Institute of Nutritional Science

Justus Liebig-University Giessen, Germany

and

Section of Microbiology and Biochemistry

Geisenheim Research Center, Germany

Impact of protease activity of yeasts on wine fermentation and formation of volatile and non-volatile metabolites

Thesis submitted in partial fulfilment of

the requirements for the degree of

Doctor oeconomiae trophologiaeque

(Dr. oec. troph.)

Submitted by
Ni-orn Chomsri
Lampang/Thailand
2008

This Ph.D. work was approved by the committee (Faculty 09: Agricultural and Nutritional Sciences, Home Economics and Environmental Management) of Justus Liebig-University Giessen, as a thesis to award the Doctor degree of oeconomiae trophologiaeque (Dr. oec. troph.)

1. Supervisor: Professor Dr. Hans Brueckner
2. Supervisor: Professor Dr. Doris Rauhut
3. Supervisor: Professor Dr. Sylvia Schnell

Date of disputation: 10.07.2008

Contents

List of Tables

List of Figures

Abbreviations and symbols

A_{600}	Absorbance at wavelength 600 nm
BSA	Bovine serum albumin
d	day
DMDC	Dimethyl dicarbonate
DMDS	Dimethyl disulphide
DMS	Dimethyl sulphide
EC	Enzyme Commission
FAN	Free alpha amino nitrogen
g	Gram
g	gravity
GABA	Gamma-aminobutyric acid
GC-MS	Gas chromatography-mass spectrometry
FTIR	Fourier transform infrared spectroscopy
HDM	Hefe-Differenzierungs-Medium
l	Litre
kDa	Kilodalton
mg	Milligram
min	Minute
ml	Millilitre
MWCO	Molecular weight cutoff
PI	Protein isolated
N	Nitrogen
NC-IUBMB	Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
NCR	Nitrogen catabolite repression
n.d	not detectable
nm	nanometer
n.q.	Not quantifiable
NTU	Nephelometric Turbidity Units
OD	Optical density
PR	Pathogenesis related
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SGJ	Synthetic grape juice
TA	Total acidity
TCA	Trichloroacetic acid
TSS	Total soluble solid

v/v	volume by volume
WON	without nitrogen
WN	with nitrogen
YNB	Yeast nitrogen base

1. INTRODUCTION

1.1 Statement and significance of the study

The ability of yeasts to release proteases has been observed by many researchers because of their potential to degrade haze proteins in wine and to generate assimilabe nutrient sources for microorganisms (Sturley & Young, 1988; Ogrydziak, 1993; Dizy & Bisson, 2000; Delfini & Vormica, 2001). These proteases can influence the nitrogen containing compounds in must and wine (Alexandre *et al.* 2001; Martinez-Rodríguez et al., 2001 b). It is well established that non-*Saccharomyces* yeasts possess higher extracellular protease activity than *S. cerevisiae*. In these surveys, protease activity of *Saccharomyces* and *non-Saccharomyces* yeasts was detected in a wide range. For example, Lagace and Bisson (1990) evaluated a set of non-*Saccharomyces* yeasts and found that the greatest proteolytic activity was secreted by *Kloeckera apiculata* strains, whereas week activity was claimed for this species by Charoenchai et al. (1997). As reviewed by Ogrydziak (1993), the differences can be probably explained by varying growth factors and assay conditions.

Surprisingly, the experiments on yeast proteases were mostly carried out in synthetic media, and substrates, e.g. haemoglobin, casein which are not found in natural grape juice were used for protease activity assay. As the result of that, research on catalytic activity of proteinases of non-*Saccharomyces* yeasts in enological circumstances is still lacking. On the contrary, several enological trials were carried out to study the potential of proteolytic activity of *S. cerevisiae* yeasts (Desportes, et al., 2001; Eriksson & Fenyo, 2005; Feuillat, 2005). These studies demonstrate that during yeast autolysis protease A, an intracellular enzyme, plays an important role in the degradation of polypeptides and proteins to amino acids and peptide residues. The authors found that peptides were released during yeast autolysis. These peptides are recognized as enologically important components due to the role as nutrients for lactic acid bacteria in malolactic fermentation. They could also interact with phenolic compounds, which can influence the fining procedure, flavour and stability (Alexandre et al., 2001).

