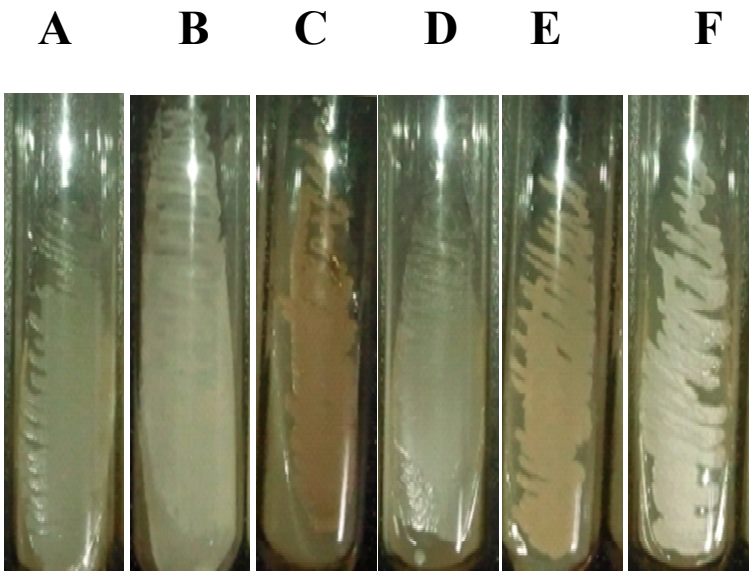

Fig. 3.1 Colony characteristics of the yeast isolates. **A:** creamy, smooth and glossy; *Hanseniaspora sp.,* **B:** white and dry; **C**: pale brown and dry; *Pichia* sp., **D**: creamy, smooth and butyrous; **E**: yellowish and mucoid; **F**: white, smooth and butyrous.

Light microscopy studies revealed that cells having creamy, smooth, glossy appearance showed apiculate cell morphology (Fig. 3.2 A, B, C, D). For the colonies having white dry appearance globose to ovoidal morphology was observed (Fig. 3.2 E, F). Pale brown and dry colonies of *Pichia* sp. Showed formation of pseudomycelium (Fig. 3.2 G, H, I). For the colonies appearing creamy smooth and buytrous, cells were round and showed budding indicating that they could belong to

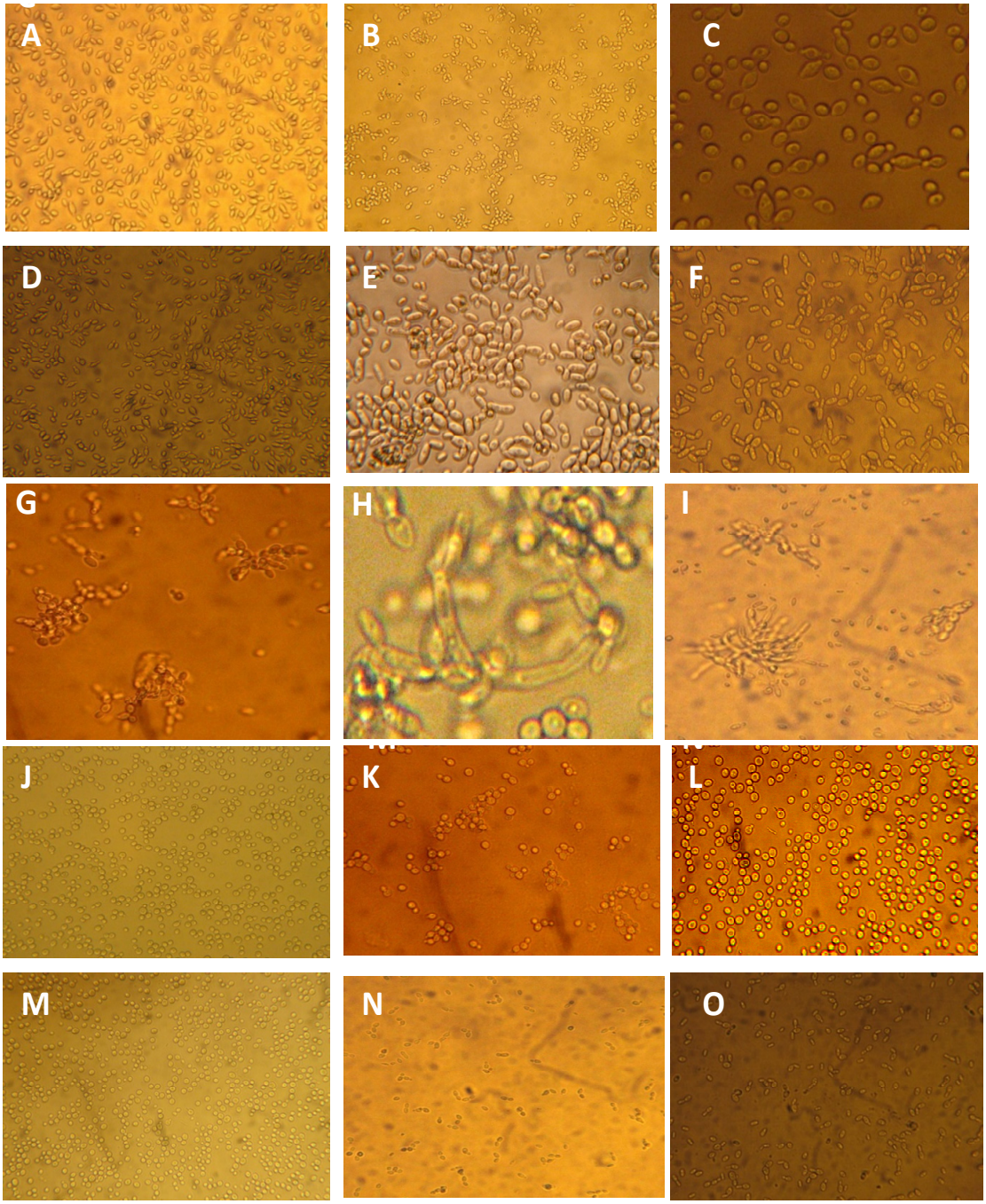

Fig.3.2 Light microscopic images of the yeast isolates

Saccharomyces genera (Fig. 3.2 J, K, L, M). Cells from yellowish mucoid colonies were small and elongated (Fig. 3.2 N, O).

3.3.2 Biochemical tests

In biochemical tests, ability of the yeasts to ferment and assimilate fifteen different sugars along with nitrate and nitrite was studied and the results were used for identification of the yeasts (Lodder, 1970; Kurtzman and Fell, 1998) (Table 3.3).

Table 3.3 Assimilation and fermentation of different carbon compounds by the 152 yeast isolates

Colony type[a]; Grape variety[b]	Isolate No.	Fermentation[c]										Assimilation[d]							
		D-Glu		D-Gal		Suc		Mal		Raffi		D-Glu	D-Gal	Rha	Mal	Suc	Cello	Lact	Sali
		A	G	A	G	A	G	A	G	A	G								
A, I	74	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	75	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	80	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	82	+	+	w	-	-	-	-	-	+	-	+	-	-	+	-	+	-	+
A, I	83	+	+	-	-	-	-	-	-	+	-	+	-	-	+	-	+	-	+
A, I	92	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	93	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	97	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	98	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	99	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	100	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	101	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	102	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	119	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	120	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	122	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	126	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	127	+	+	w	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	145	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	147	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, I	148	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+

A, I	149	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	44	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	45	+	+	-	-	-	-	-	-	w	-	+	-	-	-	-	+	-	+
A, II	47	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	48	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	50	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	51	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	55	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	56	+	+	-	-	-	-	-	-	+	-	+	-	-	+	-	+	-	+
A, II	58	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	130	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	131	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	132	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	133	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	150	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	151	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, II	152	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III,	26	+	+	-	-	-	-	-	-	-	-	+	-	-	w	-	+	-	+
A, III	28	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	30	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	35	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	38	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	39	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, III	41	+	+	-	-	-	-	-	-	-	-	+	-	-	w	-	+	-	+

A, IV	2	+	+	+	-	w	-	-	-	-	-	+	-	-	w	-	+	-	+
A, IV	5	+	+	-	-	+	+	-	-	-	-	+	-	-	-	-	+	-	-
A, IV	8	+	-	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+
A, IV	9	+	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+	-	+
A, IV	10	+	+	-	-	+	-	-	-	-	-	+	-	-	-	-	+	-	+
A, IV	18	+	+	+	+	-	-	-	-	w	-	+	-	-	-	-	+	-	+
A, IV	21	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	+	-	+
A, IV	22	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, IV	23	+	+	+	-	-	-	-	-	-	-	+	-	w	-	-	+	-	+
A, V	105	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	106	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	108	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	109	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	111	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	113	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	114	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	115	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	117	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	135	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, V	142	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, VI	62	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, VI	63	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, VI	64	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, VI	65	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, VI	66	+	+	+	-	-	-	+	-	+	-	+	w	-	-	-	+	-	+
A, VI	67	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+

A, VI	69	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, VI	71	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
A, VI	72	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
B, I	52	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	121	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	123	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, I	124	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, II	46	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, II	49	+	+	+	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-
B, III	27	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, III	40	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, IV	3	+	-	-	-	w	+	-	-	-	-	+	-	-	-	-	-	-	-
B, IV	4	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, IV	11	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, IV	15	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, IV	16	+	+	-	-	w	+	-	-	-	-	+	-	-	-	-	-	-	-
B,IV	17	+	+	-	-	w	+	-	-	-	-	+	-	-	-	-	-	-	-
B, IV	19	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, V	134	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, V	136	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, V	144	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, IV	20	w	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
B, VI	68	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	76	+	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-
C,I	77	w	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	78	+	+	-	-	-	-	-	-	-	-	+	-	-	-	+	+	-	-
C,I	79	w	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	81	w	-	w	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	86	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	87	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	88	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	91	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,I	125	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C, IV	12	+	-	-	-	w	+	-	-	-	-	+	-	-	-	-	+	-	-

C,IV	13	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,IV	14	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-
C,V	107	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,V	137	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-
C,V	141	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
D,I	84	+	+	-	-	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,I	85	+	+	w	-	+	+	+	+	+	-	+	-	-	+	+	-	-	-
D,I	89	+	+	-	-	+	+	+	+	+	+	+	+	-	+	+	-	-	-
D,I	94	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,I	95	+	+	+	+	+	+	+	+	+	-	+	+	-	+	-	-	-	-
D,I	96	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,I	103	+	+	w	+	+	+	w	+	+	+	+	+	-	+	+	-	-	-
D,I	104	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,I	128	+	+	+	+	+	w	+	+	+	-	+	+	-	+	+	+	-	+
D,I	129	+	+	+	+	+	w	+	-	+	-	+	+	-	+	+	+	-	+
D,II	57	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	-	-
D,II	59	+	+	w	+	+	+	w	+	+	+	+	+	-	+	+	-	-	-
D,II	60	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	-	-
D,II	61	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	-	-
D, III	25	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	-	-	-
D,III	29	+	+	w	+	+	+	w	+	+	+	+	+	-	+	+	-	-	-
D,III	31	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D,III	32	+	+	w	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D,III	33	+	+	+	-	+	-	+	+	+	-	+	+	-	+	+	-	-	-
D,III	34	+	+	w	+	+	+	w	+	+	+	+	+	-	+	+	-	-	-
D,III	36	+	+	w	-	+	+	+	+	-	-	+	+	-	+	+	-	-	-
D,III	37	+	-	w	+	w	+	w	+	-	-	+	+	-	-	+	+	-	-
D,III	42	+	+	+	-	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,III	43	+	+	+	+	+	-	+	+	-	-	+	+	-	+	+	-	-	-
D,VI	70	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,VI	73	+	+	w	+	+	+	w	+	+	+	+	+	-	+	+	-	-	-
D,V	110	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,V	112	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	-	-	-
D,V	116	+	+	w	+	+	+	w	+	+	+	+	+	-	+	+	-	-	-

D,V	138	+	+	+	-	+	+	+	w	-	+	+	+	-	+	+	+	-	+
D,V	139	+	+	+	+	+	w	+	+	+	-	+	+	-	+	+	+	-	+
D,V	143	+	+	+	-	+	+	+	-	+	-	+	+	-	+	+	+	-	+
E, I	146	+	+	-	-	-	-	-	-	-	-	+	-	-	-	-	+	-	+
E, II	53	+	+	-	-	-	-	w	+	-	-	+	+	-	+	+	+	-	-
E, II	54	+	-	-	-	-	-	-	-	+	-	+	+	-	+	+	-	-	-
E, IV	6	+	+	w	+	-	-	+	+	-	-	+	+	-	+	+	-	-	-
E, IV	7	+	-	-	-	+	-	-	-	-	-	+	+	-	+	+	-	+	-
E, IV	1	+	+	-	-	w	+	+	+	-	-	+	+	-	+	+	+	-	-
E, IV	24	+	-	-	-	+	+	-	-	-	-	+	+	-	+	+	-	-	-
E,V	140	+	+	+	w	+	+	+	-	+	-	+	+	-	+	+	+	-	-
F,I	90	+	-	w	-	w	+	-	-	-	-	+	+	+	+	+	+	-	+
F,I	118	+	+	+	-	+	+	+	-	+	-	+	-	-	-	+	-	-	-

Colony type[a]: **A:** Creamy, smooth and glossy, **B**: White and dry, **C**: Pale brown and dry, **D**: Creamy, smooth and butyrous, **E**: Yellowish and mucoid, **F**: White, smooth and buytrous.

Grape Variety[b] **:**I, Shiraz ; II, Cabernet; III, Zinfandel; IV, Banglore Blue; V, Sauvignon Blanc.VI Chenin Blanc

Fermentation[c]: 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[d]: L-Rha, Rhamnose; Cello, Cellobiose; Lact, Lactose; Sali, Salicin

3.3.2.1 Fermentation and assimilation of sugars

All the 152 isolates assimilated and fermented glucose while none of the isolates assimilated lactose, nitrate and nitrite. Total 74 isolates assimilated cellobiose and salicin while galactose, rhamnose and sucrose were not assimilated by them. Twenty isolates could assimilate and ferment only glucose while 16 isolates showed weak glucose fermenting ability and could not assimilate or ferment any other sugar. Ten isolates assimilated galactose, sucrose and maltose and did not assimilate rhamnose, cellobiose or salicin. Fermentation of five sugars i.e. glucose, galactose, sucrose, maltose and raffinose, was observed in 32 isolates. The results of biochemical tests are given in Table 3.3 with details. Seven genera were identified on the basis of their cultural and biochemical characteristics.

3.3.2.2 Cluster analysis

Cluster analysis of 152 yeast isolates was carried out on the basis of results obtained for 15 biochemical tests and their ability to form pseudomycelium with comparable standard strains reported in the literature. The dendrogram generated by cluster analysis showed two major branches (I and II) based on sucrose and maltose fermentation. In the first branch a group of 113 isolates were clustered (1-5) on the basis of lack of fermentation and assimilation of sucrose and maltose. Cluster 1 and 2 had 38 isolates based on non-assimilation of cellobiose and salicin and they were separated on the basis of pseudomycelium formation. In first cluster, 9 isolates were grouped with *I. orientalis*, 11 isolates were grouped with *I. terricola* and single isolate with *C. diversa* and *T. delbrukii* based on the absence of pseudomycelium formation. In second cluster out of 16 isolates showing pseudomycelium formation, 8 isolates were grouped with *P. membranifaciens*, 6 isolates with *P. manshurica* and one isolate each with *P. kluyveri* and *P. fermentans*. Remaining 75 isolates from 1st branch which could assimilate cellobiose and salicin were spread in clusters based on maltose assimilation and galactose fermentation (3-5). Among them, 74 isolates were grouped with different *Hanseniaspora* sp. and one isolate with *D. hansenii* (Fig.3.3). Thirty nine isolates from second branch, capable of fermenting and assimilating sucrose and maltose, were separated as cluster 6 and 7 based on raffinose assimilation. From these, 30 isolates were grouped with *S. cerevisiae* in cluster 6 and nine isolates were grouped with *C. quercitrusa, C. azyma* and *Z. steatolyticus* in cluster seven.

