Tulasi Satyanarayana · Gotthard Kunze
Editors

Yeast Diversity in Human Welfare

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Preface

Yeasts are eukaryotic microbes placed in the kingdom Fungi, under the phyla Ascomycota and Basidiomycota with approximately 2000 species described till date. These are estimated to constitute 1–1.5% of the fungal species described, and the number of existing yeast species is expected to exceed that of the described ones. In case yeasts make up 1–1.5% of the estimated fungal species extant on Earth of three million species, the yeast species would be between 30,000 and 45,000. Extensive efforts are needed to understand the diversity of yet to be cultured yeast species. Yeasts are mostly unicellular, although some species develop multicellular characteristics by forming pseudohyphae. Most yeasts reproduce asexually by mitosis, and many do so by the asymmetric division process called budding and a few by fission.

By fermentation, the yeast species *Saccharomyces cerevisiae* and others have been converting carbohydrates to carbon dioxide and alcohols for thousands of years, and carbon dioxide has been used in baking and the alcohol in alcoholic beverages. It is also a centrally important model organism in modern cell biology research, and is one of the most thoroughly investigated eukaryotic microbes. Researchers have used it to gather information about the biology of the eukaryotic cell and ultimately human biology. Other species of yeasts like *Candida albicans* are opportunistic pathogens and known to cause infections to humans. Yeasts have recently been used to generate electricity in microbial fuel cells, and to produce ethanol for the biofuel industry.

Certain strains of some yeast species produce proteins called yeast killer toxins, which allow them to eliminate competing strains. This may cause problems for wine making, but could potentially be used to advantage by using killer toxin-producing strains to make wine. Yeast killer toxins may find medical applications in the treatment of yeast infections.

Yeasts occur in the environment, and particularly in sugar-rich materials. For instance, naturally occurring yeasts are found on the skins of fruits and berries and plant exudates. Some yeasts are also found in association with soil and insects. The ecological function and biodiversity of yeasts have not yet been adequately understood. Yeasts are also present in the gut flora of mammals and some insects.

Even deep-sea environments also host some yeasts. An Indian investigation on 7 bee species and 9 plant species found 45 yeast species belonging to 16 genera to colonize the nectaries of flowers and honey stomachs of bees. Most were members of the genus *Candida*; the most common species in honey was *Dekkera intermedia* and in flower nectaries, *Candida blankii*. Yeast-colonizing nectaries of the stinking hellebore have been found to raise the temperature of the flower, which may aid in attracting pollinators by increasing the evaporation of volatile organic compounds. Black yeast has been observed as a partner in a complex relationship between ants, their mutualistic fungus, a fungal parasite of the fungus and a bacterium that kills the parasite. The yeast has a negative effect on the bacteria that normally produce antibiotics to kill the parasite, so may affect the ants' health by allowing the parasite to spread.

Some species of yeasts are opportunistic pathogens, which can cause infection in people with compromised immune systems. *Cryptococcus neoformans* and *Cryptococcus gattii* are significant pathogens of immuno-compromised individuals. They are the species primarily responsible for cryptococcosis, a fungal disease that occurs in about one million HIV/AIDS patients, causing over 600,000 deaths annually. Yeasts of the genus *Candida* cause oral and vaginal infections in humans called candidiasis. The pathogenic yeasts of candidiasis in probable descending order of virulence for humans are: *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. guilliermondii*, *C. viswanathii*, *C. lusitaniae*, and *Rhodotorula mucilaginosa*. *Candida glabrata* is the second most common pathogenic yeast after *C. albicans*, causing infections of the urogenital tract, and of the bloodstream (candidemia).

The useful physiological properties of yeast have led to their use in the field of biotechnology. Fermentation of sugars by yeast is the oldest and largest application of this technology. Many types of yeasts are used for making many foods: baker's yeast in bread production, brewer's yeast in beer fermentation, and yeast in wine fermentation and the production of xylitol.