The production of wine using mixed yeast culture fermentation has been extensively studied during the last two decades. There is an increase of evidence that metabolites of non-*Saccharomyces* yeasts contribute positively to the quality of complex wines. On the other hand, it should be noted that in vinification metabolites of non-*Saccharomyces* yeasts might be detrimental. Thus, the use of mixed yeast cultures in fermentation should be prudent. Using multicultures in winemaking consisting either of the *Saccharomyces* species (Grossmann et al., 1996; Eglinton et al., 2000; Hayasaka et al., 2007) or of combination with *Saccharomyces* and non-*Saccharomyces* strains (Jeune et al., 2006; Brunner, 2006) contributes to the complexity of aroma when compared to the use of a single strain.

Therefore, aroma compounds produced by non-*Saccharomyces* yeasts have been widely studied for some strains. No extensive studies have been conducted about an impact of nitrogen-containing compounds on their growth in grape juice.

In general treatments are carried out to lower the protein content in grape juice and wine, because they can cause haze and precipitate in wine. When this occurs during processing, additional treatments are required and that results in delay. If it occurs after bottling, consumers will reject wine and that results in economic loss. This frequently occurs in white wines. It is rarely found in red wines because they have high quantities of tannins which can react with unstable proteins to form insoluble tanno-protein compounds (Colagrande et al., 1994; Charpentier, 2004; Cosme et al., 2008). This reaction leads to a drastic reduction in the protein levels. Many studies attempted to use proteases to limit these proteins related to haze forming in wine (Water et al., 1995a, 1995 b; Pocock et al., 2003; Water et al., 2005). In addition these authors have reported about grape proteins which were identified as pathogenesis related (PR) proteins. They concluded that PR proteins were produced in grape berries when infected by fungi. It has been thought that yeast proteases could not degrade the PR proteins because of their inherent resistance (Pocock et al., 2000; Rensburg and Pretorius, 2000; Pocock, et al, 2003). On the other hand, Rensburg and Pretorius (2000) indicated that incubation of a protease concentrate from *K. apiculata* with Chenin blanc and Chardonnay wines showed some degradation of the wine proteins.

Yeast protease may liberate amino acids and peptides from grape protein during fermentation which can benefit growth of microorganisms during or after alcoholic fermentation. Another aspect is that yeast cells may release nitrogen containing metabolites to the media. The composition of amino acids peptides and proteins in wine is based on grape related compounds transferred and transformed during the winemaking process and breakdown products through the protease activity from yeasts and compounds released by yeasts. Fornairon-Bonnefond et al., (2002) and Oganesjanz et al. (2007) pointed out that the composition of amino acids, peptides, and proteins can have an influence on flavour and mouthfeel of wine. However, knowledge of these molecules related to non-*Saccharomyces* yeasts which are normally prevalent at early stage of wine fermentation is lacking. Since certain peptides are responsible for tastes, they may affect certain characters of wine quality like wine taste (bitter, sweet and umami) and mouth feel (wine body or fullness). In some studies, wine from spontaneous fermentation or inoculation of non-*Saccharomyces* yeasts in combination with *Saccharomyces* yeasts were preferred, this could possibly linked to such an effect. The use of non-*Saccharomyces* yeasts for wine fermentation could be a tool to meet demands of consumers better. In this regard, inoculation with non *Saccharomyces* yeasts may become the future strategy to improve fullness of wine, particularly white wine. However, first, scientists must carry out large, controlled studies in which appropriate mixed

yeast cultures can produce desirable nitrogen-containing metabolites in wine. This will be a challenging task because many factors have to be considered and controlled. More specific information and better identification of nitrogen containing compounds is required to understand their influence.

1.2 Objectives

This study is based on the hypothesis that protease activity of non-*Saccharomyces* can influence the composition of amino acids, peptides and proteins in wine. This work should contribute to the basic understanding of the role of non-*Saccharomyces* in winemaking. To examine the hypothesis the following objectives have been defined:

- screening of *Saccharomyces* and non-*Saccharomyces* yeasts isolated from grapes and wines for their proteolytic activity
- evaluation of the behavior of extracellular proteases produced by wine yeast species in defined and enological conditions
- assessment of certain volatile and non volatile metabolites produced by yeasts exhibiting proteolytic activity during fermentation
- study of the influence of selected yeast strains in mixed cultures during winemaking on differences in the composition of amino acids, polypeptides and proteins.