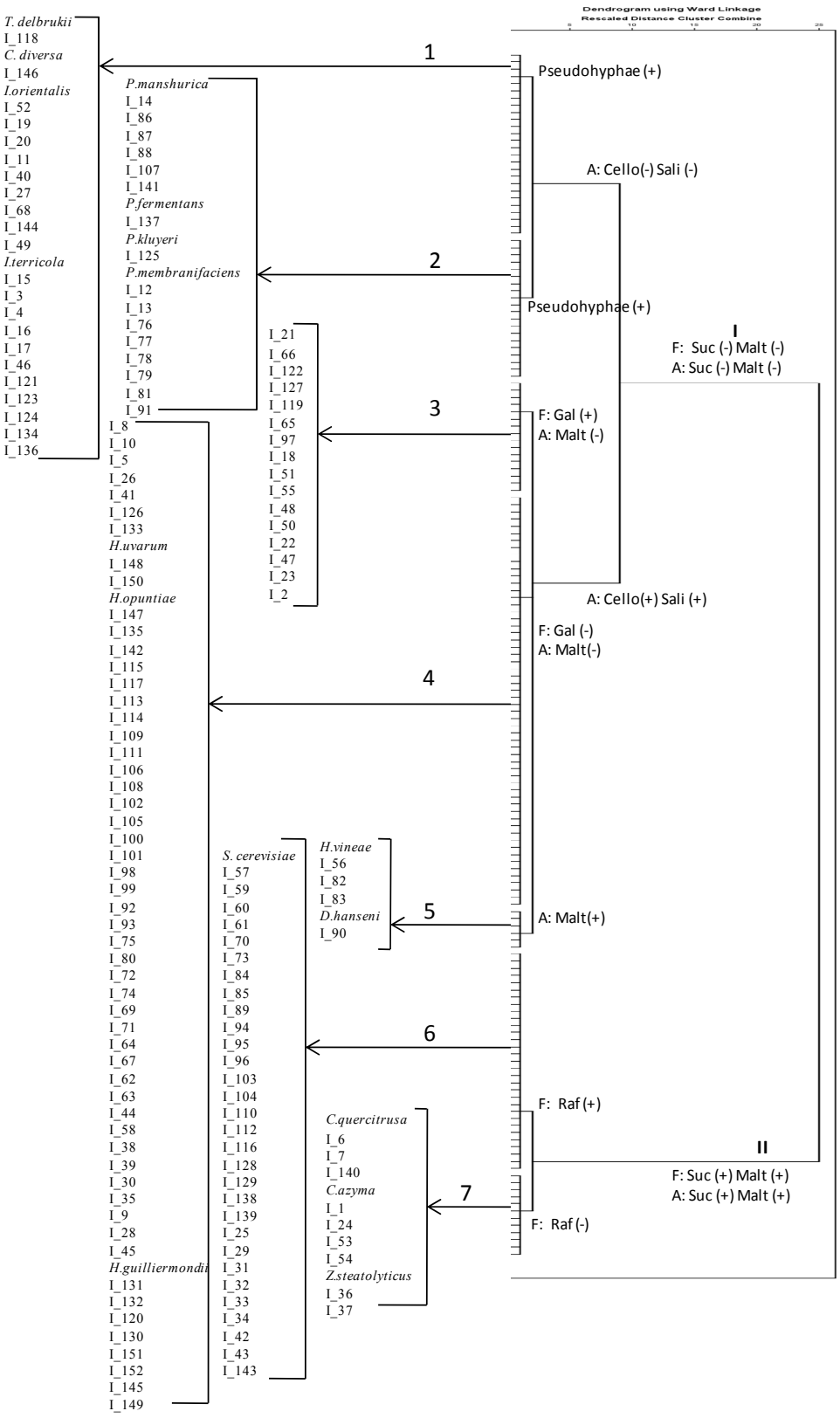

Fig.3.3 Cluster analysis of the 152 yeast isolates with standard strains

Standard strains used for comparison included - *Candida azyma* CBS 6826, *Candida quercitrusa* CBS 8602, *Candida diversa* CBS 4074, *Debaroyomyces hansenii* CBS 767, *Hanseniaspora guilliermondii* CBS 479, *H. uvarum* CBS 314, *H. viniae* CBS 313, *H. opuntiae* CBS 8733, *Issatchenkia orientalis* CBS 5147, *I. terricola* CBS 8131, *Pichia membranifaciens* CBS 107, *P. fermentans* CBS 187, *P. kluyveri* CBS 7145, *P. manschurica C*BS 7324, *Saccharomyces cerevisiae* CBS 1171, *Torulaspora delbrukii* CBS 1146, *Zygoascus steatolyticus* CBS 4028.

All *S. cerevisiae* were identified clearly on the basis of their morphological and biochemical characters. As both morphology and biochemical tests were not useful to differentiate *Hanseniaspora, Issatchenkia* and *Pichia* at the species level, molecular identification was carried out to further resolve these genera.

3.3.3 Molecular identification

3.3.3.1 Amplification of ITS1-5.8S-ITS2 rDNA region

The ITS1-5.8S-ITS2 region of the 152 isolates was obtained by amplification using ITS1 and ITS4 fungal primers (White et al., 1990). The PCR product was purified using the (Qiagen purification kit) Gel electrophoresis of purified PCR products showed differences in size of ITS1-5.8S-ITS2 region of different genera (Fig 3.4). All 152 yeast

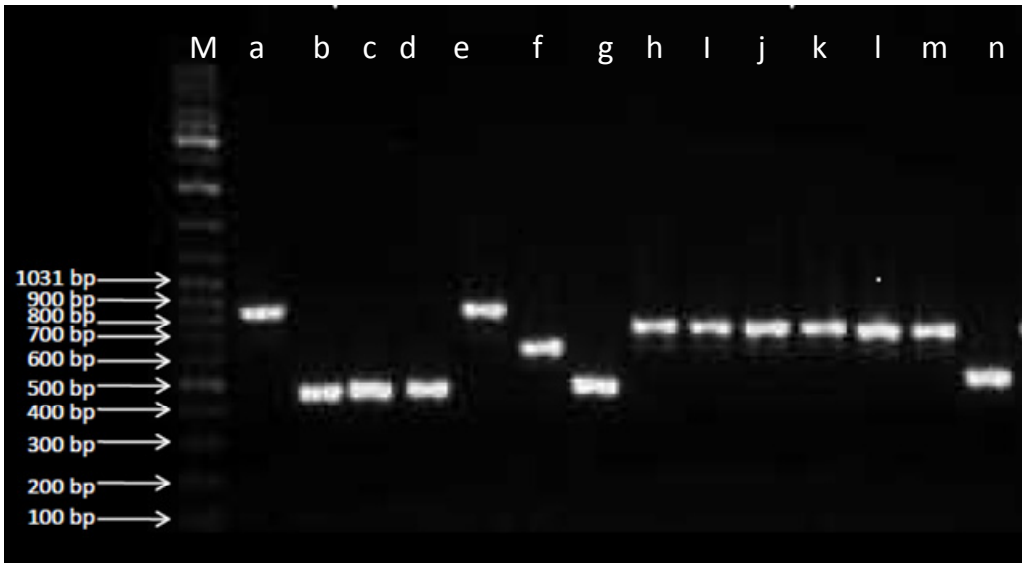

Fig. 3.4 Electrophoretic analysis of PCR products of ITS1-5.8S-ITS2 region of rDNA from **a;** I_25, **b;** I_12**, c;** I_13, **d;** I_14, **e;** I_118, **f;** I_16, **g;** I_1, **h;** I_5, **I;** I_18, **j;** I_56, **k;** I_147, **l;** I_8, **m;** I_36, **n;** I_20; **M**; marker.

isolates had amplicon size between 361-750bp.

3.3.3.2 Sequencing and data analysis

The amplicons obtained were sequenced using same primers with ABI 3730 analyser. ITS1-5.8S-ITS4 sequences from the 152 strains were used to generate a BLAST analysis. On the basis of the significant sequence alignments from the BLAST search the strain identification was carried out. The sequence identity with the closest sequence was used to identify the isolates and identified as 17 different yeast species belonging to eight genera (Table 3.4). Most isolates (117) had high identity (≥ 97%) with the type strains. Among the remaining 34 isolates, 18 had identities ranging between 90-96% while 17 had lower identities (79-89%). On the basis of sequence identity *Candida* genera was resolved into three species *C. azyma* (4 isolates), *C. quercitrusa* (3 isolates) and *C. diversa* (1 isolate). Only one isolate each were of *Debaromyces hansenii* and *Torulaspora delbrueckii,* whereas two isolates were identified as *Zygoascus steatolyticus*. *Hanseniaspora* isolates were resolved into 4 species, *H. guilliermondii* (56 isolates), *H. uvarum* (10 isolates), *H. opuntiae* (5 isolates) and *H. vineae* (3 isolates). *Issatchenkia* isolates were resolved into 2 species, *I. orientalis* (9 isolates) and *I. terricola* (11 isolates). *Pichia* isolates were separated into 4 species, *P. membranifaciens* (8 isolates), *P. manshurica* (6 isolates), *P. fermentans* (1 isolate) and *P. kluyveri* (1 isolate). Thirty isolates were identified as *S. cerevisiae.* 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.

3.3.3.3 Amplification of D1/D2 region of 26S rDNA region

The use of D1/D2 26S rDNA amplification has the potential to markedly increase the accuracy of yeast identification (Kurtzman and Robnett, 1998). The amplification was carried out using NL1 and NL2 primers (O'Donnell, 1993). No difference in the size of the amplicons was observed for the PCR products by gel electrophoresis.

3.3.3.4 Sequencing and data analysis

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 isolates. The identification was carried out on the basis of sequence identity with the reported closest sequences in BLAST search.

Table 3.4 Percent identity of ITS sequences of the 152 isolates with type strain

Yeast species	Type strain	Isolate No	% identity	Variety*
C. azyma	EF533997	1, 24, 53, 54	99, 99, 99, 100	IV, IV, II, II
C. quercitrusa	AM160627	6, 7, 140	97, 99, 98	IV, IV, V
C. diversa	KC509573	146	95	I
D. hansenii	EF061757	90	100	I
H. guilliermondii	EF449522	2, 5, 8, 9, 10, 21, 22, 23	99, 99, 94, 99, 99, 99, 99, 99	IV
		26, 28, 30, 35, 38, 39, 41	99, 99, 99, 99, 99, 99, 99	III
		44, 47, 48, 50, 51, 55, 58	99, 99, 99, 99, 98, 99, 97	II
		62, 63, 64, 65, 66, 67, 69, 71, 72	98, 98, 99, 99, 83, 98, 99, 99, 99	VI
		74, 75, 80, 92, 93, 97, 98, 99, 100, 101, 102, 119, 122, 127	99 (All), 84, 99, 79	I
		105, 106, 108, 109, 111, 113, 114, 115, 117, 135, 142	99 (All), 98, 99, 86	V
H. uvarum	AM160628	18, 45, 130, 131, 132, 151, 152	80, 98, 94, 95, 98, 100, 100	IV, II (All)
		120,145,149	98, 100, 100	I (All), V
H. vineae	AY046201	56, 82, 83	96, 92, 89	II, I, I
H. opuntiae	KC870065	126, 133, 147, 148, 150	90, 97, 99, 99, 99	I (All), II
I. orientalis	EF198013	11, 19, 20, 27, 40, 49, 52, 68, 144	98, 92, 76, 97, 99, 80, 90, 100	IV (All), II, II, VI
I. terricola	EF648009	3, 4, 15, 16, 17, 46	97, 99, 99, 99, 99, 97	IV (All), II
		121, 123, 124	90, 94, 99	I
		134, 136	85, 99	V
P. membranifaciens	DQ198964	12, 13, 76, 77, 78, 79, 81, 91	89, 89, 79, 99, 98, 90, 100, 99	IV, IV, I (All)
P. manshurica	FM199959	14, 86, 87, 88	88, 96, 93, 96	IV, I (All)
		107, 141	88, 94	V
P. fermentans	FN376418	137	90	V
P. kluyveri	JX188203	125	88	I
S. cerevisiae	AM262831	25, 29, 31, 32, 33, 34, 42, 43	100, 99, 100(All)	III
		57, 59, 60, 61, 70, 73	100, 99, 100, 100, 98, 100	II (All),VI,VI
		84, 85, 89, 94, 95, 96, 103, 104, 128, 129	100, 100, 100, 99, 100, 87, 99, 96, 100, 100	I
		110,112, 116, 138, 139, 143	100, 100, 100, 94, 97, 99	V
T. delbrukii	KJ160641	118	88	I
Z. steatolyticus	AY447033	36, 37	99, 99	III

Grape variety* I, Shiraz ; II, Cabernet; III, Zinfandel; IV, Banglore Blue; V, Sauvignon Blanc; VI, Chenin Blanc.

All isolates whose ITS1-5.8S-ITS2 region were previously showing less than 97% percent similarity with type strain were showing 99-100% similarity for sequences of 26S rDNA region with the same type strains confirming the results. Thus, 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-ITS2 region sequences for 152 isolates were submitted to National Centre for Biotechnology Information (NCBI) GenBank data library. Yeast strains with accession number are listed in Annexure I.

3.3.3.5 Phylogenetic analysis

The Phylogenetic tree for the sequences was generated by maximum parsimony method using MEGA6 software. Bootstrap was performed for 100 replicates and there were 356 positions in final dataset. The Phylogenetic tree was in accordance with the observations of Kurtzman et al. (2011). The 152 isolates were resolved into 17 species belonging to eight genera. The topology of the phylogenetic tree showed 2 branches that diverged from the main node (Fig. 3.5). In the first branch, *Z. steatolyticus* diverged early and branched close to *S. pombe.* Second branch displayed the presence of 5 sub-branches containing 150 isolates. In the first sub-branch, 30 isolates of *S.cerevisiae* were grouped together while 74 isolates from *Hanseniaspora* genera were grouped together in second subbranch, with *H. guillermondii*, *H. uvarum*, *H. vineae* and *H. opuntiae* sharing the same branch point along with *T. delbrukii.* In the third and fourth sub branches isolates belonging to *Candida* and *Debaromyces* genera were grouped together along with respective type strains. *Issatchenkia* and *Pichia* diverged from the same node as fifth sub-branch. Members from *Pichia* group, *P. fermentans*, *P. manshurica, P. kluyveri and P. membranifaciens* showed highest diversity amongst themselves

Among the 152 identified species, the largest diversity of yeast species was found in Shiraz grape variety (11 species) followed by Cabernet (7 species), Bangalore Blue (8 species), Sauvignon Blanc (6 species), Zinfandel (4 species) and Chenin Blanc (3 species).

C. azyma and *C. quercitrusa* were reported for the first time from grape berries of Bangalore Blue and Cabernet varieties. *C. quercitrusa* was previously reported to be associated with insects, while *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 (Suzuki et

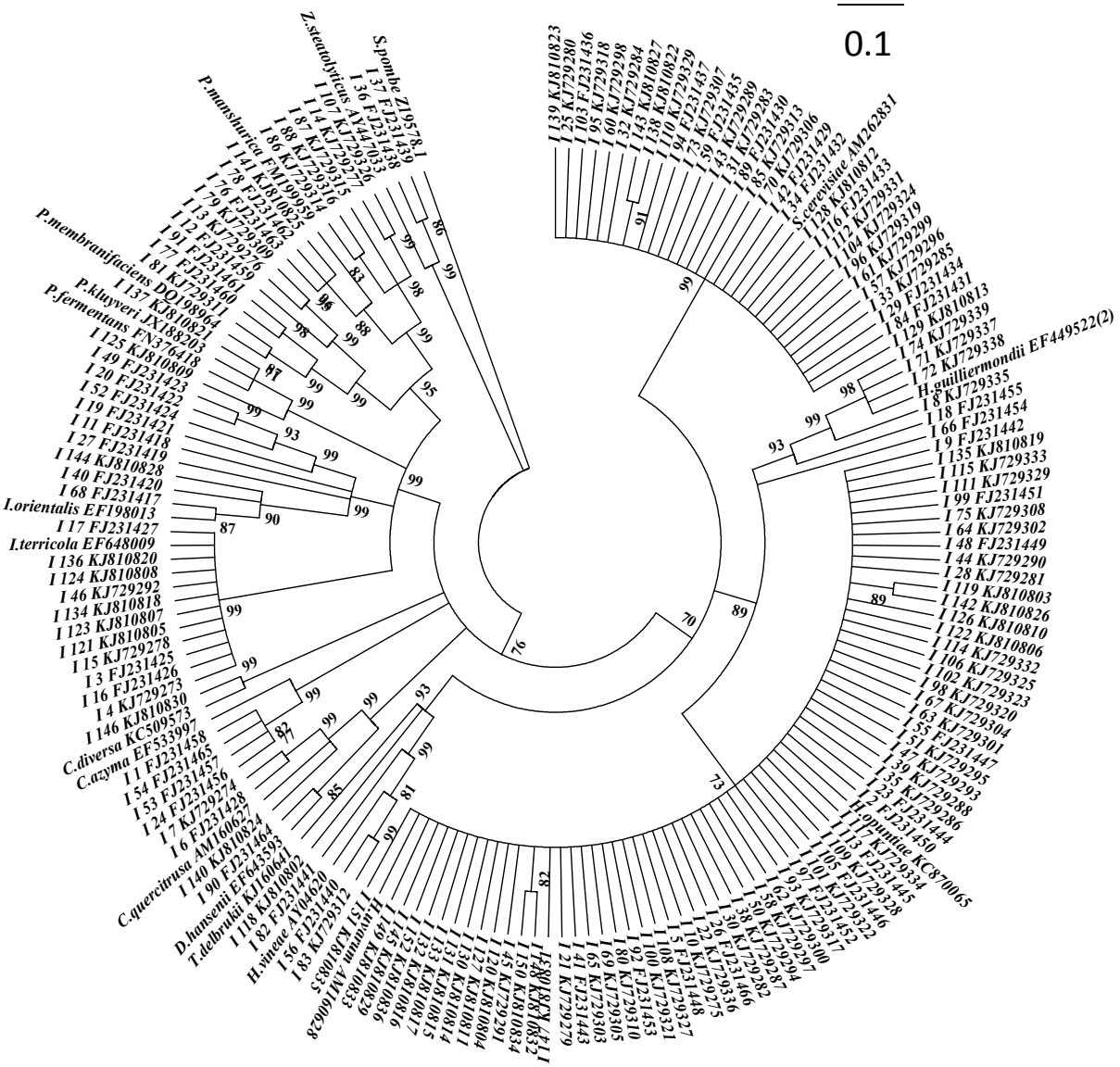

Fig. 3.5 Phylogenetic tree representing 152 grape isolates and their closest related species

al., 1999; Lachance et al., 2001). It would be interesting to study the association of yeast flora with the local soil flora and change in cropping pattern as both Pune and Sangli districts supports sugarcane farming along with viticulture. Different *Candida* sp. were associated with distinctive wine properties such as *Candida albidus* with Slovene wine, *Candida vanderwaltii* and *Candida amapae* with wines from Tacoronte-Acentejo and Valle de Gu¨ imar regions of Spain (Combina et al., 2005; Gonzalez et al., 2006; Raspor et al., 2006). The potential to produce region specific wines from Bangalore Blue and Cabernet varieties could be explored.

Species namely, *H. guilliermondii, H. viniae, H. uvarum, I. orientalis, I. terricola, S. cerevisiae, P. membranifaciens* and *Z. steatolyticus* have been reported in

various vine microflora studied from different parts of the world. *Hanseniaspora* sp. was predominantly present among the strains isolated from all the six vine varieties grown in regions of our study. Increased incidence of the apiculate yeast from the mature grape berries have also been reported Fleet (2003). *S. cerevisiae* isolates were detected from all of the vine varieties except Bangalore Blue. Most studies have indicated low occurrence of *S. cerevisiae* in the grape juice and must (Mortimer and Polsinelli, 1999), while, 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.