Some yeasts can find potential application in the field of bioremediation. One such yeast, *Yarrowia lipolytica*, is known to degrade palm oil mill effluent, TNT (an explosive material), and other hydrocarbons such as alkanes, fatty acids, fats, and oils. It can also tolerate high concentrations of salt and heavy metals, and is being investigated for its potential as a heavy metal biosorbent. *Saccharomyces cerevisiae* has potential to bioremediate toxic pollutants like arsenic from the industrial effluents. Bronze statues are known to be degraded by certain species of yeast. Different yeasts from Brazilian gold mines accumulate free and complexed silver ions.

Yeast is used in nutritional supplements popular with health-conscious individuals and those following vegetarian diets. It is often referred to as "nutritional yeast" when sold as a dietary supplement. Nutritional yeast is deactivated yeast, usually *S. cerevisiae*. It is an excellent source of protein and vitamins, especially B-complex vitamins as well as other minerals and cofactors required for growth. It is also naturally low in fat and sodium. Some brands of nutritional yeast, though not all, are fortified with vitamin B_{12}, which is produced separately by bacteria.

In 1920, the Fleischmann Yeast Company began promoting yeast cakes in a successful "Yeast for Health" campaign. They initially emphasized the importance of yeast as a source of vitamins, good for skin and digestion. Their advertising later claimed a much broader range of health benefits. Nutritional yeast has a nutty, cheesy flavor that makes it popular as an ingredient in cheese substitutes. It is often used by vegetarians in the place of Parmesan cheese. Another popular use is as a topping for popcorn. It can also be used in mashed and fried potatoes, as well as in scrambled eggs. It comes in the form of flakes or as a yellow powder similar in texture to cornmeal. In Australia, it is sometimes sold as "savory yeast flakes." Though "nutritional yeast" usually refers to commercial products, inadequately fed prisoners have used "home-grown" yeast to prevent vitamin deficiency.

Some probiotic supplements use the yeast *Saccharomyces boulardii* for maintaining and restoring the natural flora in the gastrointestinal tract. *S. boulardii* has been shown to reduce the symptoms of acute diarrhea, reduce the chance of infection by *Clostridium difficile*, reduce bowel movements in diarrhea patients, and reduce the incidence of antibiotic-, traveler's-, and HIV/AIDS-associated diarrheas.

Yeasts are able to grow in foods with a low pH (5.0 or lower) and in the presence of sugars, organic acids, and other easily metabolized carbon sources. During their growth, yeasts metabolize some food components and produce metabolic end products. This causes the physical, chemical, and sensible properties of a food to change, and the food is spoiled. The growth of yeast within food products is often seen on their surfaces, as in cheeses or meats, or by the fermentation of sugars in beverages, such as juices, and semi-liquid products, such as syrups and jams. The yeast of the genus *Zygosaccharomyces* has had a long history as spoilage yeasts within the food industry. This is mainly because these species can grow in the presence of high sucrose, ethanol, acetic acid, sorbic acid, benzoic acid, and sulphur dioxide, representing some of the commonly used food preservation methods. The major spoilage yeast in enology is *Brettanomyces bruxellensis*.

Several yeasts, in particular *S. cerevisiae*, have been widely used in genetics and cell biology, largely because this is a simple eukaryotic cell, serving as a model for all eukaryotes including humans, for studying fundamental cellular processes such as the cell cycle, DNA replication, recombination, cell division, and metabolism. Yeasts are easily manipulated and cultured in the laboratory, which has allowed the development of powerful standard techniques, such as yeast two-hybrid, synthetic genetic array analysis, and tetrad analysis. Many proteins important in human biology were first discovered by studying their homologues in yeast, which include cell cycle proteins, signaling proteins and protein-processing enzymes.