2. LITERATURE REVIEW

2.1 Yeasts

Yeasts are single cell microorganisms classified in the kingdom Fungi. Regarding the morphology, yeasts can be differentiated from bacteria by their larger cell size and their oval, elongate, elliptical, or spherical cell shapes. Typical yeast cells range from 5 to 8 μm in diameter, with some being even larger (Barnett et al., 2000; Jay et al., 2005). Yeasts can grow over wide ranges of acid pH and with specific treatment they can produce alcohol up to 21% and grow in the presence of 55-60% sucrose (Thomas & Ingledew, 1992 a). Yeast colonies show colours from creamy, to pink, to red are produced by yeasts (Kurtzman, and Fell, 2006). Approximately 1,500 (Kurtzman, 1998) and 678 species (Barnett et al., 2000) of yeasts have been described, most of which reproduce by budding, although in a few cases by binary fission. Of these, *S. cerevisiae* is one of the main sources for commercial production of enzymes with application in food industry, as well as for different kinds of biochemical analyses (Ganeva et al., 2002).

In addition to baking and traditional alcoholic fermentations, yeasts have been used in a broad applications: (i) fermenting lactose to ethanol, to produce lactose-free milk for sufferers from lactose intolerance; (ii) producing protein from alkanes and paper-pulp waste; (iii) producing various alditols, such as glycerol or D-glucitol; (iv) providing enzymes, such as □-fructofuranosidase (invertase), α-and β-galactosidase and lipase (v) biocontrol agents because of their antifungal activity (Barnett et al., 2000; Kurtzman & Droby, 2001; Fleet 2007). Cell biomass (food and fodder yeasts) (Boze, et al., 1992; Demain et al., 1998; Buzzini & Vaughan-Martini, 2006), yeast probiotics (Fleet, 2006; Edwards-Ingram et al., 2007), production of ingredients and additives for food processing (Fleet, 2006) are other aspects for the utilization of yeasts. The presence and metabolism of yeasts can also have detrimental effects, e.g. food spoilage and yeast-related health (Fleet, 1992; Caruso et al., 2002; Loureiro and Malfeito-Ferreira, 2003).

Current research about yeasts has extremely increased understanding of ecology and biology of yeasts and provided information which can assist in developing of yeast applications (Barnett, 2007). Yeasts have an enormous impact on wine production. They are responsible for the conversion of fermentable sugars into alcohol and other by products. It is now recognized that wine fermentation involves the development and activity of a number of different yeast species that originate from both the grape and the winery environment (Heard, 1999). Grape must is relatively complete in nutrient content, but having a low pH and a high sugar content, it imposes strong selective pressure on microbial species such that only several species of yeasts and other microorganisms can proliferate (Henschke, 1997).

Therefore, extreme diversity of yeast population and species can occur in spontaneous fermentation. For example 2 *Saccharomyces* and 10 non-*Saccharomyces* strains were found during fermentation of four spontaneous fermentations (Sturm et al., 2006). The authors reported that non-*Saccharomyces* species was presented 30% of the total isolates. It was obviously demonstrated that grape processing influenced variability of species present during fermentation.

The early stages of the spontaneous fermentation are characterized by the growth of *Kloeckera apiculata, Hanseniaspora uvarum, Hansenula anomala, Candida stellata, Candida pulcherrima* and several other species (Heard & Fleet, 1986). Sensitivity to ethanol then limits the growth of these yeasts to the first 1-2 days of fermentation (Fleet & Heard, 1993). Consequently, strains of *S. cerevisiae* eventually dominate in fermentation as the concentration of ethanol increases (Heard & Fleet, 1985). A similar growth pattern is also suggested by many other studies (Heard & Fleet, 1985; Jemec et al., 2001; Fleet & Heard, 2002; Ruek, 2005; Maro et al., 2007; Stoebeln, 2007). This growth development in the spontaneous fermentation may be illustrated for a representative structure as suggested by Heard & Fleet (1986) and Dittrich & Grossmann (2005) (**Figure 2-1**). Additional selection may be exerted by sulphur dioxide, which is widely used for its antioxidant and antimicrobial properties, on the growth of undesirable oxidative yeasts (Henick-Kling et al., 1994; Renouf et al., 2006; Roussis et al., 2007). Furthermore, as the must begins to undergo fermentation, anaerobic conditions are established, certain nutrients become depleted, and the increasing ethanol concentration imposes additional selective pressure on microbial species (Henschke, 1997; Swiegers et al., 2005).