3.4 Distribution of *Saccharomyces* and non-*Saccharomyces* yeasts based on region, variety and agricultural practices

Depending on the geographical locations or regions, the number and population of the grapes microflora varies significantly (Barata et al., 2012). Yeast diversity was found to be higher in vineyards from Italy, Spain and China followed by France, India, Argentina and Portugal, whereas yeast diversity was low for vineyards from Australia, Brazil, Canada, Greece and Japan.

Ten species of *Candida, Debaromyces, Hanseniaspora, Issachenkia, Pichia* and *Saccharomyces* genera were isolated from grapes grown in Sangli region. Ten species from *Candida, Hanseniaspora, Issatchenkia, Pichia, Torulaspora* and *Saccharomyces* genera were found on the berries grown in Nashik region, whereas twelve species belonging to six genera namely, *Candida, Hanseniaspora, Issatchenkia, Pichia, Zygoascus* and *Saccharomyces* were obtained from the fruit grown in Pune region (Table 3.5). *P. fermentans, P. kluyveri,* and *T. delbrueckii* were specifically associated with Nashik region. *C. azyma* and *P. membranifaciens* were isolated from the both Sangli and Pune region. *C. quercitrusa, H. opuntiae* were found in Nashik and Pune region. Isolates of *H. guilliermondii, H. uvarum, I. orientalis, I. terricola, P. manshurica* and *S. cerevisiae* were observed in all the three regions.

Variety specific associations of yeast flora were also observed (Table 3.5). *H. guilliermondii* strains were found on almost all varieties whereas other species such as *H. opuntiae* and *H. vinae* were isolated from only two grape varieties, Shiraz and Cabernet. *S. cerevisiae* strains were detected on all the grape varieties studied except Bangalore Blue. Species found to be associated with specific variety were- *C. diversa*,

D. hansenii, T. delbrueckii, P. kluyveri with Shiraz variety, *P. fermentans* with Sauvignon Blanc and *Z. steatolyticus* with the Zinfandel grape variety.

Table 3.5 Region and variety based variation in the yeast diversity

Yeast genera	Sangli				Nashik				Pune		
	SH	CAB	SB	CB	SH	CAB	SB	BB	ZF	SH	CAB
C.quercitrusa							1	2			
C. azyma		2						2			
C.diversa										1	
D. hansenii	1										
H. guilliermondii	11	7	9	9	3		2	8	7		
H. vineae	2	1									
H.opuntiae					1	1				2	1
H.uvarum		1				3		1		2	3
I. orientalis		2		1			1	3	2		
I. terricola		1			3		2	5			
P. mansturica	3		1					1		1	
P. membranifaciens	6							2			
P.fermentens							1				
P.kluyveri					1						
S. cerevisiae	8	4	3	2	2		3		8		
T.delbrukii					1						
Z.steatolyticus									2		
No.of genera	4	4	3	3	5	1	5	4	4	3	1
No.of species	6	7	3	3	6	2	6	8	4	4	2
	10species /6 genera				10species/6 genera				12species /7genera		

SH- Shiraz; CAB- Cabernet; BB- Bangalore Blue; ZF- Zinfandel; SB- Sauvignon Blanc; CB- Chenin Blanc

First report | Region & variety specific | Region specific | variety specific | Predominant genera

Thus, the 152 yeasts isolates obtained from the grape berries of six vine varieties from three regions of Maharashtra, India were identified by biochemical analysis and molecular techniques into 17 different species belonging to eight genera. Yeast diversity was extensively studied for the Indian vineyards. The indigenous yeast flora associated with the grapes differed among vine varieties as well as regions.

3.4.1 Effect of pesticides used in vineyards on natural diversity of yeasts

It is well known that the colonisation of yeasts on grape surfaces is influenced by several environmental and nutritional factors, the varieties and degree of maturation of the grapes and the agrochemical treatments. Several studies have highlighted that fungicides significantly influence the yeast species composition and their fermentation ability, prolonging the lag phase (Regueiro et al., 1993; Mlikota et al., 1996). However, only a few studies have evaluated the influence of fungicide

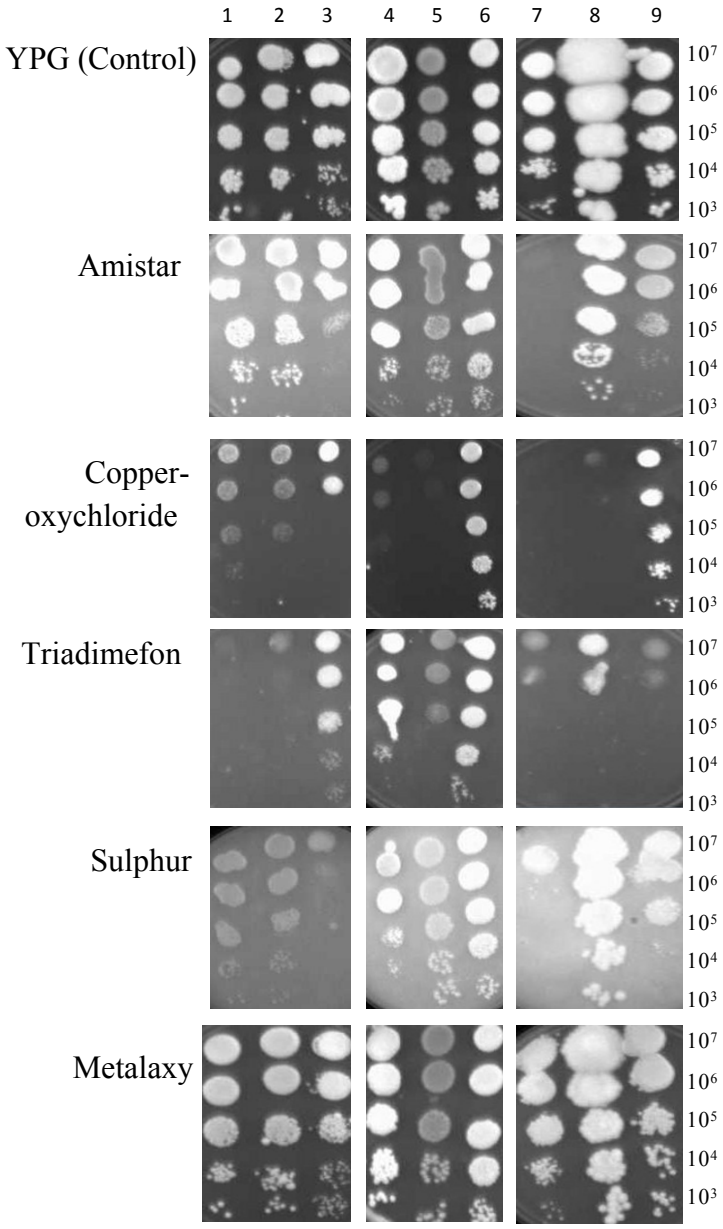

Fig. 3.6 Effect of pesticides on yeast isolates from different genera; 1-*Zygoascus steatolyticus* (I_36), 2-*Saccharomyces cerevisiae* (I_25), 3-*Debaromyces hansenii* (I_90), 4- *Pichia membranifaciens* (I_13), 5-*Hanseniaspora guillermondii* (I_5), 6-*Issachenkia orientalis* (I_11), 7- *Candida azyma* (I_1), 8-*Torulaspora delbrukii* (I_118),9- *Issachenkia terricola* (I_15)

treatments on colonisation of yeast on wine grapes (Guerra et al., 1999).

Single representative isolate from each genera was used for this assay to check the sensitivity or resistance to the pesticides commonly used in the vineyards. Growth was not observed for any of the cultures, using differnt cell concentrations for difecanazole, mancozeb, hexaconazole, tubecanazole, pecanazole indicating their toxicity. *Candida azyma* was sensitive to all the pesticides tested except that it was able to grow (at 10^7 cfu/mL) in presence of sulphur. Metalaxyl exhibited no effect and Amistar exhibited weak inhibition at lower cell concentrations (10^7cfu/mL) against the tested yeast isolates. *Debaromyces* isolate was sensitive to copper oxychloride at all cell concentrations and it exhibited sensitivity for other pesticide at lower cell concentration (Fig. 3.6).

Viviani-Nauer et al. (1995) found that pesticides decreased yeast population and diversity in fermenting musts. No adverse effect on ability of fermentation of *S. cerevisiae* by 6 different fungicides, while stimulation in fermentation ability of *K. apiculata* was observed Cabras et al. (1999). Guerra et al. (1999) concluded that pesticide affects diversity and frequency of *S. cerevisiae* and other species by comparing two different groups of pesticides. Several fungicides did not affect yeast numbers nor must fermentation, even when applied in the day of harvest Oliva et al. (2007). Recent studies related to the differences in farming practices and its impact on the diversity of microflora on the vines tempt us to conclude that organic farming leads to higher biodiversity, both in *S. cerevisiae* and in *non- Saccharomyces* yeasts (Cordero-Bueso et al., 2011).

B. Role of non-*Saccharomyces* yeasts in wine fermentation

Although microbial diversity of grapes including yeasts, bacteria and filamentous fungi play an important role in the wine making and determination of chemical composition of wine, yeasts have major influence due to their role in alcoholic fermentation and production of different enzymes (Fleet, 2003; Fugelsang, 1997). During fermentation, especially spontaneous fermentations, there is a sequential succession of yeasts (Fleet and Heard, 1993).

The primary role of wine yeast is to catalyze the rapid, complete and efficient conversion of grape sugars to ethanol, carbon dioxide and other minor, but important metabolites. Most of these biological catalysts originate from the grape, the indigenous micro flora or the yeast and bacteria present during wine making. During the first step of the fermentation, low-fermentative yeasts catalyze some important reactions in must, which improves the final flavor of wines. Pectin, cellulose, glycans, hemicelluloses, proteins and lignin are major structural components of grapes. Enzymatic degradation of these compounds may improve different stages of vinification, for instance by enhancing the yield and clarification of the must, increasing color extraction, and improving filtration of the wine, thereby enhancing the wine quality (Charoenchai et al., 1997). Not all of the enzymes important in fermentation are obtained from the grape and those that are present may not be fully effective under in situ conditions. Therefore, it is important to promote growth of non-*Saccharomyces* yeasts in fermenting must as source of these enzymes.

To understand the role of different non-*Saccharomyces* yeasts in wine fermentation, production of different enzymes namely, pectinase, protease, β-1, 3 glucanase and β glucosidase were checked. Wine fermentation for Shiraz variety was done in the laboratory and various parameters including yeasts succession, enzyme levels in the must and chemical analysis of final product was carried out.

3.5 Production of different enzymes by the yeast isolates

To check the potential of individual yeasts for production of hydrolytic enzymes, all the 152 yeast isolates were grown in YPG media and the levels of pectinase, protease, β-1, 3 glucanase and β glucosidase were estimated. Isolates of *C. quercitrusa, P. fermentens* and few isolates from *Hanseniaspora* genera were found to be potential producers of all four enzymes. The remaining isolates exhibited either one or two enzyme activities from protease, β-1, 3 glucanase and β-glucosidase. Out of the four

enzymes evaluated, pectinase was exhibited by all the isolates. Strains of *Z. steatolyticus*, *P. membranifaciens*, *P. manshurica, H. guillermondii* and *C. quercitrusa* showed high pectinase activities (> 20000 U/mL, Table 3.6).

β-1,3-glucanase activity was observed in 98 isolates. Among them, *Hanseniaspora* and *Saccharomyces* species exhibited β-1, 3-glucanase activity in the range of 1110-14232 U/mL and 673-4043 U/mL, respectively (Table 3.6). Isolates of *I. terricola* also showed high glucanase activity (2656-27279 U/mL). Out of the 16 *Pichia* isolates only one isolate exhibited β-1, 3 glucanase activities.

Among the 17 isolated species, protease activitie (22-3512 U/mL) was observed only in 43 strains belonging to 6 species i.e. *C. azyma, C. quercitrusa, H. guillermondii*, *Z. steatolyticus, P. manschurica* and *P fermentans* (Table 3.6). Highest activity was observed in *C. azyma* (3512 U/mL).

β-glucosidase activity was observed in 88 isolates with higher activities being observed in strains belonging to *Issatchenkia* and *Saccharomyces* genera (Table 3.6). Except one strain from each *S. cerevisiae* and *Issatchenkia*, all isolates exhibited β-glucosidase. Among the 152 isolates, highest glucosidase (1010 U/mL), pectinase (34999 U/mL), glucanase (27279 U/mL) and protease (10810 U/mL) activity were observed for isolates *I. terricola* I_134, *P.manshurica* I_87, *I. terricola* I_123, and *H. guilliermondii* I_122 respectively.

Commercial wine yeast strain, *S. cerevisiae* EC1118 grown in YPG was also studied for production of all for enzymes (Table 3.6). All the enzyme activities could be detected within 48 h except protease.

Van Rensburg and Pretorius (2000) emphasized the pivotal role of enzymes endogenous from grapes and also from natural flora of the berries in the wine making. The enzymes like pectinases, glucanases, xylanases and proteases are involved in the clarification and processing of wine. Non-*Saccharomyces* species are important contributors to the final taste and flavor of wines due to their capacity to produce different enzyme activities such as protease, β-glucosidase, esterase, pectinase and lipase (Esteve-Zarzoso et al., 1998). The hundred and fifty plus isolates reported in the present study also exhibited a potential to produce all four enzymes in YPG medium (Table 3.6). Protease activity was observed in strains of *Candida pulcherima, K. apiculata* and *Pichia anomala* (Charoenchai et al., 1997; Fernandez et al., 2000). Proteases from *Candida olea, Candida lipolytica, Candida pulcherrima* and *K. apiculata* were found to reduce wine turbidity (Lagace and Bisson, 1990). Dizy and

Bisson (2000) demonstrated that species belonging to the genus *Kloeckera*/ *Hanseniaspora* were the highest producers of proteolytic activity in the must and affected the protein profile of the finished wines too.

Table 3.6 Enzyme activities (range) produced by the yeast isolates*

Species	Pectinase (U/ml)	β- 1,3-glucanase (U/ml)	Protease (U/ml)	β-glucosidase (U/ml)
C. azyma (4)	11026-17037 (4)	4162-5946 (4)	1102-3512 (3)	ND
C. diversa (1)	8216 (1)	8796 (1)	ND	216 (1)
C. quercitrusa (3)	6508-23101 (3)	713-911 (2)	701-1851 (3)	64-568 (3)
D. hansenii (1)	33398 (1)	3250 (1)	ND	338 (1)
H. guilliermondii (56)	2205-27458 (56)	1110-8697 (41)	117-1956 (32)	15-154 (14)
H. uvarum (10)	4054-15365 (10)	1347-14232 (8)	ND	17-53 (4)
H. vinae (3)	10492-12377 (3)	3092-9157 (2)	ND	152-229 (2)
H. opuntiae (5)	3414-10883 (5)	3162-13145 (5)	ND	26-220 (3)
I. orientalis (9)	1920-15528 (9)	396-8697 (7)	ND	13-548 (8)
I. terricola (11)	7860-18531 (11)	2656-27279 (10)	ND	298-1010 (11)
P. fermentans (1)	17179 (1)	1779 (1)	1866(1)	138 (1)
P. kluyveri (1)	10350 (1)	ND	ND	141 (1)
P. membranifaciens (8)	1643-26996 (8)	ND	ND	35-289 (3)
P. manshurica (6)	4801-34999(6)	ND	22-529 (2)	41-419 (5)
S. cerevisiae (30)	2347-18282 (30)	673-4043 (16)	ND	24-583 (29)
T. delbrueckii (1)	11524 (1)	ND	ND	188 (1)
Z. steatolyticus (2)	17250-44335 (2)	ND	2278-2439 (2)	99 (1)
S. cerevisiae (inoculum)	15426	4593	ND	138

* Values in parentheses indicate the number of isolates. ND – Not detected in any of the isolates

Most of the strains which exhibited β- glucosidase activity did have high levels of glucanase activity. Similar trend was also noted in the present study (Table 3.6). Ability of glucanase to hydrolize *p*-nitrophenyl-β-D-glucanopyroside, a substrate of β- glucosidase has been reported (Strauss et al. (2001). In a recent study, a glucanase secreting strain of *Aureobasidium pullulans* was shown to be associated with grapes (Bauermeister et al., 2015).

Candida, Pichia, Saccharomyces, *Zygosaccharomyces* are known producers of pectinase, with significant activity being observed in *Saccharomyces fragilis* (*Kluyveromyces fragilis*) and *Candida tropicalis* (Blanco et al., 1999; Fellows and Worgan, 1984; Fernandez-Gonzalez et al., 2004; Kotomina and Pisarnitskii, 1974; Roelofsen, 1953; Sanchez et al., 1984). *S. cerevisiae* and strains of *Candida stellata, Kloeckera apiculata,* and *Pichia membranifaciens* were reported to produce glucanases (Strauss et al., 2001). Extracellular secretion of β-1,3- glucanase in culture medium was seen in *Hanseniaspora uvarum* and *Hanseniaspora valbynsis* (Ahmed and phaff, 1968). β-1, 3- Glucanase from *Delftia tsuruhatensis* was reported to be a useful tool to prevent slime production and undesirable yeast growth during vinification (Blattel et al., 2010). Glucosidases are present in all grape varieties, and their concentrations vary according to the variety (Gunata et al., 1985). Glucosidases that hydrolyse non-volatile glycosidic precursors of the grape exhibited a role in improving the aroma and flavor of wine (Pombo et al., 2011). Recently Renault et al. (2015) also reported that *T. delbrueckii* along with *Saccharomyces* could be useful for ester formation which can directly enhance the aroma of wine.

The results show the potential of non-*Saccharomyces* yeast isolates for enzyme production and also highlights the possibility of considering these autochthonous strains for mixed culture fermentation along with starter strain.