Saccharomyces cerevisiae was announced to be the first eukaryote to have its genome on April 24, 1996, comprising 12 million base pairs, fully sequenced as part of the Genome Project. At that time, this was the most complex organism to have its full genome sequenced at that time, and took 7 years with the efforts of more than 100 laboratories. The second yeast species to have its genome sequenced was *Schizosaccharomyces pombe*, which was completed in 2002. It was the sixth eukaryotic genome sequenced that comprised 13.8 million base pairs. By 2012, over 30 yeast species have had their genomes sequenced and published. A total of

approximately 24,200 novel genes were identified, the translation products of which were classified together with *S. cerevisiae* proteins into about 4700 families, forming the basis for interspecific comparisons. The analysis of chromosome maps and genome redundancies revealed that the different yeast lineages have evolved through a marked interplay between several distinct molecular mechanisms, including tandem gene repeat formation, segmental duplication, a massive genome duplication, and extensive gene loss.

Yeast species have been genetically engineered to efficiently produce various drugs by a technique called metabolic engineering. *S. cerevisiae* is easy to genetically engineer; its physiology, metabolism, and genetics are well known, and it is amenable for use in harsh industrial conditions. A wide variety of chemicals in different classes can be produced by engineered yeast, including phenolics, isoprenoids, alkaloids, and polyketides. About 20 biopharmaceuticals are produced in *S. cerevisiae*, including insulin, vaccines for hepatitis, and human serum albumin.

The advances in modeling and synthetic biology tools and how these tools can speed up the development of yeast cell factories have been recently made. Metabolic engineering strategies for developing yeast strains for the production of polymer monomers: lactic, succinic, and cis, cis-muconic acids have been attempted. *S. cerevisiae* has already firmly established itself as a cell factory in industrial biotechnology and the advances in yeast strain engineering will stimulate the development of novel yeast-based processes for production of chemicals in the near future. Strategies are being developed for metabolic engineering of ethanologenic yeasts for the production of bioethanol from complex lignocellulosic residues. Recent examples of yeast metabolic engineering have shown that evolutionary potential of cells should not be underestimated in strain improvement. Evolutionarily evolved strains can form suitable starting points for inverse metabolic engineering approaches too. For developing an understanding of the cell as a whole, sophisticated computational methods capable of integrating copious amounts of data/information are required.

This book is an attempt in bringing together the scattered information on various aspects of the utility of yeast diversity for human welfare into one volume. This includes recent developments made in the past few decades on these aspects. The chapters have been written by experts, who have done a commendable job of reviewing the developments made in recent years. We wish to thank all the contributors. The views expressed by authors are their own. We sincerely hope and wish that the book will be useful for teachers, scientists, researchers and students of biology, microbiology, mycology, and biotechnology.

We wish to appreciate and thank the efforts made by Springer in publishing the book for disseminating knowledge on the utility of yeast diversity for human welfare.

New Delhi, India — Prof. Tulasi Satyanarayana
Gatersleben, Germany — Prof. Gotthard Kunze

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Tulasi Satyanarayana after obtaining M.Sc. and Ph.D. at the University of Saugar (India), Tulasi Satyanarayana had postdoctoral stints at the Paul Sabatier University and National Institute of Applied Sciences, Toulouse, France. In 1988, he joined the Department of Microbiology, University of Delhi South Campus as Associate Professor and became Professor in 1998. His research efforts have been focused on understanding the diversity of yeasts, and thermophilic fungi and bacteria, their enzymes and potential applications, heterotrophic carbon sequestration and metagenomics. He has published over 250 scientific papers and reviews and edited six books. He has two Indian patents to his credit. He has been conferred with Dr. G.B. Manjrekar award of the Association of Microbiologists of India in 2004, Dr. V.S. Agnihotrudu award of Mycological Society of India in 2009 and Malaviya award of Biotech Research Society of India in 2012 for his distinguished contributions.

Gotthard Kunze studied biology at the Ernst Moritz Arndt University in Greifswald. He got a postdoctoral fellowship and a position as scientific assistant at the Department of Biology of the University. In 1986 he joined as a research associate at the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben. Since 1998, he has been a Visiting Professor at the University of Greifswald and Professor at the Technical University Anhalt at Köthen since 1998. During this period, he focused his research activities on yeast genetics (construction of new yeast host vector systems, heterologous gene expression, thermo- and osmo-resistance in nonconventional yeasts and microbial yeast biosensors). Professor Gotthard Kunze is the author of about 182 publications, editor of two books, and teaches at the universities of Greifswald and Köthen.