The role of non-*Saccharomyces* yeasts is currently the subject of considerable interest in relation to wine flavour complexity (Soden et al., 1999; Fleet, 2003). Regarding the positive potential, the use of mixed inoculants in wine fermenation has been extensively studied. On the other hand, it must take into account that some species can produce metabolites of desirable quality as well as objectionable concentrations of metabolites leading to unwanted characters. Therefore, *S. cerevisae species* is preferred to inoculate into must due to a controlled fermentation is favoured in commercial winemaking. Scientific and technological understanding of the role of *Saccharomyces* and non-*Saccharomyces* yeasts in winemaking has been available from a large amount of research studies. However, further studies related to the practical use of mix cultures are required to obtain a more fundamental knowledge of improvement and development of wine quality.

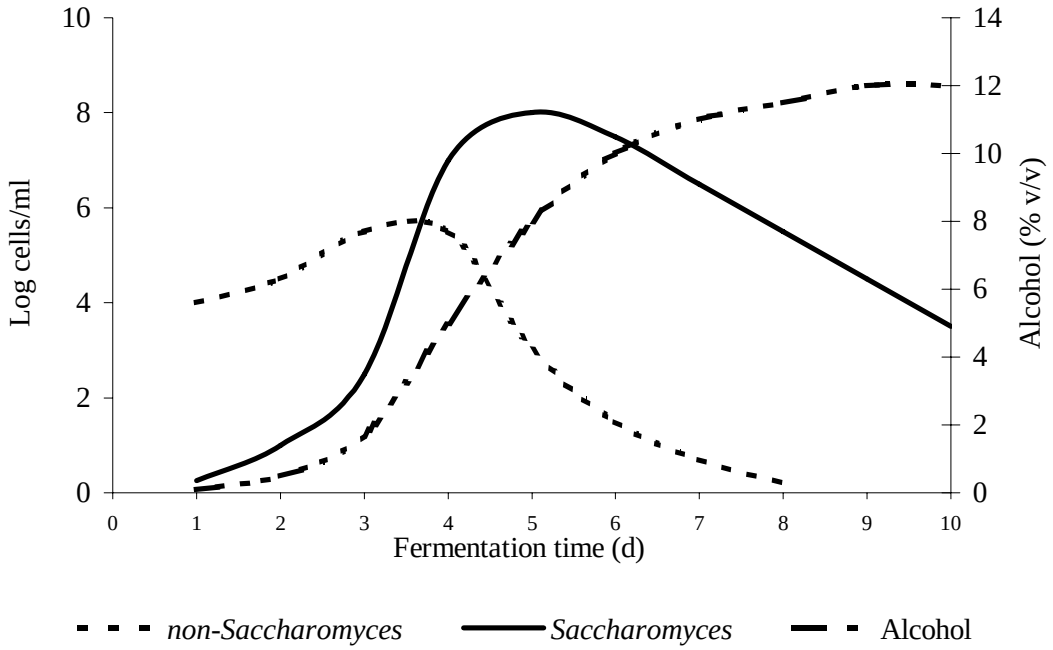


Figure 2-1 Generalized growth of yeasts during spontaneous fermentation of wine (modified from Henick-Kling, 1994; Dittrich and Grossmann, 2005)

Nomenclature and importance of enological yeasts

The classification and taxonomies of 678 yeasts have currently been recognized and described by Barnett et al. (2000). Those versatile yeasts, a genus of *Saccharomyces* and 19 genera of non-*Saccharomyces* are associated with winemaking (Fleet, 1999; Dittrich & Grossmann, 2005; Jolly et al., 2006; Roeder, 2007). Yeasts that are present in uninoculated grape juice are named by different terms such as natural, native, wild, wine or indigenous yeasts (Soden et al., 1999). *S. cerevisiae* is prevalent on the surface of winery equipment, whereas the indigenous wine yeasts on grapes are considered to be non-*Saccharomyces* species (Fleet & Heard, 2002; von Wallbrunn, 2007). Therefore, in wine production, yeast species may be divided into two broad groups, i.e. *Saccharomyces* and non-*Saccharomyces* groups (Jolly et al., 2006; von Wallbrunn, 2007).