3.6 Ethanol tolerance test for natural yeast flora and spoilage yeasts

In order to influence the wine quality, the non-*Saccharomyces* yeasts should be capable of tolerating ethanol and grow in the fermenting must. Hence, 17 representative yeast isolates from the seven genera were evaluated for tolerance to ethanol (1-13%) in vitro, in YPG medium. Most of the natural yeast flora could tolerate up to 6% 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%, while only *D. bruxellensis* was able to grow at 13% ethanol after 48 h. The tolerance levels of these yeasts may vary in the fermenting must.

Hanseniaspora, Candida, Pichia, Kluyveromyces, Metchnikowia and *Issatchenkia* sp. generally found in grape juice can not tolerate ethanol exceeding 5-7%, thereby their number declines or they die off as the fermentation progresses

beyond mid-stage (Heard and Fleet, 1988). In case of *S. cerevisiae,* no loss in viability was observed in presence of 15% ethanol, even after 12 days. *S. pombe, Z. bailii* and *Z. fermentati* are known for high ethanol tolerance (>10%) and are reported to be present in winery environments (Fleet, 2000; Romano and Suzzi, 1993).

3.7 Wine fermentation using Shiraz variety grapes

Fig. 3.7 Laboratory scale wine fermentation using Shiraz variety grapes

Laboratory scale fermentation of Shiraz variety grapes was carried out at 20ºC for 15 d with naturally occuring non-*Saccharomyces* yeasts from the grapes and *S. cerevisiae* (EC1118) as added inoculum to produce wine (Fig. 3.7). During the fermentation, succession of yeasts and different enzyme activities were monitored.

3.7.1 Succession of yeasts during fermentation

Qualitative and quantitative changes in the yeast community were monitored for 15 d during fermentation. Initially (0 d) *Hanseniaspora* sp., *Issatchenkia* sp., *Pichia* sp., *Torulaspora* sp. along with *S.cervisiae* was present in the must. The count of *Hansenispora* sp. was $1.6x10^4$ cells/mL in grape must and it reached to $2x10^6$ cells/mL on 3rd d. On 6th day of fermentation, it further increased upto 10^7 cells/mL. The cell concentration decreased with increase in ethanol concentration. From the five species present on 0 day, *Torulaspora* sp. could not be detected on 3rd day, *Pichia* sp. disappeared on 6th day, and *Issatchenkia* disappeared on 9th day whereas only *S. cerevisiae* was present in the sample even after 9th day 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 day of fermentation, *Issatchenkia* sp. and *Pichia* sp. count reached to $7x10^6$ cells/mL and 5.2×10^5 cells/mL, respectively. Inoculum of *S. cerevisiae* was $1x10^6$ cells/mL on 0 day which first increased to $8.1x10^6$ and then

decreased to, $4x10^8$, $1.6x10^8$, $1.6x10^7$ and $8x10^5$ cells/mL on 3rd, 6th, 9th, 12th, and 15th day, respectively.

Non-*Saccharomyces* yeasts, mainly *H. uvarum* and *C. stellata* have been reported to dominate first stages of Garnatxa and Xarel.lo grapes fermentation (Beltran et al., 2002). For Merlot variety, *K. apiculata, C. stellata* and *S. cerevisiae* were dominant yeasts with lower levels of *Pichia* and *Rhodotorula* sp. in musts. As the fermentation progressed, rapid decrease and disappearance of *Pichia* and Rhodotorula was observed. *K. apiculata* and *C. stellata* initially proliferated upto 10^7 cells/mL, and then disappeared. *S. cerevisiae* dominated alcoholic fermentation with count upto 10^9 cells/mL and continued to survive during malolactic fermentation. Interestingly, *Pichia membranefaciens* appeared during malolactic fermentation and was detected in final wine at 10^4cells/mL (Fleet et al., 1984). *Hanseniaspora, Rhodotorula, Cryptococcus* and *Debaryomyces* genera were present in the must, but only apiculate yeasts of *Hanseniaspora* genera completed spontaneous fermentation of *Tinta Roriz* grapes (Moreira et al. 2011). Initial phases of spontaneous fermentations are mainly dominated by *Kloeckera* and *Candida* sp., followed by *Metschnikowia*, *Pichia* and occasionally *Brettanomyces*, *Kluyveromyces*, *Schizosaccharomyces*, *Torulaspora*, *Rhodotorula* and *Zygosaccharomyces* (Clemente-Jimenez et al., 2004).

3.7.2 Enzyme levels during Shiraz 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 for reducing the cost and improve the wine quality. Monitoring of these enzymes during fermentation could also be useful in preventing stuck fermentation. These enzyme levels are not necessarily constant throughout the fermentation process (Maturano et al., 2012).

During the fermentation of Shiraz variety, fluctuations in the enzyme levels were detected with all the four enzymes being detected in significant quantity during early days of fermentation. The levels of protease, pectinase and glucosidase decreased in late stage of fermentation (Fig. 3.8). Pectinase levels were high (3558

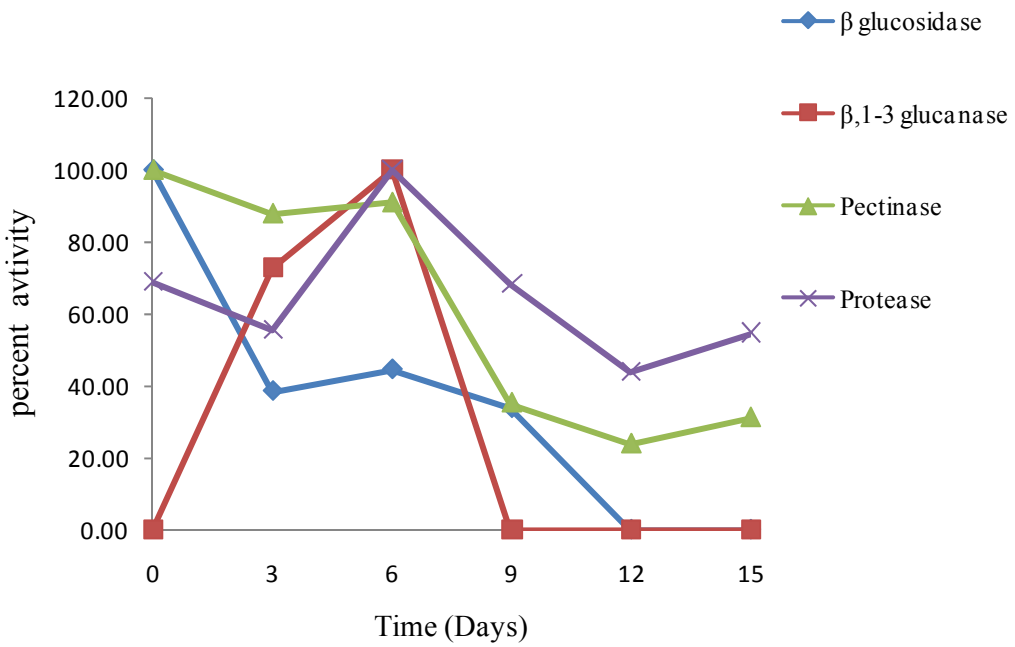

Fig. 3.8 Enzyme activities during Shiraz grapes fermentation

U/ml) and increased till 6th d (4390 U/ml), which then decreased by >60% from 9th d onwards. Glucanase was absent on 0 d, which then increased gradually and was maximum, i.e. 122 U/ml on 6th d. It was not detected in samples after 9th d. Maximum level of β-glucosidase activity (36 U/ml) was on 0 d. Protease level was also maximum (568 U/ml) on 6th d and decreased in late stage of fermentation. Glucosidase activity decreased as fermentation advanced and was absent in last stage of fermentation. Increase (till 6th day) and fall (day 9 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 an important source for enzymes of enological interest. The inference was also supported by enzyme activities exhibited by the isolates during fermentation in YPG medium.

Zamuz et al. (2004) reported the levels of different enzyme activities during fermentation. In a study of fermentation using Tempranillo variety (Zamur et al 2004), initial pectinase activity was 40 nmole/ml/h (0 day), which increased to 60 nmole/ml/h on 5th day and later decreased to 20 nmole/ml/h on 7th day and remained the constant till 26th d. β-1, 3 glucanase activities ~ 50 nmole/ml/h were detected during initial stage of fermentation, whereas, β-glucosidase was detected on 7th and 8th day (~10nmol/mL/h). Extracellular β-glucosidase activity produced by

Saccharomyces and non-Saccharomyces strains could hydrolyze monoterpene glucosides present in the grape juice possibly influencing the aromatic characteristics of the wine (Fia et al., 2003). Glucanases were also reported to exhibit activity against wine spoilage yeasts such as *Cryptococcus*, *Dekkera*, *Pichia* and *Zygosaccharomyces* (Enrique et al., 2010) preventing their growth during fermentation.

3.7.3 Chemical analysis of grape juice and wine

Chemical Analysis of the grape juice obtained from Shiraz variety grapes, Shiraz wine prepared in the laboratory (test wine) was done and various parameters were studied. Sugar concentration in the juice 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 that in grape juice (566 mg/L, 896 mg/L, respectively). Wine color density was two fold higher (4.49 a.u) in test wine than grape juice (2.18 a.u.). A commercially available Shiraz variety wine was also tested and all wine color parameters of test wine and marketed wine were found to be comparble. Alcohol concentration in test wine and markated wine was 12.8% and 14% respectively.

Table 3.7 Analysis of Shiraz grape juice, test wine and marketed wine

Parameter	Grape juice	Test wine	Marketed wine
Sugar	261.1 g/L	4.19 g/L	2.8 g/L
Ethanol	ND	12.8%	14 %
Titrable acidity	ND	6.34 g/L	5.97 g/L
Total phenolic content	566.66 mg/L	1340 mg/L	1651.3 mg/L
Total flavonoid content	407.29 mg/L	462.5 mg/L	765.3 mg/L
Tannin content	896.87 mg/L	1159.3 mg/L	1893.7 mg/L
Glycerol content	ND	4.92 g/L	5.91 g/L
pH	3.71	3.76	3.89
Wine color			
Wine color density	2.18 a. u.	4.49 a. u.	4.55 a. u.
Wine color hue	1.00	1.17	1.16
Estimate of SO_2 resistant pigments	0.92 a. u.	1.70 a. u.	2.06 a. u.
Total red pigments	0.06 a. u.	0.10 a. u.	0.076 a. u.
Modified wine color density	3.74 a. u.	4.49 a. u.	4.54 a. u.
Modified wine color hue	1.32	1.12	1.17

ND; Not detected

Glycerol content in test wine was 4.92g/L and 5.91 g/L in markated wine sample which is rsponsible for mouth feel. No significant difference was observed in pH of grape juice, test wine and marketed wine sample (Table 3.7).

3.7.3.1 Phenolic compounds in Shiraz variety

Various enzymes play an important role in efficient extraction of desirable grape pigments and other phenolic compounds in grape pulp. Due to presence of different enzymes, non-*Saccharomyces* yeasts could positively contribute to the analytical and sensorial composition of wine by releasing different flavor active secondary

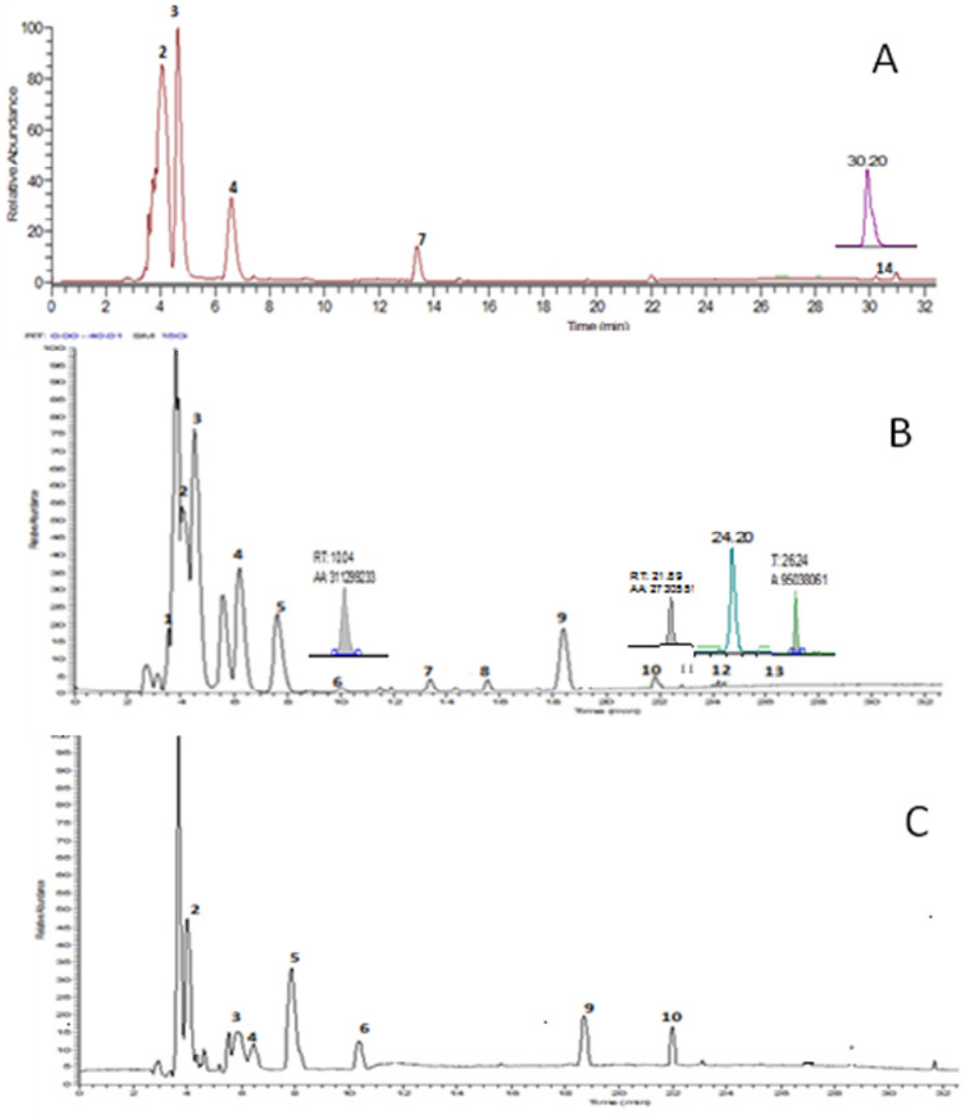

Fig. 3.9 HR-LCMS chromatogram of Shiraz grape juice (A); Test wine (B); Marketed wine (C) 1-Caffeic acid, 2- Tartaric acid, 3- Malic acid, 4- Citric acid, 5-Succinic acid, 6-Gallic acid, 7-Glutationyl caffeoyl tartaric acid, 8-Catechin, 9-Fragment of piceatannol, 10-Syringic acid, 11- p-coumaric acid, 12-Quercetin 3 glucoside, 13- Resveratrol, 14- Vanilic acid β-d-glucopyranoside. (Mass peaks are given in Annexure II)

metabolites including phenolic compounds, alcohols, esters, acids, aldehydes, volatile sulphur compounds, ketones (Lambrechts and Pretorius, 2000). For instance, glucosidases are present in all grape varieties, and their concentrations vary according to the variety (Gunata et al., 1985). β glucosidases that hydrolyse non-volatile glycosidic precursors of the grape help in improving the aroma and flavor of wine (Pombo et al., 2011).

Altogether, 14 polyphenols and organic acids were identified from both the samples based on the comparison of retention time and MS-MS spectra (Fig. 3.9). The organic acids identified were malic acid, tartaric acid, citric acid and succinic acid. Among polyphenolic compounds, gallic acid, syringic acid and vanilic acid were detected while hydroxybenzoic acids. *p*- Coumaric acid (a hydroxycinnamic acid) catechin (a flavanol) and resveratrol (a stillbene) were other polyphenolics that were found. Tartaric acid, malic acid and citric acid were detected in grape juice test wine and marketed wine sample. 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, syringic acid, succinic acid, citric acid, fragment piceatannol and p-coumaric acid were detected in both marketed wine and test 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. Caffeic acid (32.6 mg/L), *P*-coumaric acid (8.2 mg/L), quercetin-3-glucoside, catechin (5.97 mg/L), resveratrol (18.28 mg/L) and glutationyl caffeoyl tartaric acid were detected only in test wine (Table 4.8). Catechin reacts with tannin and is important for primary flavor whereas resveratrol has antimicrobial, antioxidant and anticancer properties. *P*-coumaric acid is also a known antimicrobial and antioxidant. The difference in the test and marketed wine may be attributed to non-*Saccharomyces* yeasts due to the practice/fact that during industrial production, potassium metabisulphite is added to the grape juice to inhibit microorganisms other than *S. cerevisiae*.

Relative percentage of few compounds was calculated with respect to the concentration of tartaric acid (Table 3.8). 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).

Table 3.8 Organic acids and polyphenols detected in grape juice, test wine and marketed wine of Shiraz variety

Compound	Peak No	RT (min)	Mass m/z	Molecular formula	Shiraz GJ mg/L	Test wine mg/L	Marketed wine mg/L
Caffeic acid*	1	3.54	179	$C_9H_8O_4$	ND	32.6	ND
Tartaric acid	2	4.05	149	$C_4H_6O_6$	13310	1620	2150
Malic acid	3	4.50	133	$C_4H_6O_5$	10140	2340	2120
Citric acid	4	6.22	191	$C_8H_8O_7$	33.99	62.44	14.76
Succinic acid*	5	7.63	117	$C_4H_6O_4$	ND	39.78	98.76
Gallic acid	6	9.97	169	$C_7H_6O_5$	ND	19	35
Glutationyl caffeoyl tartaric acid*	7	13.37	616	$C_{23}H_{27}N_3O_{15}S$	13.02	35.47	ND
Catechin	8	15.53/19.19	289	$C_{15}H_{14}O_6$	ND	5.9	ND
fragment piceatanol*	9	18.38	175	-	ND	27.86	55.67
Syringic acid*	10	21.84	197	$C_9H_{10}O_5$	ND	31.85	31.65
P-coumaric acid	11	22.87	163	$C_9H_8O_3$	ND	8.2	ND
Quercetin 3-glucocide*	12	24.20	463	$C_{21}H_{20}O_{12}$	ND	18.72	ND
Resveratrol	13	26.24	227	$C_{14}H_{12}O_3$	ND	18.2	ND
Vanilic acid β-d-glucopyranoside*	14	30.24	329	$C_{14}H_{18}O_9$	2.02	ND	ND

* Relative percentage with respect to tartaric acid (%); ND; Not detected

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 (Brezna et al., 2010; Nykanen, 1986). *Hanseniaspora guilliermondii, H. uvarum* and *Pichia anomala* were able to produce ethyl acetate, geranyl acetate, isoamyl acetate and 2-phenylethyl acetate (Rojas et al. 2001). 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).