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Diversity of Natural Yeast Flora of Grapes and Its Significance in Wine Making

Sarika S. Mane, Vandana Ghormade, Santosh G. Tupe and Mukund V. Deshpande

Abstract The biodiversity of yeasts associated with grapes has been studied in different regions of wine producing countries throughout the world. Most of the species associated with the wine environment are similar, while some species are specifically associated with specific regions. Though *Saccharomyces cerevisiae* is primarily used for fermentation of grape juice, its occurrence is low on grape berries. Non-*Saccharomyces* yeasts belonging to the genera *Torulaspora, Hanseniaspora, Pichia, Candida, Issatchenkia, Metschnikowia* etc. are in abundance in grape musts and may dominate the early stages of fermentation. Subsequently, *S. cerevisiae* proliferates, becomes dominant and completes the wine fermentation. Therefore, yeasts diversity associated with the grapes and must significantly contribute to the quality and varietal character of wine. In present review, the diversity of yeasts associated with vineyard, winery, succession of yeasts during fermentation and their role in wine quality is discussed. The knowledge will be useful to monitor and control the fermentation with respect to quality and spoilage.

Keywords Natural yeast flora of grapes · Non-*Saccharomyces* yeasts · *Saccharomyces cerevisiae* · Wine fermentation

1 Introduction

The earliest known wine was made in Mesopotamia around 3500 BC (Robinson 2006). However, chemical analyses of organic residues on ancient pot sherds indicated that grape juice was deliberately being fermented in China as early as

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7000 BC (McGovern et al. 2004). According to historical mural paintings and ancient pottery, the Egyptians, Phoenicians and Greeks were also quite willing winemakers and consumers. The Romans are assumed to have acquired the ability for cultivating grapes and winemaking from the Greeks and spread it into central and northern Europe. European pioneers in the 16th and 17th century introduced the grape vine into South, Middle and North America. 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) and is followed closely by Spain (4.46 billion litres), and France (4.41 billion litres).

2 Grape Varieties Used for Winemaking

Worldwide different grape varieties are used for wine production. So far 1368 vine varieties have been documented across the globe. 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, 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, which is mainly because of the presence of different secondary metabolites responsible for the principal flavor compounds in grape must (Lambrechts and Pretorius 2000). For instance, the varietal differences impart characteristic flavor and aroma to the wine, like reminiscent of blackcurrants or cedar wood 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.

3 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 10–18 °C to improve the retention of aromas; whereas red wines are fermented at higher temperatures between 18–29 °C to achieve good extraction of phenolic compounds. An initial temperature of 20 °C 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 convert 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.

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 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. 2012a).

4 Microbial Diversity of Phylloplane

The microbial communities of phylloplane are diverse comprising of different genera of bacteria, filamentous fungi, yeasts, algae, and, less frequently, protozoa and nematodes too. The yeasts usually colonize rapidly on the leaves. Number of yeasts were reported by Chand-Goyal and Spotts (1996) from the apple and pear fruit surface. *Aureobasidium pullulans, Cryptococcus albidus* and *Rhodotorula glutinis* were found on fruits in most of the studied pear orchards. Other yeasts colonizing pear fruit surfaces were *Cryptococcus infirmo-miniatus, Cryptococcus*

laurentii, Debaryomyces hansenii, Rhodotorula aurantiaca, Rhodotorula fujisanensis, Rhodotorula minuta and *Sporobolomyces roseus*. Slavikova et al. (2009) isolated 150 plus strains belonging to 11 genera from 5 fruit trees, namely apple, cherry, apricot, peach and plum leaves. Most common were *A. pullulans, C. laurentii* and *Metschnikowia pulcherima* while *Hanseniaspora uvarum, Pichia anomala, R. glutinis* and *Saccharomyces cervisiae* were less frequent.