Improving and enhancing wine sensorial quality through the combination of *Saccharomyces* and non-*Saccharomyces* is of increasing interest for winemakers. Hence, understanding the role of these yeasts and their interaction in the fermentation process should bring great benefit to winemaking.

Saccharomyces yeasts

Saccharomyces yeasts have a unicellular, globose, and ellipsoid to elongate shape. Multilateral (multipolar) budding is typical for vegetative reproduction (Vaughan-Matini and Martini, 1998). A number of 16 species is characterized under the genus *Saccharomyces* as excellently proposed by Barnett et al. (2000). Of these, *S. cerevisiae* is one of the main sources for commercial production of enzymes with application in food industry, as well as for different kinds of biochemical analyses (Ganeva et al., 2002). This yeast is also the most well studied and widely provided in the market in association with wine production. Nomenclature of *Saccharomyces* yeasts is that according to Barnett et al.(2000) with the teleomorphic (perfect) names and 2 anamorphic (imperfect) names is given in **Table 2-1**.

Non-*Saccharomyces* yeasts

The presence of non-*Saccharomyces* species becomes more important in winemaking than in the former time, although *S. cerevisiae* is principally responsible for the alcoholic fermentation. At different stages of the spontaneous fermentation different phenotypes of the non-*Saccharomyces* yeasts are represented (Romano et al., 1997). They produce the metabolites which can contribute to the final taste and flavour of wines (Esteve-Zarzoso et al., 1998; Rainieri & Pretorius, 2000). Therefore, in recent years wine researchers have realized that non-*Saccharomyces* yeasts can improve quality of wine more than previously thought (Sommer et al., 2007). Numerous enological researches associated with non-*Saccharomyces* yeasts were conducted to study about their production of metabolites. A dominant characteristic of non-*Saccharomyces* yeasts is to produce great amount of components like esters, higher alcohols, acetic acid, acetoin, thus volatile metabolites of these yeasts have been mainly focused. These components can make a contribution to the desirable fermentation bouquet of wine and on the other hand, they can also be considered detrimental to the wine quality (Heard & Fleet, 1986; Ciani & Maccarelli, 1998; Rojas et al., 2003; Paraggio, 2005).

Table 2-1 List of the *Saccharomyces* yeasts, with their teleomorphic and anamorphic names.

Teleomorphic name (sexual state)	Anamorphic name (asexual state)
Saccharomyces barnettii	
Saccharomyces bayanus	
Saccharomyces castellii	
Saccharomyces cerevisiae	*Candida robusta*
Saccharomyces dairenensis	
Saccharomyces exiguus	*Candida holmii*
Saccharomyces kluyveri	
Saccharomyces kunashirensis	
Saccharomyces martiniae	
Saccharomyces paradoxus	
Saccharomyces pastorianus	
Saccharomyces rosinii	
Saccharomyces servazzii	
Saccharomyces spencerorum	
Saccharomyces transvaalensis	
Saccharomyces unisporus	

As a matter of fact, 19 genera of non-*Saccharomyces* yeasts are relevant to vinification (Swiegers et al., 2005; Dittrich & Grossmann, 2005; Jolly et al., 2006). The teleomorphic and anamorphic names are listed in **Table 2-2** as described in system by Barnett et al. (2000); Kurtzman & Fell (2000). Regarding the fermentative ability and aerobic obligation, non-*Saccharomyces* found in grape must and during fermentation may be divided into three groups: (Jolly et al., 2006): (i) aerobic yeasts, e.g. *Pichia* spp., *Debaromyces* sp., *Rhodotorula* spp., *Candida* spp., and *Cryptococus albidus* ; (ii) apiculate yeasts with low fermentative acitivity, e.g. *H. uravrum*, *Kloeckera apis*, *K. javanica* ; and (iii) fermentative yeast, e.g. *K. marxianus*, *Torulaspora* spp., and *Zygosaccharomyces* spp.