Non-*Saccharomyces* yeasts including genera *Candida*, *Debaryomyces*, *Hanseniaspora*, *Hansenula*, *Kloeckera*, *Metschnikowia*, *Pichia*, *Schizosaccharomyces*, *Torulaspora* and *Zygosaccharomyces* have been reported to produce high concentrations of some fermentation compounds, such as acetic acid, glycerol, esters and acetoin that influence the sensory quality of wine (Fleet et al., 1984)

In conclusion non-*Saccharomyces* yeasts such as *H. guilliermondii, H. uvarum, H. opuntiae* and *Issatchenkia terricola, Pichia* sp. 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. Uses of winery waste for possible value addition to the wine Industry

Grape is the world's largest fruit crop with more than 70 million tons produced annually in 2010. Worldwide ~80% of the total crop is used in wine making and pomace represents approximately 13-25% of the weight of grapes processed. Huge amount of wastes such as pomace and yeast lees is generated as by-product of wine industry. Grape pomace finds limited use in animal feed or as manure. Presence of anti-nutritional factors such as phenolic components, which inhibit the ruminal symbionts (Botella et al., 2007) and inhibitory effect on seed germination puts limitation to its effective utilization. Management and disposal of this huge quantity of wine industry residual waste poses a serious environmental problem (Bustamante et al., 2008) and remains a major challenge to the wine industry.

In India wine industry comparatively is in nascent stage and now coming up as an organized sector. It is important to use pomace for different purposes such as pharmaceutically important and human health-promoting bio-actives development and to maximize by-product recovery along with minimizing the secondary wastes of wine fermentations. The process would not only boost wine industry tremendously but also reduce the load on environmental pollution. In our efforts for valorization of grape pomace, polyphenolic enriched fraction exhibiting broad spectrum biological activities were isolated and the residual pomace was effectively used as a SSF substrate for the production of hydrolytic enzymes production using different fungal and yeast cultures. Another waste from winery, yeast lees was effectively used for the isolation of chitin and chitosan polymers. The results are discussed in following sections.

3.8 Physicochemical analysis of grape pomace

The pomace for two red (Shiraz and Cabernet) and two white (Chenin Blanc and Sauvignon Blanc) were collected from a winery located in Nashik district. Initial moisture content of the pomace samples was reduced to 4.3-5.7 % by shade drying. Total phenolic content was estimated with Folin-Ciocalteu reagent and expressed as milligrams of gallic acid equivalent (GAE).

Table 3.9 Physicochemical analysis of grape pomace

Parameters	Shiraz	Cabernet	Chenin Blanc	Sauvignon Blanc
pH	3.4	3.6	3.2	3.5
Moisture content (%)	5.7	4.4	4.4	4.81
Total sugar (g/kg)	104	200	127	249
Phenolic content (g/kg)	26.3	23.1	18.21	15.53
Protein (g/kg)	115.62	154.37	84.37	124.37
Nitrogen (%)	1.85	2.47	1.35	1.99
C:N ratio	22.12	18.53	32.27	22.80

Pomace of Shiraz (26.3 g/Kg) and Cabernet (23.1 g/Kg) red grape varieties were found to have more phenolic contents as compared to the white grape varieties, Sauvignon Blanc (15.53 g/Kg) and Chenin blanc (18.21 g/Kg). The value for phenolic contents (246.3 ± 0.9 mg gallic acid equivalent/g) of red grape pomace was comparable to the dried extract of red grape poamce (Jariyapamornkoon et al., 2013). Sri Harsha et al. (2013) reported that total phenolics content in red grape skins extract was in the range of 12.1–53.6 g/kg gallic acid equivalent. Protein content of all pomace samples was in the range of 84.37-154.37 % which was comparable with 109.8 g/kg from winery waste (Lucas et al., 2008). All pomace samples had nitrogen content in the range of 1.35 - 2.47% (Table 3.9). The nitrogen percentage reported for the grape pomace is in the range of 2.14–3.74 (Ioannis, 2006).

3.9 Extraction of polyphenolic enriched fraction

Grape pomace of all varieties of grapes was shade dried. Skin with pulp and seeds was separated manually and processed for the extraction of polyphenolics and fat. Lipids were extracted in petroleum ether, whereas polyphenolics were extracted in methanol and n-butanol.

The fat content of all skin and seed extacts were in the range of 1.18-2.15 % and 9.84-34.88%, respectively. The fat content was significantly higher in seed extracts as compared to the skin extracts for all the samples. Deng et al. (2011) reported that fat content from skin extracts of different red varieties was in the range of 1.14%-6.63%. Polyphenolic enriched fractions from skin-pulp and seeds were designated with sample codes mentioned in the Table 3.10.

Table 3.10 Fat and Polyphenolic extracts obtained from skins and seeds of different grape varieties pomace

Name of variety	Sample (Code)	Petroleum ether fraction (Fat %)	Polyphenolics enriched fraction (%)
Shiraz	Skin-pulp (Sh-P)	1.78	3.08
	Seed (Sh-S)	34.88	1.84
Cabernet	Skin-pulp (Ca-P)	1.18	2.62
	Seed (Ca-S)	10.38	2.00
Chenin Blanc	Skin-pulp (Ch-P)	1.08	2.54
	Seed (Ch-S)	12.37	1.88
Sauvignon	Skin-pulp (Sa-P)	2.15	2.26
Blanc	Seed (Sa-S)	9.84	1.06

3.10 Biological activities of polyphenolic enriched fraction

Different biological activities such as antioxidant, antiglycation, antibacterial, antifungal and anticancer were carried out for the polyphenolic enriched fractions obtained after extraction.

3.10.1 Anticancer activity

The antiproliferative effect of all the polyphenolics enriched fractions obtained from skins and seeds were evaluated against SiHa (Squamous cell carcinoma, cervix) cell line by MTT assay. The absorbance in MTT assay is a measure of mitochondrial activity of viable cells obtained by the reduction of tetrazolium salt (MTT) to purple colored water soluble formazan. The absorbance thus reflects the cellviability. A dose dependent decline in the absorbance was noted for all the samples. IC_{50} values for all the fractions are given in Table 3.11. All the fractions except Cabernet skin-pulp fraction (Ca-P) exhibited pharmaceutically significant cytotoxic activity against SiHa cells. Shiraz skin-pulp extract with IC_{50} 16 µg/mL showed most potent cytotoxicity. This may be due to maximum polyphenolics contents (3.08%) in the extract. Ye et al. (1999) assessed the cytotoxicity of grape seeds proanthocyanidin extract (GSPE) against MCF-7 human breast cancer cells, A-427 human lung cancer cells, CRL-1739 human gastric adenocarcinoma cells and K562 chronic myelogenous leukemic cells at 25 and 50 mg/L concentrations. GSPE caused concentration- and time-dependent cytotoxicity to MCF-7 breast cancer, A-427 lung cancer and gastric adenocarcinoma

cells. With GSPE 25 mg/L and 50 mg/L and 72 h incubation, MCF-7 cell growth inhibition was 43% and 47%, respectively. Kaur et al. (2006) studied the effect of grape seed extract (GSE) on human colorectal cancer HT29 and LoVo cells in culture for proliferation, cell cycle progression, and apoptosis. GSE (25-100 mg/mL) caused a significant dose- and time-dependent inhibition of cell growth with concomitant increase in cell death.

Table 3.11 Anticancer activities of different polyphenolics enriched fractions against SiHa cell line

Fraction	IC_{50} value (µg/mL)
Sh-P	16
Ca-P	500
Ch-P	55
Sa-P	25
Sh-S	34
Ca-S	62
Ch-S	61
Sa-S	31

3.10.2 Total Antioxidant activity

Total antioxidant activities of the extracts, expressed as µg of ascorbic acid equivalents/ mg of fraction are given in Table 3.12. Total antioxidant activities for skin fractions of different varieties were in the range 7.8 - 12.1 µg of ascorbic acid/ mg of fraction. In case of the seed fractions, total antioxidant activities ranged from 52.4 to 90.5 µg of ascorbic acid/ mg of fraction indicating the potential of grapes seeds as rich source of antioxidants for dietary supplement. Phenolic constituents are known to react with active oxygen radicals such as hydroxyl, superoxide anion and lipid peroxy radical and exert antioxidant activity. Antioxdant activities of all the fractions correlated with their phenolics content.

Llobera and Canellas (2007) reported that total antioxidant activities for the pomace and stem extract of Manto Negro red grape variety were 61 and 187 mg of ascorbic acid equivalent/g, respevtively. Ishiwata et al. (2004) have reported antioxidant activities for dried grapes in the range 13.26 - 28.83 mg of ascorbic acid equivalent/g of dried material.

Table 3.12 Total antioxidant activity and antiglycation activity of the polyphenolics enriched fractions

Fraction	Total antioxidant activity (µg of ascorbic acid/ mg of fraction)	Antiglycation activity after 24 h (%)*
Sh-P	7.8	18.00
Ca-P	12.1	29.09
Ch-P	8.1	11.81
Sa-P	10.9	29.09
Sh-S	90.5	25.45
Ca-S	52.4	37.00
Ch-S	59.6	42.72
Sa-S	75.9	30.90

*Calculated considering inhibition of glycation caused by 100 µg aminoguanidine (10 mM) as 100 %.

3.10.3 Antiglycation Activity

Increased protein glycation and the subsequent build-up of tissue advanced glycation endproducts (AGEs) contribute towards the pathogenesis of diabetic complications. There is interest in compounds with anti-glycation activity as they may offer therapeutic potential in delaying or preventing the onset of diabetic complications.

Skin fraction shows antiglycation activity after 24 h is in the range of 11-29% while for seed fraction, it is in the range of 25-42%. Antiglycation potential of all the skin and seed fractions after 24 h of incubation is mentioned in Table 3.12. Seed fractions showed better antiglycation activity than skin fractions.Grape skins have been reported to exhibit antioxidant and anti-glycation activities because of their anthocyanins and proanthocyanidins content (Teixeira et al., 2014).

Sri Harsha et al. (2013) demonstrated that anti-glycation effect of red grape skins from ten different winemaking processes was higher than that of commercial nutraceutical preparations. Even white grape skin exhibited potent antiglycation activity (250-711 mmol aminoguanidine Eq/kg). Sri Harsha et al. (2014) indicating the potential application of these extracts in functional foods targeting wellbeing of diabetic and elderly people.

3.10.4 Antimicrobial activities

Antimicrobial activities of the different fractions were evaluated against different pathogenic bacteria, yeasts and wine spoilage yeasts. No antibacterial and antifungal activity was observed for skin-pulp fractions except for the fraction from Shiraz variety, which caused ~40% inhibition of *M. bovis* and *B. subtilis* at 100 µg/mL. However, seed fractions from both red and white varieties showed antibacterial and antifungal activities (Table 3.13 and Table 3.14).

Table 3.13 Antibacterial acitivties of different polyphenolics enriched fractions (% inhibition at 100 µg/mL)

Fractions	*Mycobacterium tuberculosis* H37Ra		*Mycobacterium bovis* BCG	*Staphylococcus aureus*	*Bacillus subtillus*
	Dormant	Active			
Sh-P	ND	ND	41.3	ND	39.8
Ca-P	ND	ND	ND	ND	ND
Ch-P	ND	ND	ND	ND	ND
Sa-P	ND	ND	ND	ND	ND
Sh-S	68.66	61.73	40.7	ND	ND
Ca-S	ND	ND	41.4	44.3	ND
Ch-S	37.08	ND	38.8	62.4	ND
Sa-S	36.44	ND	ND	97.1	52.7

ND- Not detected

Table 3.14 Antifungal activities (MIC µg/mL) of different polyphenolics enriched fractions

Pathogenic fungi and Wine spoilage yeasts	Sh-S	Ca-S	Ch-S	Sa-S
Candida albicans	> 2000	> 2000	> 2000	> 2000
Cryptococcus neoformans	> 2000	> 2000	> 2000	1500
Dekkera bruxellensis	> 2000	> 2000	> 2000	> 2000
Schizosaccharomyces pombe	500	500	500	500
Torulaspora delbrukii	> 2000	500	> 2000	> 2000
Metchnikowia pulcherima	1000	500	> 2000	> 2000
Zygosaccharomyces rouxii	> 2000	500	> 2000	> 2000

In a recent study, Oliveira et al. (2013) reported moderate antimicrobial activity (MIC 1500- 2000 µg/mL), for pomace of Shiraz grapes against *S.aureus*, *B. cereus, E. coli, P.aeruginosa*. Syrah variety showed less activity than Cabernet Sauvignon extracts, with inhibition against *S. aureus* and *Listeria monocytogenes* not exceeding 90% and 45% at concentrations of 500 and 62.5 µg/mL, respectively (Loroto et al., 2014). The antimicrobial activity of fermented pomace was either as effective as or significantly better than whole fruit grape extracts (Thtmothe et al., 2007). Reported minimum inhibitory concentration (MIC) of the methanolic extract of red grape pomace was found to be 900 ppm for *Bacillus subtilis*, *Bacillus cereus* and *Bacillus coagulans* and 1000 ppm for *Staphylococcus aureus* (Amarowicz and Weidner, 2009).

Rotava et al. (2009) showed that phenolic compounds from defatted grape (*Vitis vinifera*) seed extracts inhibited the growth of *Staphylococcus aureus* and *Escherichia coli*, while no effects was observeds for *Salmonella* sp. For antifungal effect, Oliveira et al (2013) reported >2000 ppm as the MIC of Shiraz pomace extract against *C. albicans*. The extracts may be useful as natural food preservatives or as a source of active pharmaceutical ingredient (Xia et al., 2010).

3.11 Solid state fermentation

Solid-state fermentation (SSF) holds great potential for the production of different microbial enzymes, and has advantages over submerged fermentation, like agro industrial residues can be used as substrates, is cost effective and requires less water and energy.

In present study, solid state fermentation for chitinolytic enzyme production from *Myrothecium verrucaria* was carried out using grape pomace: chitin (3:1) as a substrate. *M. verrucaria* is a saprophytic fungus that secrets high level of cuticle degrading enzymes complex (CDE), comprising of chitinases, N-acetyl-β-D-glucosaminidase (NAGase), lipase, β,1-3 glucanase and protease. This enzyme complex is known to degrade cuticle of the insect as well as cell wall of fungi which shares chitin as the common component, and thus holds promise as an environmentally safe biocontrol product. With Jowar: chitin (3:1) as substrate, the chitinases and NAGase activities obtained were observed to be 1.01 U/g and 11.84 U/g, respectively. After optimizing the chitin media and inoculum level, maximum chitinases and NAGase levels obtained with grape pomace: chitin substrate were 0.94

U/g and 10.9 U/g, respectively (Table 3.15). Results obtained with grape pomace were comparable to Jowar substrate and indicated its potential for use as a SSF substrate. In a previous report by Binod et al. (2007), various substrates such as wheat bran, rice bran, soyabean meal, coconut oil cake, groundnut oil cake, and prawns shell powder singly and in combination with chitin were used for production of chitinases using *Penicillium aculeatum* NRRL 2129 and chitinases activities obtained were in the range of 0.05-3.2 U/g. *Trichoderma longibrachiatum* IMI 92027 (ATCC 36838) gave the highest yield (5.0 IU/g of dry substrate) after 3 d of fermentation on wheat bran-crude chitin (9:1 mixture) medium under soild stte fermentation (Kovacs et al., 2004).

Table 3.15 Optimization for cuticle degrading enzyme activities using *M. verrucaria* in Solid state fermentation

Combinations	Enzyme activities (U/g)		
	Protease	Chitinases	NAGase
Jowar 7.5 g + Chitin 2.5 g + 5 ml chitin media + Inoculum 1 mL	6.8 ± 0.48	1.01 ± 0.01	11.84 ± 0.59
Grape pomace 7.5 g + Chitin 2.5 g + 6 ml chitin media + Inoculum 1 mL	4.45 ± 0.07	0.45 ± 0.2	4.4 ± 0.07
Grape pomace 7.5 g +Chitin 2.5 g + 6 ml chitin media + Inoculum 3 mL	2.8 ± 0.33	**0.83** ± 0.09	**7.6** ± 0.28
Grape pomace 7.5 g + Chitin 2.5 g + 7 ml chitin media + Inoculum 1 mL	1.85 ± 0.7	0.48 ± 0.12	4.14 ± 0.32
Grape pomace 7.5g + Chitin 2.5 g + 7 ml chitin media Inoculum 3 ml	2.7 ± 0.07	**0.94** ± 0.09	**10.9** ± 0.49
Grape pomace 7.5 g + Chitin 2.5 g + 8 mL chitin media + Inoculum 1 mL	2.4 ± 0.47	0.45 ± 0.3	7.08 ± 1.04
Grape pomace 7.5g + Chitin 2.5g + 8 mL chitin media + Inoculum 3 mL	2.7 ± 0.07	**0.83** ± 0.02	**10.22** ± 0.57

In a similar experiment, grape pomace was used as SSF substrate for the production of pectinases by different yeasts isolated from natural wine fermentation. Pectinases constitute a group of enzymes which degrade the pectin present in most plants with main application in food industries for clarification and extraction of fruit juices. The pectinase levels produced by the wine yeast isolates namely, *P.*

membrenifaciens, S. cerevisiae, Z. steatolyticus, D. hansenii and *H. guilliermondii* were 650, 610, 750, 630 and 580 U/g, respectively.