Nakase et al. (2006) reported the presence of *H. uvarum, Kluyveromyces marxianus, Pichia amethioina, Pichia chambardii, Pichia farinose, Pichia kluyveri, Pichia membranaefaciens, S. cerevisiae, Lachancea kluyveri* (Synonyms*: Saccharomyces kluyveri, Torulaspora kluyveri*) and *Zygosaccharomyces rouxii* in 17 cultivars of bananas from Java, Indonesia. While Gana et al. (2014) observed different yeast species such as *Brandoniozyma complexa, Candida wangnamkhiaoensis, Debaryomyces nepalensis, Hypopichia burtonii, Kodamaea ohmerii, P. anomala, Pseudozyma hubeiensis, Psuedozyma prolific* and *Pseudozyma pruni* on the surface of banana from Philippines. The presence of different yeasts was attributed to the geographical differences.

Xue et al. (2006) isolated 8 *Metschnikowia* strains under 3 different species, *M. sinensis, M. zizyphicola* and *M. shanxiensis* from the surface of jujube fruits (*Zizyphus jujube*) collected in China. Phylogenetically by 26S rDNA D1/D2 domain sequence analysis, it was suggested that these three novel species could be clustered in a clade together with *M. fructicola, M. andauensis, M. pulcherrima* and *M. chrysoperlae*.

Janisiewicz et al. (2014) reported that there was a significant change in the natural yeast flora on plum surface during development/ripening. The presence of *Rhodotorula, Sporidiobolus* and *Aureobasidium* was significantly higher than *Cryptococcus* throughout the fruit development. However, on the mature fruit *Hanseniaspora, Pichia, Zygosaccharomyces* and *Wickerhamomyces* species were observed. The natural yeast flora of the fruit, especially *A. pullulans* and *R. phylloplana* exhibited antagonistic activity against *Monilinia fructicola*, a fungus that causes brown rot.

On grapes, bacteria and unicellular and filamentous fungi with different physiological characteristics have been reported. 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. 2012b), whereas 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. 2012b; 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. *Plasmopara 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. 2012a). Besides, *Erysiphe* and *Fusarium* were also observed on grapes (Diguta et al. 2011). 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.

5 Natural Yeast Flora of Grapes

5.1 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. Targets for wine yeast genetic improvements are: better fermentation performance, efficient wine processing, control of wine-spoiling microorganisms, and quality improvement.

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. Whereas, 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. Whereas, *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 and 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. Barbosa et al. (2014) studied phenotypic and metabolic diversity of 20 commercial *Saccharomyces* strains used in different countries. According to their findings there was a relationship between nitrogen availability, yeast cell growth and sugar utilization during wine fermentation which can be additional criteria for strain selection. Brice et al. (2014) reported that the differences in nitrogen requirement between *S. cerevisiae* strains results from a complex allelic combination. They identified four genes namely *MDS3, GCN1, ARG81* and *BIO3* for which allelic variations were found to be associated with the differences in fermentation under nitrogen limiting conditions.

5.1.1 *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 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).

Two successive processes, namely, alcoholic fermentation of must by yeast and second, biological aging are involved in producing sherry wine. 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). Diaz et al. (2013) using quantitative real-time PCR reported that *S. cerevisiae* remained active at the end of the fermentation along with *M. pulcherrima*, *R. mucilaginosa*, *P. kluyveri*, *P. membranifaciens*.

5.2 *Non*-Saccharomyces *Yeasts*

Grape berry surface provides 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.

5.2.1 *Non*-Saccharomyces *Yeasts Associated with Fermenting Must*

The solid portion of must is called pomace. The grape must i.e. grape juice with skin, seeds and stems of fruits, has low pH, high sugar content. Availability of the oxygen, and/or ethanol concentration 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). The clarification of white must (centrifugation, enzyme treatments, cold settling) also reduces the initial population of yeasts (Fleet 1990; Lonvaud-Funel 1999; Pretorius 2000).