Table 2-2 List of the Non-*Saccharomyces* yeasts, with their teleomorphic and anamorphic names

Teleomorphic name (sexual state)	**Anamorphic name (asexual state)**
Citeromyces	
Citeromyces matritensis	*Candida globosa*
Debaryomyces	
Debaryomyces nepalensis	*Candida naganishii*
Debaryomyces hansenii	*Candida famata*
Dekkera	
Dekkera anomala	*Brettanomyces anomalus*
Dekkera bruxellensis	*Brettanomyces bruxellensis*
Hanseniaspora	*Kloeckera*
Hanseniaspora uvarum	*Kloeckera apiculata*
Hanseniaspora guilliermondii	*Kloeckera apis*
Hanseniaspora occidentails	*Kloeckera javanica*
Hanseniaspora osmophila	*Kloeckera corticis*
Issatchenkia	
Issatchenkia occidentalis	*Candida sorbosa*
Issatchenkia orientalis	*Candida krusei*
Kluyveromyces	
Kluyveromyces thermotolernas	*Candida dattila*
Lodderomyces	-
Metschnikowia	
Metshnikowia pulcherrima	*Candida pulcherrima*
Pichia	
Pichia anomala	*Candida pelliculosa*
Saccharomycodes	-
Shizosaccharomyces	-
Torulaspora	
Torulaspora delbrueckii	*Candida colliculosa*
Zygoascus helleicus	*Candida hellenica*
Zygosaccharomyces	-
-	*Cryptococcus*
-	*Rhodotorula*

Synopsis of common genera of non-*Saccharomyces* yeasts in vinification

Brettanomyces (Teleomorphic name is *Dekkera*). These asporogenous yeasts form ogival cells and terminal budding, and produce acetic acid from glucose only under aerobic conditions (Dittrich & Grossmann, 2005). This genus may be found during barrel aging and in wine after bottling (Silva et al., 2004) They cause spoilage of wine (Loureiro & Malfeito-Ferreira, 2003; Roeder et al., 2007), particularly *D. bruxellensis* contributes to the formation of biogenic amines in red wines (Caruso et al., 2002). Growth of these yeasts results negative characteristics of “animal”, “farmyard”, or “mousy” taints. In winemaking, “Brett” character from this genus is identified as mousy and medicinal-like. Compounds like tetrahydropyridines and 4-ethy phenol are mainly involved in the above mentioned off-flavours (Grbin et al., 1995; Grbin & Henschke, 2000; Gafner, 2003 b; Barata et al., 2008)

Candida. The Genus *Candida* represents the biggest number of species in the yeast characteristic of Barnett et al. (2000). Members of this genus are prevalent on grape surface in general as well as in juice. Many species of this genus are also involved in wine fermentation, particularly the initial stages of fermentation (Heard & Fleet, 1986). The ascomycetous imperfect species involved in vinification are grouped in this genus, including the former genus *Torulopsis*. For *C. stellata* (*Torulopsis stellata*), a 12% alcohol tolerance (Combina et al., 2005) and up to 14 g/l of glycerol production (Ciani & Picciotti, 1995; Ciani and Ferraro, 1998) has been reported. Additionally, this species is recognized as a fructophilic yeast (Jolly et al., 2007). It depleted fructose in fermenting Chardonnay must after 15 days but not glucose (Soden et al., 2000). *C. pulcherrima* is a high producer of esters (Clemente-Jimenez et al., 2004). In addition, *C. pulcherrima* showed an antagonistic effect on other yeasts including *S. cerevesiae* (Panon, 1997; Nguyen & Panon, 1998), while this was not observed in other fermentation studies (Jolly et al., 2003 a ,2003 b). On the other hand, *Metschnikowia pulcherrima* the asexual state of *C. pulcerrima* can be effective in protecting grapes against post harvest rot caused by *Botrytis cinerea* and other postharvest pathogens (Sipiczki, 2006).

Cryptococcus. This genus represents the anamorph of *Filobasidiella* and other *Basidiomycetes* (Jay et al., 2005). Yeasts of this genus could be found on grapes (Longo et al., 1991; Yanagida et al., 1992; Rementeria et al., 2003). *Cryptococcus albidus* is known as wine spoilage yeast (Enrique et al., 2007). Dittrich and Grossmann, 2005 noted that *C. albidus* can be found in slimy wine, although the fermentative ability is lacking (Fell & Statzell-Tallman, 1998).