Mrudula and Anitharaj (2011) used rice bran, wheat bran, lemon peel, banana peel, sugarcane baggase, and orange peel as substrate for the production of pectinases using *Aspergillus niger*, and the activities obtained were in the range of 335-1240 U/g. In another study (Kumar et al. 2011) with wheat bran and corn bran as substrate, the pectinase activity observed was 179.83 U/g.

3.12 Isolation of chitin and chitosan from yeast lees

Yeast lees is another waste generated during wine making. Yeast lees obtained after fermentation was processed by alkali extraction for the isolation of chitin and chitosan. The chitin and chitosan content obtained were 165.4 mg/g (16.5%) and 16 (1.6%) mg/g of dry lees. Physiochemical characterization (degree of deacetylation) of chitosan was carried out using Fourier Transform Infrared Spectroscopy (FTIR), which revealed characteristic absorption bands at 1650 cm^{-1} (amide I) and 1450 cm^{-1} (amide II), 1074 cm^{-1} (C-N stretching) and at 3368 cm^{-1} (O-H stretching) (Fig. 3.10). The degree of deacetylation of chitosan extracted from yeast lees was 79.30 %, while for commercial chitosan (Sigma), it was 80.38%. Chitosan with a high degree of deacetylation has high positive charges and is more suitable for food applications as a

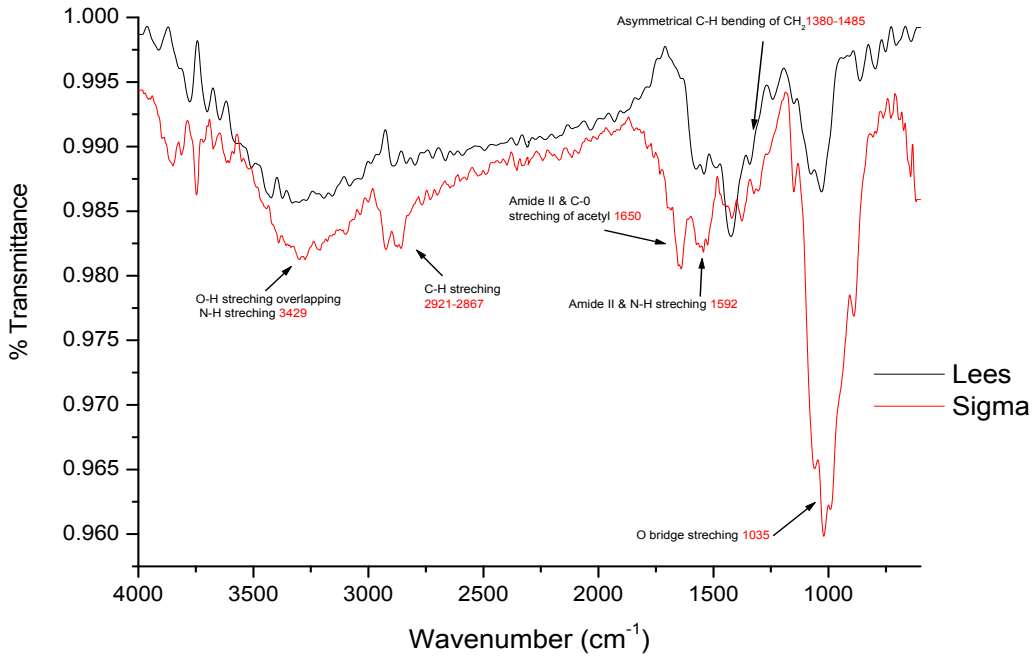

Fig. 3.10 FT-IR spectra for chitosan obtained from yeast lees and sigma chitosan

coagulating or chelating agent, a clarifying agent or an antimicrobial agent (Crestini et al. 1996).

The results for yeast lees were comparable with the chitin and chitosan content from other yeast sources. Chitin and chitosan contents of *H. guillermondii* biomass were 20.38 and 172.2 mg/g, respectively. While, for *I. orientalis* biomass, chitin and chitosan obtained were 15.33 and 170.60 mg/g. Pochanavanich and Suntornsuk (2002) isolated chitin and chitosan from different fungal sources, among them for *Zygosaccharomyces rouxii* and *C. albican*s chitosan contents were 36 mg/g (3.6%) and 44 mg/g (4.4%), respectively. For other filamentous fungi including *Aspergillus niger* and *Rhizopus oryzae* chitosan content were in the range of 11-14%. The chitin content of the *A. niger* mycelia was reported to be 42% (Knorr, 1991).

Thus, in present study, possible use of winery waste for value addition to the wine industry by evaluating the biological activities of polyphenolic enriched fraction and utilization of residual pomace as substrate in SSF for enzyme production was demonstrated. Secondly, yeast lees, was used for the isolation of cell wall polymers, chitin and chitosan, which have diverse medical and agricultural applications.

Chapter 4

Summary and conclusions

Literature review explains the important role of *Saccharomyces* and non-*Saccharomyces* yeast flora from 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 reports about yeast diversity associated with Indian vineyard and winery micro-flora. Besides, huge amount of waste such as pomace and yeast lees is generated as by-product of wine industry. Grape pomace finds limited use in animal feed or as manure therefore to reduce environmental load as pollutant, management of such waste is remain a major challenge to the wine industry. Based on this background, investigations were made as per the objectives defined earlier.

The wine industry has expanded significantly over the past decade. In the last few years' Indian wine market has shown significant growth of about 25-30% per annum. In India, Maharashtra is largest grape growing state and accounts for most of the wineries with 84% of India's wine production. Four different red grape varieties Bangalore Blue, Cabernet, Shiraz, Zinfandel and two white varieties Chenin Blanc and Sauvignon Blanc were collected from Nashik, Pune and Sangli regions of Maharashtra, India, for the study of yeast flora. The sugar concentration of different grape juices was between 161 - 270 g/L and acidity ranged from pH 3.5-4.5. A total of 152 yeasts were isolated from the six different grape varieties. The numbers of isolates from each variety were: 24, Bangalore Blue; 19, Zinfandel; 25, Cabernet; 48, Shiraz; 24, Sauvignon Blanc and 12, Chenin Blanc. Identification of natural yeasts was carried out on the basis of morphology, colony characteristics, biochemical tests and molecular techniques.

Six different types of colony morphologies were observed in 152 isolates. Seventy four isolates had a creamy and glossy colony and apiculate cell morphology typical of *Hanseniaspora* species. Twenty isolates exhibited white dry colonies. Sixteen isolates with pale brown and dry colony morphology also produced pseudomycelia typical of *Pichia* sp. Creamy, smooth and butyrous colonies were

observed for thirty two isolates. Eight isolates had yellowish mucoid colonies. Two isolates exhibited white, smooth butyrous colonies. In light microscopy, apart from probable *Hanseniaspora* and *Pichia* isolates, all other yeasts showed globose or ovoidal shape with budding morphology.

Fifteen assimilation and fermentation tests were performed for identification of the yeasts. All 152 isolates assimilated and fermented glucose while none of the isolates assimilated lactose, nitrate and nitrite. Total 74 isolates assimilated cellobiose and salicin while galactose, rhamnose and sucrose were not assimilated by them. Twenty isolates could assimilate and ferment only glucose while 16 isolates were weak glucose fermenting and could not assimilate and ferment any other sugar. Ten isolates assimilated galactose, sucrose and maltose but did not assimilate rhamnose, cellobiose and salicin. Fermentation of five sugars i.e. glucose, galactose, sucrose, maltose and raffinose, was observed in 32 isolates.

Cluster analysis of 152 yeast isolates was carried out on the basis of results obtained for 15 biochemical tests and their ability to form pseudomycelium with comparable standard strains reported in the literature. The dendrogram generated by cluster analysis showed two major branches (I and II) based on sucrose and maltose fermentation. In the first branch a group of 113 isolates were clustered (1-5) on the basis of lack of fermentation and assimilation of sucrose and maltose. Cluster 1 and 2 had 38 isolates based on non-assimilation of cellobiose and salicin and they were separated on the basis of pseudomycelium formation. In first cluster, 9 isolates were grouped with *I. orientalis*, 11 isolates were grouped with *I. terricola* and single isolate each with *C. diversa* and *T. delbrukii* based on the absence of pseudomycelium formation. In second cluster out of 16 isolates showing pseudomycelium formation, 8 isolates were grouped with *P. membranifaciens*, 6 isolates with *P. manshurica* and one isolate each with *P. kluyveri* and *P. fermentans*. Remaining 75 isolates from 1st branch which could assimilate cellobiose and salicin were spread in clusters based on maltose assimilation and galactose fermentation (3-5). Among them, 74 isolates were grouped with different *Hanseniaspora* sp. and one isolate with *D. hansenii.* Thirty nine isolates separated in a second branch on the basis of fermentation and assimilation of sucrose and maltose from. From this, 30 isolates were grouped with *S.cerevisiae* in cluster 6, four isolates were grouped with *C.azyma*, 3 isolates were groped with *C.quercitrusa* and two isolates with *Z.steatolyticus* on the basis of raffinose assimilation.

Seven genera were identified on the basis of cultural and biochemical tests. Further molecular identification was carried out to identify the isolates up to species level (White et al., 1990). The amplicons obtained were sequenced using ITS 1 and ITS 4 primers with ABI 3730 analyser. ITS1-5.8S-ITS4 sequences from the 152 strains were used to generate a BLAST analysis. On the basis of the significant sequence alignments from the BLAST search the strain identification was carried out. The sequence identity with the closest sequence was used to identify the isolates and identified as 17 different yeast species belonging to eight genera (Table 3.5). Most isolates (117) had high identity (≥ 97%) with the type strains. Among the remaining 34 isolates, 18 had identities ranging between 90-96% while 17 had lower identities (79-89%). On the basis of sequence identity *Candida* genera was resolved into three species *C. azyma* (4 isolates), *C. quercitrusa* (3 isolates) and *C. diversa* (1 isolate). Only one isolate each were of *Debaromyces hansenii* and *Torulaspora delbrueckii,* whereas two isolates were identified as *Zygoascus steatolyticus*. *Hanseniaspora* isolates were resolved into 4 species, *H. guilliermondii* (56 isolates), *H. uvarum* (10 isolates), *H. opuntiae* (5 isolates) and *H. vineae* (3 isolates). *Issatchenkia* isolates were resolved into 2 species, *I. orientalis* (9 isolates) and *I. terricola* (11 isolates). *Pichia* isolates were separated into 4 species, *P. membranifaciens* (8 isolates), *P. manshurica* (6 isolates)*, P. fermentans* (1 isolate) and *P. kluyveri* (1 isolate). Thirty isolates were identified as *S. cerevisiae.* 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 Phylogenetic tree was generated for the sequences by maximum parsimony method using MEGA6 software. Bootstrap was performed for 100 replicates and there were 356 positions in final dataset. The 152 isolates were resolved into 17 species belonging to eight genera. The Phylogenetic tree was in accordance with the observations of Kurtzman et al (2011). The topology of the Phylogenetic tree showed 2 branches that diverged from the main node. In the first branch *Z steatolyticus* diverged early and branched close to *S. pombe.* Second branch displayed the presence of 5 sub-branches containing 150 isolates. In the first sub-branch, 30 isolates of *S.cerevisiae* grouped together While 74 isolates from *Hanseniaspora* were grouped together, with *H. guillermondii*, *H uvarum*, *H. vineae* and *H. opuntiae* sharing the same branch point along with *T.delbrukii.* In the second, third and fourth sub branches isolates belonging *Candida* and *Debaromyces* genera were grouped

together along with their type strains. *Issatchenkia* and *Pichia* diverged from the same node as fifth sub branch. Members of *Pichia* group *P. fermentens*, *P. manschurica, P. kluyveri*, *P. membranifaciens*, showed the highest diversity amongst themselves.

In conclusion seventeen species belonging to eight different genera were isolated and identified from six grape varieties collected from three regions of India. *C. azyma* isolated from Bangalore Blue and cabernet variety was reported as grape yeast flora for the first time. 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. Variety and region specific associations of yeast flora were also observed in the present study. Ten species belonging to *Candida, Debaromyces, Hanseniaspora, Issachenkia, Pichia* and *Saccharomyces* genera were found on grapes from Sangli region. Ten species belonging to six genera i.e. *Candida, Hanseniaspora, Issachenkia, Pichia, Torulaspora* and *Zygoascus* were found on grapes from Nashik region. Twelve species belonging to six genera were found on grape varieties from Pune region.

H. guilliermondii strains were found on almost all varieties whereas *H.opuntiae, H.uvarum* and *H.vinae* were isolated from only Shiraz and Cabernet varieties. *S. cerevisiae* were detected on all the grape varieties studied except Bangalore blue. *C. diversa, D. hansenii, T. delbrueckii* were specifically associated with the Shiraz variety grapes. Among the 152 identified species, the largest diversity of yeast species was found in Shiraz (11 species) followed by Bangalore Blue (8 species), Cabernet (7 species), Sauvignon Blanc (6 species), Zinfandel (4 species) and Chenin Blanc (3 species).

Along with the grape variety and region from which samples were collected agricultural practices such as use of pesticides may alter the yeast diversity. In the present study all the commonly occurring genera from grapes were studied for their sensitivity against different pesticides used in vineyards. Most of the less abundant genera such as *Candida, Zygoascus, Pichia, Issachenkia, Hanseniaspora* and *Saccharomyces* were sensitive to the tested pesticides.

Non-*Saccharomyces* yeasts are known to produce different enzymes responsible for extraction of flavor and phenolics in the wine which could improve wine quality. In order to ascertain the potential of individual species for production of different enzymes, all the 152 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 of *C. quercitrusa, P. fermentens* and few isolates from *Hanseniaspora* genera 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 152 isolates, highest glucosidase (1010 U/mL), pectinase (34999 U/mL), glucanase (27279 U/mL) and protease (10810 U/mL) activity were observed for isolates *I. terricola* I_134, *P.manshurica* I_87, *I. terricola* I_123, and *H. guilliermondii* I_122 respectively. β-glucosidase activity was observed in 88 isolates belonging to *Issatchenkia, Pichia* and *Saccharomyces* genera. In case of 15 *Pichia* isolates, it was interesting to note that though β- glucosidase activity was present in all the isolates, glucanase activity was not detected. *Issatchenkia* sp. was found to be significant glucosidase producers, while β-1, 3-glucanase activity was observed in 98 isolates. β-1, 3-glucanase activities of *Hanseniaspora* and *Saccharomyces* species were in the range of 1110-14232 U/mL and 673-4043 U/mL, respectively, while isolates of *I. terricola* also showed high activity in range of 2656-27279 U/mL. Among the 17 species, protease activity was observed for 43 isolates belonging to *C. azyma, C. quercitrusa, H. guillermondii*, *Z. steatolyticus, P. manshurica* and *P fermentans*. Highest activity was observed in *C. azyma* (3512 U/mL). All the enzyme activities were observed within 48 h except detectable levels of protease activity. The activities produced by commercial wine yeast strain, *S. cerevisiae* EC1118 in YPG were also studied. Both the natural isolates and EC1118 exhibited similar pattern of the activities. The results showed potential of non-*Saccharomyces* yeast for enzyme production and also highlighted the possibility of considering these autochthonous strains having higher enzyme activities can be used for the mixed culture fermentation along with starter strain.

To ascertain the role of non-*Saccharomyces* yeasts in wine production, fermentation with *S. cerevisiae* (added as inoculum) was carried out for red Shiraz variety and succession of yeasts and changes in enzyme activities were monitored. *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 to 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 9^{th} d of fermentation. *Issatchenkia* sp. and *Pichia* sp. were present in grape juice at $3x10^4$ and $4x10^4$ cells/mL respectively. On 3^{rd} d of fermentation, *Issatchenkia* sp. and *Pichia* sp. count reached to $7x10^6$ and $5.2x10^5$ cells/mL, respectively. Inoculum of *S. cerevisiae* was $1x10^6$ cells/mL on 0 d which first increased to $8.1x10^6$ and then decreased to, $4x10^8$, $1.6x10^8$, $1.6x10^7$ and $8x10^5$ cells/mL on 3^{rd}, 6^{th}, 9^{th}, 12^{th}, and 15^{th} d respectively.

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 fermentation. Pectinase levels were high (>4500 U/ml) and constant till 6 d, which then dropped by >60% from 9 d. Glucanase activity was not detected at 0 d, which then increased gradually and was maximum, i.e. 122 U/ml on 6 d. Glucanase activity was not detected in samples from 9, 12 and 15 d. Protease level was also maximum (568 U/ml) on 6 d and decreased in late stage of fermentation. Maximum level of β-glucosidase (36 U/ml) was on 0 d. Glucosidase activity decreased as fermentation advanced and was absent in last stage of fermentation. Increase (till 6^{th} day) and fall (day 9 onwards) in all the enzyme activities over the fermentation period was concurrent with increase in cell count and disappearance of non-*Saccharomyces* yeasts.

LC-MS analysis of phenolics 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. 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. 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 Cui et al. (2012). Other parameters were also evaluated for the quality of wine including residual sugar (4.19 gm/L), ethanol (12.8%), titrable acidity (6.34 gm/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. These results indicated that non-*Saccharomyces* yeasts play important role in determining wine quality.

The pomace for two red (Shiraz and Cabernet) and two white (Chenin Blanc and Sauvignon Blanc) were collected from a winery located in Nashik district. Initial moisture content of the pomace samples was reduced to 4.3-5.7 % by shade drying. Total phenolic content was estimated with Folin-Ciocalteu reagent and expressed as milligrams of gallic acid equivalent (GAE). The total phenolic content from each sample varied because it depends on the solvent used for the extraction, grape variety and method of extraction.