The non-*Saccharomyces* yeast population changes during cold maceration and alcoholic fermentation which can be attributed to the changes in micro-environment. For instance, Hierro et al. (2006) reported that *H. osmophila*, *C. tropicalis* and *Z. bisporus* species were predominantly found during cold maceration. Depending on

the availability of the oxygen, and/or ethanol concentration different species of yeasts become predominant in the fermenting must. Combina et al. (2005) studied non-*Saccharomyces* flora of fermenting must of Malbec variety of grapes. They reported the ubiquitous presence of *Kloeckera apiculata, C. stellata* and *Metschnikowia pulcherrima* in the spontaneous fermentation.

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 $\sim 10^3$–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 10^4 cells/mL during malo-lactic fermentations.

Nemcova et al. (2015) reported that the 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*. Assis et al. (2014) studied yeast flora of Chenin Blanc variety cultivated in the "Sao Francisco Valley" region of Brazil and observed that *Hanseniaspora opuntiae* and mixed cultures of *H. opuntiae* and *S. cerevisiae* influenced the wine quality.

Domizio et al. (2014) studied eight non-*Saccharomyces* strains, namely *Hansensiaspora osmophila*, *Lachancea thermotolerans*, *M. pulcherrima*, *Pichia fermentans*, *Saccharomycodes ludwigii*, *Starmerella bacillaris*, *Torulaspora delbrueckii* and *Zygosaccharomyces florentinus,* to check their potential to modulate the concentrations of various volatile compounds. Furthermore, these strains demonstrated a higher capacity to release polysaccharides such as mannoproteins compared to *S. cerevisiae*.

5.2.2 *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,

Table 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 et al. (2004)
Brazil	Bordeaux (red) Isabel (red)	*Hanseniaspora, Saccharomyces, Issatchenkia, Sporidiobolus*	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, Cryptococcus, Sporidiobolus*	
France	Merlot (red) Cabernet Sauvignon (red)	*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 and others*	
	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, Candida, Issatchenkia*	
	Nerod' Avola (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*	

(continued)

Table 1 (continued)

Country	Grape variety (red/white)	Associated yeast genera	References
	Shiraz (red)	*Debaryomyces, Hanseniaspora, Saccharomyces, Pichia*	
	Chenin Blanc (white)	*Hanseniaspora, Issatchenkia*	
	Sauvignon Blanc (white)	*Hanseniaspora, Pichia*	
Japan	Niagara (white)	*Kloeckera, Candida, Cryptococcus*	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, Candida, Pichia, Kloeckera, Zygosaccharomyces, Rhodotorula*	Jolly et al. (2003)
Southern Slovakia	Frankovka (red)	*Pichia, Candida, Metschnikowia, Hanseniaspora, Issatchenkia*	Brezna et al. (2010)
	Veltlin (white)		

Argentina and Portugal, while relatively low species diversity was observed in vineyards of Australia, Brazil, Canada, Greece and Japan (Table 1).

Longo et al. (1991) reported isolation of two hundred plus yeast strains from six wineries, all located in two wine regions of northwest Spain. The difference concerning yeast diversity between both regions was mainly due to their oxidative behavior. For instance, 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).

Brilli et al. (2014) assessed the long-term relationship (1997–2012) between quantitative and qualitative yeast diversity and the meteorological variables such as air temperature, relative humidity, and rainfall at one location. *Kloeckera apiculata* and *Candida zemplinina* represented almost the totality of non-*Saccharomyces* yeasts in grape and fresh musts and quantitatively well correlated with temperature 10 days before grape harvest.

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 and Nychas (2007) also studied yeast species diversity using restriction fragment length polymorphism and sequence analyses of the 5.8S internal transcribed spacer and the D1/D2 ribosomal DNA (rDNA) regions of yeasts during the fermentation with and without *Botrytis*-affected grape juice from two regions in Greece, Attica and Arcadia. *Botrytis* infection significantly affected species heterogeneity. 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.