Pomace of Shiraz (26.3 g/Kg) and Cabernet (23.1 g/Kg) red grape varieties were found to have more phenolic content as compared to the white grape varieties, Sauvignon Blanc (15.53 g/Kg) and Chenin blanc (18.21 g/Kg). Protein content of for all pomace samples is in the range of 84.37-154.37g/kg, which was comparable with 109.8 g/kg from winery waste. The fat content of all skin and seed extacts were in the range of 1.18-2.15% and 9.84-34.88%, respectively. The fat contents were significantly higher in seed extracts as compared to the skin extracts for all the samples.

Grape pomace from all the pomace sample were shade dried, skin with pulp and seeds were separated manually and processed for the extraction of polyphenolics content and fat contents. Lipids were extracted in petroleum ether, whereas polyphenolics were extracted in methanol and n-butanol. Different biological activities such as antioxidant, antiglycation, antibacterial, antifungal and anticancer were carried out for the polyphenolics enriched fractions obtained after extraction.

All the fractions except Cabernet skin-pulp (Ca-P) exhibited pharmaceutically significant cytotoxic activity against SiHa cells. Shiraz skin-pulp extract with IC_{50} 16 µg/mL showed most potent cytotoxicity. This may be due to maximum (3.08%) polyphenolics contents in the extract. Total antioxidant activities for skin fractions of different varieties were in the range 7.8 - 12.1 µg of ascorbic acid/ mg of fraction. In case of the seeds fractions total antioxidant activities ranged from 52.4 to 90.5 µg of ascorbic acid/ mg of fraction indicating the potential of grapes seeds as rich source of antioxidants for dietary supplement. Skin fraction shows antiglycation activity after 24 h is in the range of 11-29% while for seed fraction activity is in the range of 25-42%. Seeds fractions showed better antiglycation activity than skin fractions. Antimicrobial activities of the different fractions were evaluated against different pathogenic bacteria, yeasts and wine spoilage yeasts. No antibacterial and antifungal activity was observed for skin-pulp fractions except for the fraction from Shiraz

variety, which caused ~40% inhibition of *M. bovis* and *B. subtilis* at 100 µg/mL. The extracts may be useful as natural food preservatives or as a source of active pharmaceutical ingredient

Residual pomace after solvent extraction was further used as substrate in solid state fermentation (SSF) of *Myrothecium verrucaria* for production of an enzyme complex comprising of chitinases, N-acetyl-β-D-glucosaminidase (NAGase), lipase, β,1-3 glucanase and protease. SSF was carried out with grape pomace: chitin (3:1) as a substrate. The observed chitinases and NAGase levels were 0.94 U/g and 10.9 U/g, respectively. *M. verrucaria* solid state fermentation for chitinolytic enzyme production was carried out using grape pomace: chitin (3:1) as a substrate. With Jowar: chitin (3:1) as substrate, the chitinases and NAGase activities obtained was 1.01 U/g and 11.84 U/g, respectively. After optimizing the chitin media and inoculum level, maximum chitinases and NAGase levels obtained with grape pomace: chitin substrate were 0.94 U/g and 10.9 U/g, respectively. In similar experiment, grape pomace was used as SSF substrate for the production of pectinases by different yeasts isolated from natural wine fermentation. The pectinase levels produced by the wine yeast isolates namely, *P. membrenifaciens, S. cerevisiae, Z. steatolyticus, D. hansenii* and *H. guilliermondii* were 650, 610, 750, 630 and 580 U/g, respectively Another secondary waste generated by wine industry is yeast lees. Yeast lees is another waste generated during wine making. Yeast lees obtained after fermentation was processed by alkali extraction for the isolation of chitin and chitosan. The chitin and chitosan content oobtained were 165.4 mg/g (16.5%) and 16 (1.6%) mg/g of dry lees. Physiochemical characterization (degree of deacetylation) of chitosan was carried out using Fourier Transform Infrared Spectroscopy (FTIR). The degree of deacetylation of chitosan extracted from yeast lees was 79.30 %, while for commercial chitosan (Sigma), it was 80.38%.

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

- Systematic study of yeast diversity on different wine variety grapes commonly cultivated in India was carried out. In all, 152 natural yeasts associated with these six grape varieties grown in Pune, Sangli and Nashik region were isolated and identified.
- All the 152 yeast isolates were screened for the production of different enzymes of oenological importance. Higher levels of in vitro enzyme production by these yeast strains indicates 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 studied for Shiraz variety.
- Possible use of winery waste for value addition to the wine industry by evaluating the biological activities of polyphenolic enriched fraction and utilization of residual pomace as substrate in SSF for enzyme production was demonstrated.
- Results of the present study highlighted the diversity of *Saccharomyces* and non-*Saccharomyces* yeasts from Indian vineyards. *C. azyma* isolated from Bangalore Blue and cabernet variety was reported as grape yeast flora for the first time.

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Annexure I - List of accession numbers

Table I.1 Accession numbers for the sequences of yeast isolates submitted to NCBI

Yeast Isolate	Accession number	Organisms
I_1	FJ231458	*Candida azyma*
I_2	FJ231450	*Hanseniaspora guilliermondii*
I_3	FJ231425	*Issatchenkia terricola*
I_4	KJ729273	*Issatchenkia terricola*
I_5	FJ231448	*Hanseniaspora guilliermondii*
I_6	FJ231428	*Candida quercitrusa*
I_7	KJ729274	*Candida quercitrusa*
I_8	KJ729335	*Hanseniaspora guilliermondii*
I_9	FJ231442	*Hanseniaspora guilliermondii*
I_10	KJ729275	*Hanseniaspora guilliermondii*
I_11	FJ231418	*Issatchenkia orientalis*
I_12	FJ231459	*Pichia membranifaciens*
I_13	KJ729276	*Pichia membranifaciens*
I_14	KJ729277	*Pichia manshurica*
I_15	KJ729278	*Issatchenkia terricola*
I_16	FJ231426	*Issatchenkia terricola*
I_17	FJ231427	*Issatchenkia terricola*
I_18	FJ231455	*Hanseniaspora uvarum*
I_19	FJ231421	*Issatchenkia orientalis*
I_20	FJ231422	*Issatchenkia orientalis*
I_21	KJ729279	*Hanseniaspora guilliermondii*
I_22	KJ729336	*Hanseniaspora guilliermondii*
I_23	FJ231444	*Hanseniaspora guilliermondii*
I_24	FJ231456	*Candida azyma*
I_25	KJ729280	*Saccharomyces cerevisiae*
I_26	FJ231466	*Hanseniaspora guilliermondii*
I_27	FJ231419	*Issatchenkia orientalis*
I_28	KJ729281	*Hanseniaspora guilliermondii*

I_29	FJ231434	*Saccharomyces cerevisiae*
I_30	KJ729282	*Hanseniaspora guilliermondii*
I_31	KJ729283	*Saccharomyces cerevisiae*
I_32	KJ729284	*Saccharomyces cerevisiae*
I_33	KJ729285	*Saccharomyces cerevisiae*
I_34	FJ231432	*Saccharomyces cerevisiae*
I_35	KJ729286	*Hanseniaspora guilliermondii*
I_36	FJ231438	*Zygoascus steatolyticus*
I_37	FJ231439	*Zygoascus steatolyticus*
I_38	KJ729287	*Hanseniaspora guilliermondii*
I_39	KJ729288	*Hanseniaspora guilliermondii*
I_40	FJ231420	*Issatchenkia orientalis*
I_41	FJ231443	*Hanseniaspora guilliermondii*
I_42	FJ231429	*Saccharomyces cerevisiae*
I_43	KJ729289	*Saccharomyces cerevisiae*
I_44	KJ729290	*Hanseniaspora guilliermondii*
I_45	KJ729291	*Hanseniaspora uvarum*
I_46	KJ729292	*Issatchenkia terricola*
I_47	KJ729293	*Hanseniaspora guilliermondii*
I_48	FJ231449	*Hanseniaspora guilliermondii*
I_49	FJ231423	*Issatchenkia orientalis*
I_50	KJ729294	*Hanseniaspora guilliermondii*
I_51	KJ729295	*Hanseniaspora guilliermondii*
I_52	FJ231424	*Issatchenkia orientalis*
I_53	FJ231457	*Candida azyma*
I_54	FJ231465	*Candida azyma*
I_55	FJ231447	*Hanseniaspora guilliermondii*
I_56	FJ231440	*Hanseniaspora vineae*
I_57	KJ729296	*Saccharomyces cerevisiae*
I_58	KJ729297	*Hanseniaspora guilliermondii*
I_59	FJ231435	*Saccharomyces cerevisiae*
I_60	KJ729298	*Saccharomyces cerevisiae*
I_61	KJ729299	*Saccharomyces cerevisiae*

I_62	KJ729300	*Hanseniaspora guilliermondii*
I_63	KJ729301	*Hanseniaspora guilliermondii*
I_64	KJ729302	*Hanseniaspora guilliermondii*
I_65	KJ729303	*Hanseniaspora guilliermondii*
I_66	FJ231454	*Hanseniaspora guilliermondii*
I_67	KJ729304	*Hanseniaspora guilliermondii*
I_68	FJ231417	*Issatchenkia orientalis*
I_69	KJ729305	*Hanseniaspora guilliermondii*
I_70	KJ729306	*Saccharomyces cerevisiae*
I_71	KJ729337	*Hanseniaspora guilliermondii*
I_72	KJ729338	*Hanseniaspora guilliermondii*
I_73	KJ729307	*Saccharomyces cerevisiae*
I_74	KJ729339	*Hanseniaspora guilliermondii*
I_75	KJ729308	*Hanseniaspora guilliermondii*
I_76	FJ231463	*Pichia membranifaciens*
I_77	FJ231460	*Pichia membranifaciens*
I_78	FJ231462	*Pichia membranifaciens*
I_79	KJ729309	*Pichia membranifaciens*
I_80	KJ729310	*Hanseniaspora guilliermondii*
I_81	KJ729311	*Pichia membranifaciens*
I_82	FJ231441	*Hanseniaspora vineae*
I_83	KJ729312	*Hanseniaspora vineae*
I_84	FJ231431	*Saccharomyces cerevisiae*
I_85	KJ729313	*Saccharomyces cerevisiae*
I_86	KJ729314	*Pichia manshurica*
I_87	KJ729315	*Pichia manshurica*
I_88	KJ729316	*Pichia manshurica*
I_89	FJ231430	*Saccharomyces cerevisiae*
I_90	FJ231464	*Debaryomyces hansenii*
I_91	FJ231461	*Pichia membranifaciens*
I_92	FJ231453	*Hanseniaspora guilliermondii*
I_93	KJ729317	*Hanseniaspora guilliermondii*
I_94	FJ231437	*Saccharomyces cerevisiae*

I_95	KJ729318	*Saccharomyces cerevisiae*
I_96	KJ729319	*Saccharomyces cerevisiae*
I_97	FJ231452	*Hanseniaspora guilliermondii*
I_98	KJ729320	*Hanseniaspora guilliermondii*
I_99	FJ231451	*Hanseniaspora guilliermondii*
I_100	KJ729321	*Hanseniaspora guilliermondii*
I_101	KJ729322	*Hanseniaspora guilliermondii*
I_102	KJ729323	*Hanseniaspora guilliermondii*
I_103	FJ231436	*Saccharomyces cerevisiae*
I_104	KJ729324	*Saccharomyces cerevisiae*
I_105	FJ231446	*Hanseniaspora guilliermondii*
I_106	KJ729325	*Hanseniaspora guilliermondii*
I_107	KJ729326	*Pichia manshurica*
I_108	KJ729327	*Hanseniaspora guilliermondii*
I_109	KJ729328	*Hanseniaspora guilliermondii*
I_110	KJ729329	*Saccharomyces cerevisiae*
I_111	KJ729330	*Hanseniaspora guilliermondii*
I_112	KJ729331	*Saccharomyces cerevisiae*
I_113	FJ231445	*Hanseniaspora guilliermondii*
I_114	KJ729332	*Hanseniaspora guilliermondii*
I_115	KJ729333	*Hanseniaspora guilliermondii*
I_116	FJ231433	*Saccharomyces cerevisiae*
I_117	KJ729334	*Hanseniaspora guilliermondii*
I_118	KJ810802	*Torulaspora delbrueckii*
I_119	KJ810803	*Hanseniaspora guilliermondii*
I_120	KJ810804	*Hanseniaspora uvarum*
I_121	KJ810805	*Issatchenkia terricola*
I_122	KJ810806	*Hanseniaspora guilliermondii*
I_123	KJ810807	*Issatchenkia terricola*
I_124	KJ810808	*Issatchenkia terricola*
I_125	KJ810809	*Pichia klyuveri*
I_126	KJ810810	*Hanseniaspora opuntiae*
I_127	KJ810811	*Hanseniaspora guilliermondii*
I_128	KJ810812	*Saccharomyces cerevisiae*

I_129	KJ810813	*Saccharomyces cerevisiae*
I_130	KJ810814	*Hanseniaspora uvarum*
I_131	KJ810815	*Hanseniaspora uvarum*
I_132	KJ810816	*Hanseniaspora uvarum*
I_133	KJ810817	*Hanseniaspora opuntiae*
I_134	KJ810818	*Issatchenkia terricola*
I_135	KJ810819	*Hanseniaspora guilliermondii*
I_136	KJ810820	*Issatchenkia terricola*
I_137	KJ810821	*Pichia fermentans*
I_138	KJ810822	*Saccharomyces cerevisiae*
I_139	KJ810823	*Saccharomyces cerevisiae*
I_140	KJ810824	*Candida quercitrusa*
I_141	KJ810825	*Pichia manshurica*
I_142	KJ810826	*Hanseniaspora guilliermondii*
I_143	KJ810827	*Saccharomyces cerevisiae*
I_144	KJ810828	*Issatchenkia orientalis*
I_145	KJ810829	*Hanseniaspora uvarum*
I_146	KJ810830	*Candida diversa*
I_147	KJ810831	*Hanseniaspora opuntiae*
I_148	KJ810832	*Hanseniaspora opuntiae*
I_149	KJ810833	*Hanseniaspora uvarum*
I_150	KJ810834	*Hanseniaspora opuntiae*
I_151	KJ810835	*Hanseniaspora uvarum*
I_152	KJ810836	*Hanseniaspora uvarum*

Annexure II- HR-LC/MS Chromatograms

Standards

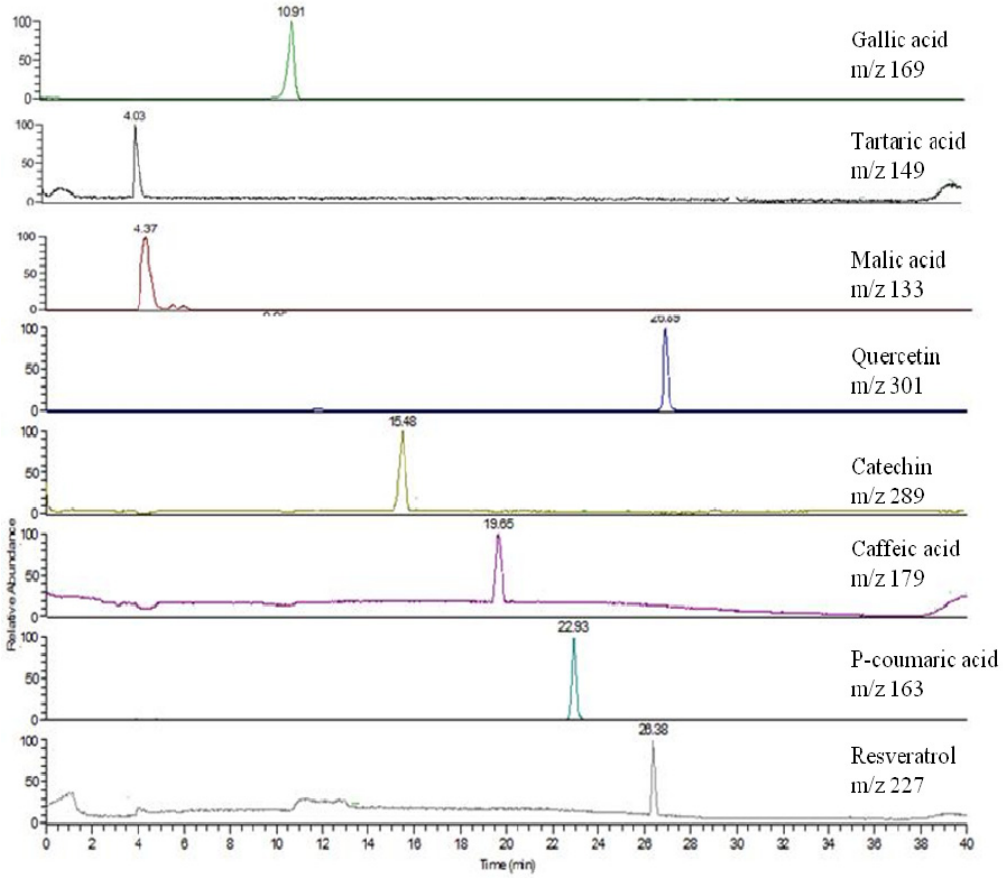

Figure II.1 Chromatograms showing peaks of standard compounds

Shiraz grape juice

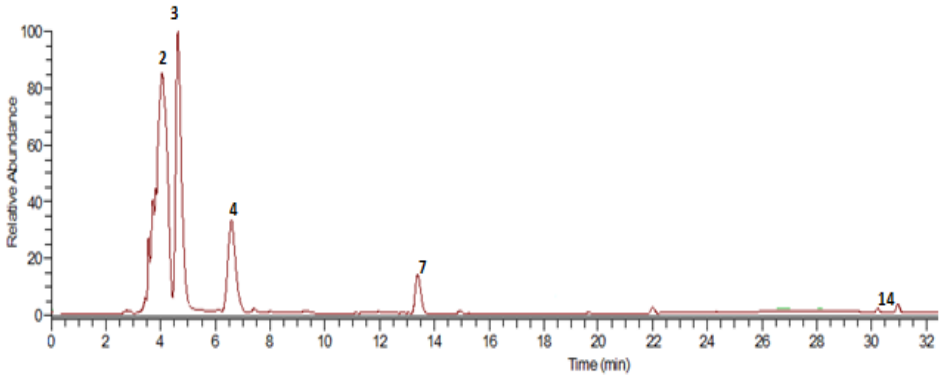

Figure II.2 HR-LCMS chromatogram of Shiraz grape juice

Table II.1 Retention time, molecular mass of identified compounds in Shiraz grape juice

Peak No	RT (min)	Mass m/z	Compound Name
2	4.05	149	Tartaric acid
3	4.62	133	Malic acid
4	6.60	191	Citric acid
7	13.39	616	Glutationyl caffeoyl tartarate
14	30.95	329	Vanilic acid β d glucopyranoside

RT; Retention time, m/z; mass:charge

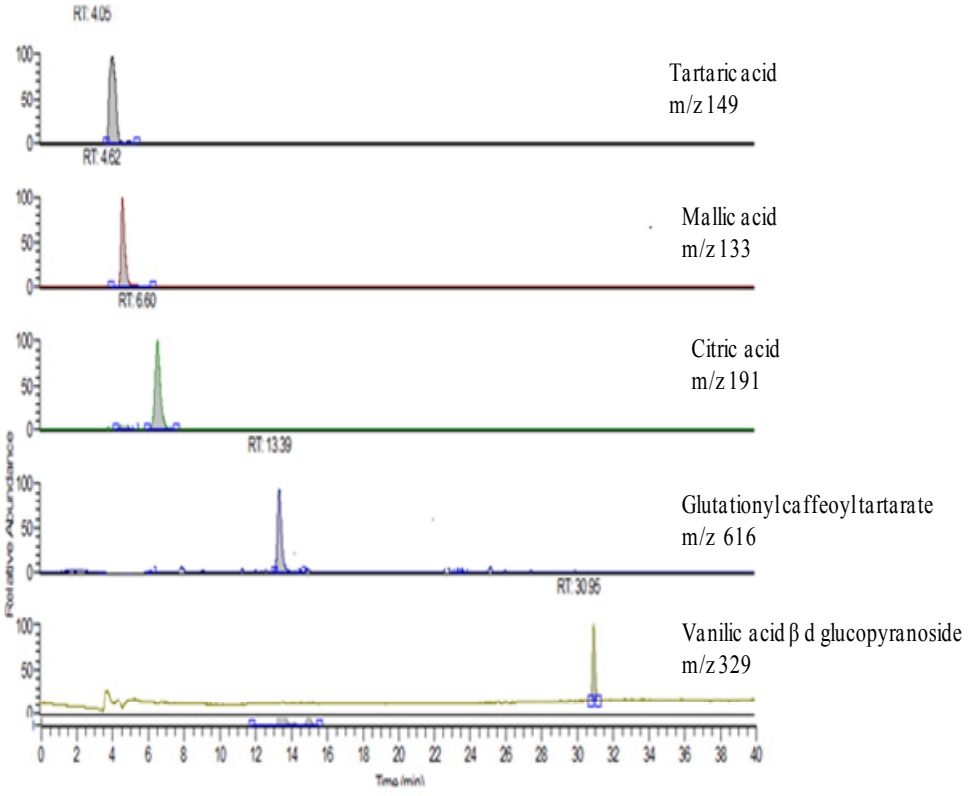

Figure II.3 HR-LCMS chromatograms showing base peaks of Shiraz grape juice

Shiraz test wine

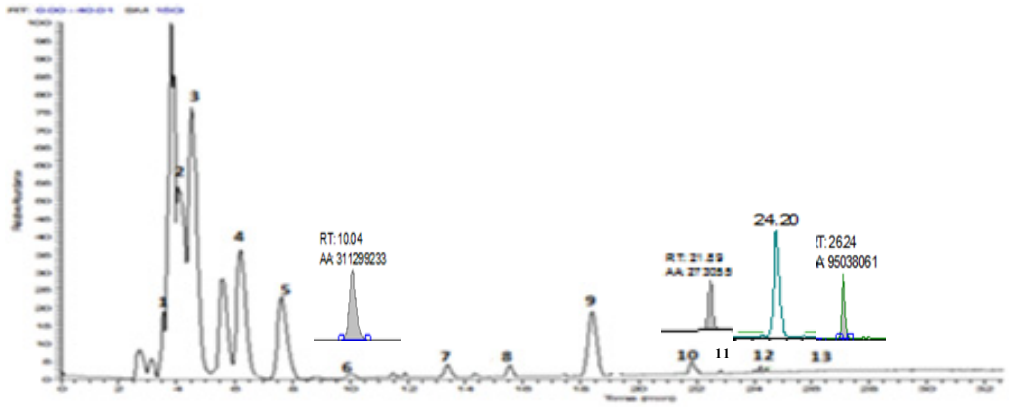

Figure II.4 HR-LCMS chromatogram of Shiraz test wine

Table II.2 Retention time, molecular mass of identified compounds in Shiraz test wine

Peak	RT(min)	m/z	Compounds
1	3.54	179	Caffeic acid
2	4.00	149	Tartaric acid
3	4.50	133	Malic acid
4	6.27	191	Citric acid
5	7.83	117	Succinic acid
6	10.04	169	Gallic acid
7	13.31	616	Glutationyl caffeoyl tartarate
8	19.27	289	Catechin
9	18.38	175	Fragment of piceatannol
10	21.98	197	Syringic acid
11	22.93	163	*p* coumaric acid
12	24.20	463	Quercetin 3 glucoside
13	26.26	227	Resveratrol

RT; Retention time, m/z; mass:charge

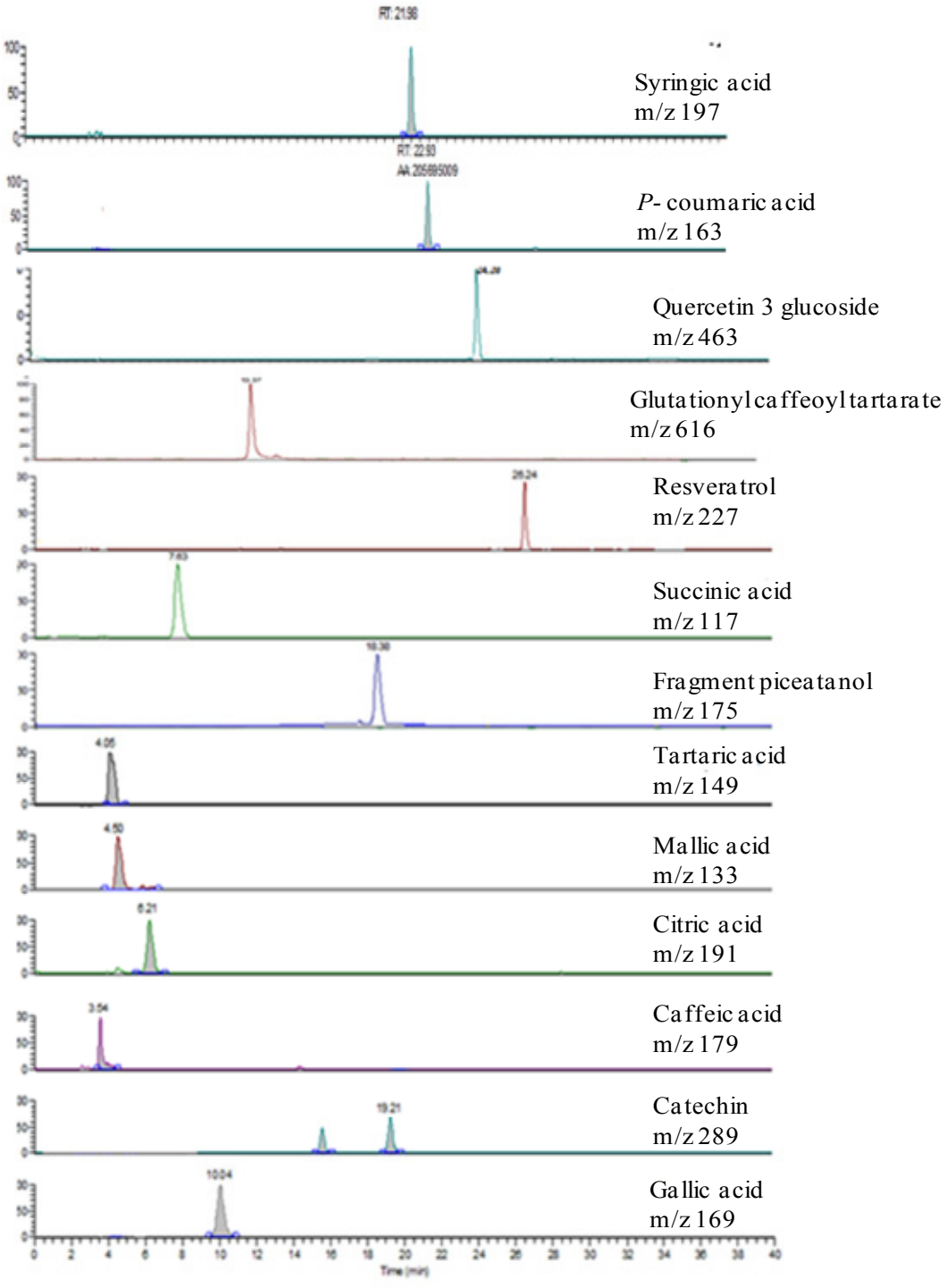

Figure II.5 HR-LCMS chromatograms showing base peaks of Shiraz test wine

Shiraz marketed wine

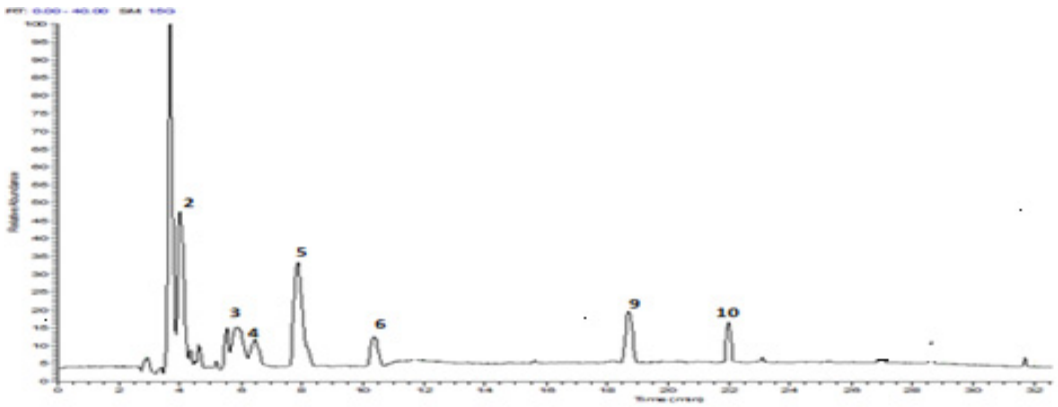

Figure II.6 HR-LCMS chromatogram of Shiraz marketed wine

Table II.3 Retention time, molecular mass of identified compounds in Shiraz marketed wine

Peak	RT(min)	m/z	Compounds
2	4.00	149	Tartaric acid
3	4.65	133	Malic acid
4	6.21	191	Citric acid
5	7.76	117	Succinic acid
6	10.36	169	Gallic acid
9	18.71	175	Fragment of piceatannol
10	21.98	197	Syringic acid

RT; Retention time, m/z; mass:charge

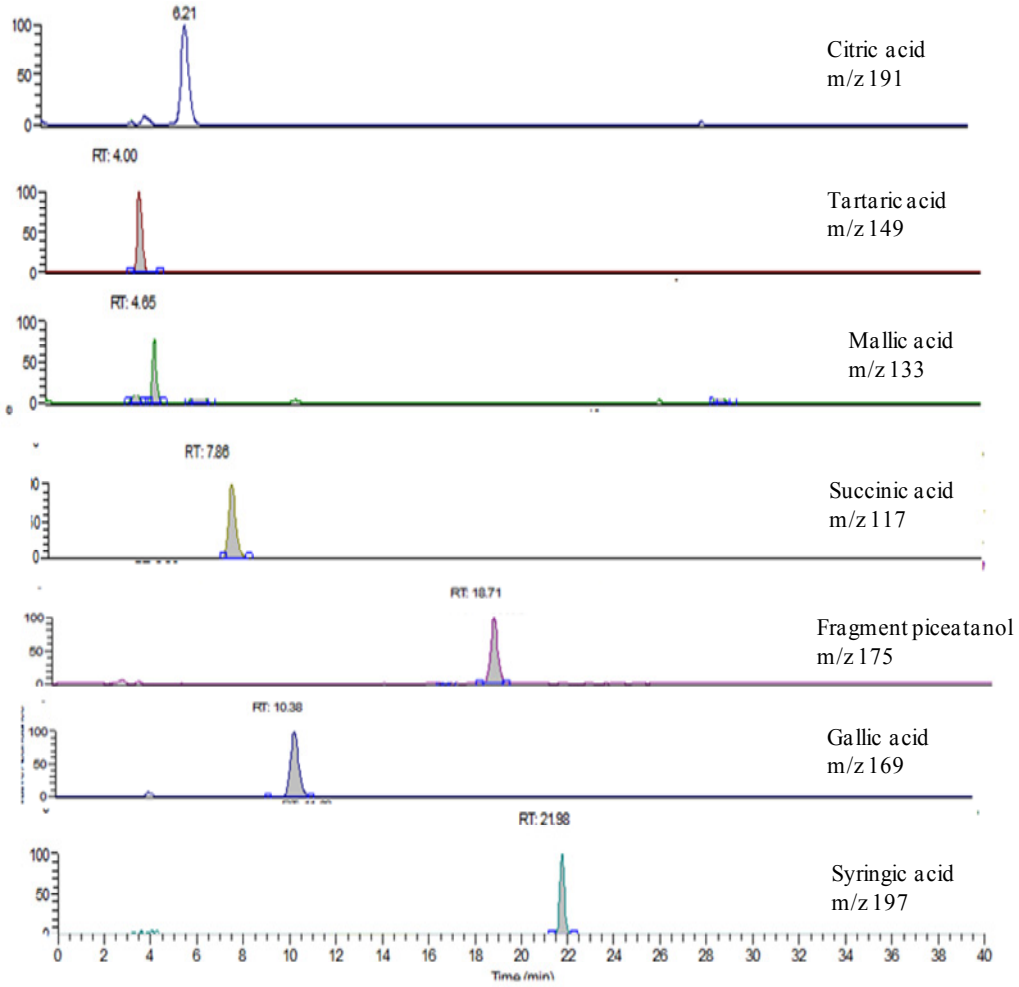

Figure II.7 HR- LCMS chromatograms showing base peaks of Shiraz marketed wine

Publications

1. Pradnya Chavan, **Sarika Mane**, Girish Kulkarni, Shamim Shaikh, Vandana Ghormade, Devidas , P. Nerkar, Yogesh Shouche, Mukund V. Deshpande (2009) Natural yeast flora from different grape varieties used for wine making in India. **Food Microbiology** 26:801-808 **(IF-3.7)**
2. Vidhate R., Ghormade V., Kulkarni S., **Mane S**., Chavan P. and Deshpande M. V. The mission mode collections of fungi with special reference to entomopathogens and mycopathogens. (2013) **Kavaka;** 41, 33-42
3. **Mane S.,** V. Ghormade, H. Munot, Y. Shouche, S. Shaikh and M. V. Deshpande. Isolation and identification of *Saccharomyces* and non-*Saccharomyces* yeasts from six wine grape varieties and their contribution in the production of hydrolytic enzymes important to wine quality. Communicated to **Mycological progress (IF-1.91)**

NCBI submission

Sequences of all the 152 yeast isolates were submitted to NCBI

Marathi articles

1. Pradnya Chavan, **Sarika Mane**, Vandana Ghormade, Shamim Shaikh, Devidas Nerkar and M.V.Deshpande (2008) **Wine nirnitimadhe Saccharomyces ani non-Saccharomyces yeast che mahatva.** In: Drakshyavrutta, Maharashtra Rajya Drakshya Bagayatdar Sangh, Pune. (August 2008).
2. **Sarika Mane**, Swati Joshi, Shamim Shaikh, Pallavi Nirmal, Pradnya Chavan and M.V.Deshpande (2010) **Bhartatil wine utpadan: Darja ani upyukatata wadhavinyacha ek marge.** In: Drakshyavrutta, Maharashtra Rajya Drakshya Bagayatdar Sangh, Pune.

Posters presented in Symposia/Conferences/Meetings, etc.

1. **S. Mane.,** P. Chavan., S. Tupe., V. Ghormade., S. Shaikh and M. V. Deshpande Importance of non-*Saccharomyces* yeasts in winemaking. Presented in Research scholar meet (RSM) 2014 at National Chemical Laboratory, Pune.

2. **Mane S. S**., Shaikh S. A., Deshpande M. V. Natural yeast diversity: Importance in winemaking. Presented in Indo-Mexico workshop held at National Chemical Laboratory on 6-8th October 2013.
3. **Mane S**., Vidhate R., Kale D., Naikwadi T., Girme A, Joshi S., Moghe A., Shaikh S., Deshpande M.V. Effective utilization of winery and vineyard waste: An eco-friendly approach for value addition to wine industry. Presented in Indo-Mexico workshop held at National Chemical Laboratory on 6-8th October 2013.
4. P. Chavan., **S. Mane.,** S. Tupe., V. Ghormade., M. V. Deshpande. Biochemical and microbial profiling of yeasts to identify their role in winemaking. Presented in Research scholar meet (RSM) 2012 at National Chemical Laboratory, Pune
5. Singh J., Nirmal P., Chavan S., Dugam S., Sable A., **Mane S**., and Deshpande M.V. Compatibility of bioagents cuticle degrading enzyme complex of *Myrothecium verrucaria and entomopathogenic* fungi used to control mealy bug infestation with chemicals applied in grape fields. Presented in 2nd ISCB symposium March 2011 at New Delhi.