Using PCR-RFLP and sequence analysis of ITS and rDNA regions, Li et al. (2010) evaluated the yeast diversity and its quantitative changes in three grape varieties cultivated in four different regions of China. Seventeen different yeast species belonging to eight genera were reported to be present 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.

To achieve unique regional qualities to the fermented wine, Sun et al. (2014) suggested the use of local strains. In this regard, the yeast flora of five grape varieties, namely Chardonnay, Cabernet Franc, Cabernet Sauvignon, Marselan, and Merlot were studied. The colony characteristics along with sequencing of the 26S rDNA D1/D2 domain were used to identify eight species of seven genera namely *A. pullulans, C. zemplinina, H. uvarum, H. occidentalis, I. terricola, M. pulcherrima, P. kluyveri*, and *S. cerevisiae*. The predominantly isolated species were *H. uvarum* and *S. cerevisiae*. They further reported the presence of six different genotypes of *S. cerevisiae* at different time points during the fermentation of Marselan variety. Earlier, Pallmann et al. (2001) used WL nutrient medium for qualitative and quantitative profiling of wine fermentation. Seventeen different colony

morphologies were correlated with six different genera such as *Candida*, *Hanseniaspora*, *Issatchenkia, Pichia, Metschnikowia* and *Saccharomyces.* Interestingly, distinct colony sub-types were identified within a single species *M. pulcherrima* which produced antimicrobial pigment, the pulcherrimin.

5.3 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, 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).

6 Profiling of Yeast Flora, Enzyme Activities and Flavor Compounds During Fermentation

6.1 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 rDNA genes was reported to be useful to analyze mixed yeast community during wine fermentation (Cocolin et al. 2000).

6.1.1 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.

The succession of non-*Saccharomyces* yeasts during natural fermentation of two varieties namely, Cabernet and Shiraz, was studied (Mane 2016). In natural fermentation of Cabernet variety *Pichia* and *Issatchenkia* were found in the initial phase (3 days) while in the fermentation of Shiraz variety both were present up to 6 days. In both the cases, *Hanseniapora* sp were observed up to 9th day of fermentation while *S. cerevisiae* up to 15th day (Mane 2016).

6.1.2 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, veraison 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).

6.2 *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 1993, 1998; 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.

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. 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). These enzyme activities improve the process of winemaking and enhance wine quality. Pectinases and β-glucanases increased juice extraction from grapes, improved wine clarification and facilitated wine filtration (Canal-Llauberes 1993; Villettaz and Duboudieu 1991). Haze formation from proteins in the finished wine may be decreased by the use of proteolytic enzymes (Waters et al. 2005). The aroma and flavor properties of wine could be enhanced by glycosidases that hydrolyse non-volatile glycosidic precursors of the grape (Pombo et al. 2011). The reduction in ethyl carbamate as well as alcohol levels was catalysed by urease and glucose oxidase, respectively (Van Rensburg

and Pretorius 2000). 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 capacity 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 2. 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.

Enzyme profiling was carried out during Shiraz and Cabernet variety fermentations. Pectinase, β-1,3-glucanase and protease activities increased from 3–6 d while β-glucosidase activity decreased after 9 d. These enzymes correlated significantly with secondary metabolites, such as total phenolics, flavonoids and tannins that are important to wine quality (Mane 2016).

From the literature, it was seen that enzyme activities were influenced by pH and temperature, presence of sugars, SO_2 and ethanol. For instance, ethanol adversely affected β-glucosidase and pectinase activities during fermentation (Maturano et al. 2012).

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). Mojsav 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. Alternately, expression of genes of polysaccharide degrading enzymes in *S. cerevisiae* was reported to be useful (Louw et al. 2006). Recombinant strains of *S. cerevisiae* were constructed using genes such as *T. reesei XYN2* xylanase, *Butyrivibrio fibrisolvens END1* glucanase, *A. niger XYN4* endo xylanase, *Erwinia chrysanthemi* pectate lyase *PEL5* and the polygalacturonase *PEH1* from *Erwinia carotovora*. The wine quality fermented with the recombinant strains was found to be comparable and acceptable (Louw et al. 2006).