Debaryomyces. These ascosporogenous yeasts sometimes produce a pseudomycelium and reproduce by multilateral budding. They are found on grapes (Jolly et al., 2006).

Hanseniaspora. Members of this genus are called apiculate yeasts whose anamorphs are *Kloeckera* spp. They exhibit bipolar budding, and consequently lemonshaped cells are produced. They can be normally found on grape berries. At the start of fermentation an initial proliferation of this genus normally occurs (Rainieri & Pretorius, 2000; Jolly et al., 2006). The genus is known for a high production of acetic acid (Ciani & Picciotti, 1995). *K. apiculata* and *H. uvarum* are often found in high numbers in grape juice (Nisiotou and Nychas, 2007).

Issatchenkia. Members of this genus produce pseudomycelia and multiply by multilateral budding. *I. orientalis* and *I. terricola (*former *Pichia terricola* are reported to be present in grape juice and wine (Clemente-Jimenez et al., 2004, Fugelsang & Edwards, 2007).

Kluyveromyces. These ascospore-forming yeasts reproduce by multilateral budding, and the spores are spherical. *K. thermotolerans* is one of the non-*Saccharomyces* yeasts widely studied in nological research. This species survived in the condition of low oxygen (Hansen et al., 2001). Furthermore, a study conducted by Kapsopoulou et al. (2005, 2006) revealed that *K. thermotolerans* produced a great amount of lactic acid in grape juice fermentation. More recently, a commercial product containing this yeast has been available for wine fermentation (Brunner, 2006; www.chr-hansen.com, 2008).

***Lodderomyces*.** This yeast reproduces asexually by multilateral budding on a narrow base (Kurtzman, 1998). This yeast can be isolated from wine (Schuller et al., 2000). During bottling process, wine could be contaminated with *L. elongisporus* (Malfeito-Ferreira et al., 1997).

***Pichia*.** This is the largest genus of true yeasts (Jay et al., 2005). They reproduce by multilateral budding, and the asci usually contain four spheroidal, hat- or Saturn-shaped spores. *Pichia* spp. typically form films on liquid media and are known to grow during fermentation or in wine improperly handled particularly on the surface of wine exposed to oxygen (Boulton & Quain, 2006). In addition they can produce off-odours during their growth, e.g. *P. anomala* is able to produce high amount of esters (Clemente-Jimenez et al., 2004). The killer protein of *P. membranifaciens* showed an effect against grey mold disease of grapevine and other yeasts is another potential for biocontrol is (Santo & Marquina, 2004).

Rhodotorula. These yeasts reproduce by multilateral budding and are non-fermenters (Dittrich & Grossmann, 2005). They produce pink to red pigments, and most are orange or salmon pink in color. This genus is seldom found during the fermentation of grape juice (Sturm et al., 2006). On the other hand, the ability to produce slime in grape juice and wine with low alcohol content can cause wine spoilage (Dittrich & Grossmann, 2005).

Saccharomycodes. *S. ludwigii* is the only species in this genus (Miller & Phaff, 1998; Barnett, 2000). *S. ludwigii* was reported to produce ethyl acetate and acetaldehyde in plenty amount which is detrimental to wine quality (Ciani & Maccarelli, 1998). This species could be isolated from natural wine fermentation (Heard & Fleet, 1986).

Schizosaccharomyces. These ascosporogenous yeasts divide by lateral fission of cross-wall formation. *S. pombe* is the most prevalent species; it is osmophilic and resistant to some chemical preservatives (Dittrich & Grossmann, 2005).

Torulaspora. Multilateral budding is its method of asexual reproduction with spherical spores in asci (Kurtzman, 1998 b). Strains of *T. delbrueckii* show considerable ability to ferment sugar (Ciani & Maccarelli, 1998). *T. delbrueckii* is the most prevalent species. *T. delbrueckii* strains, having good osmotolerance and low volatile acid production, are used for sweet wines of the Sauternes and Auslese styles (Henschke, 1997).

Zygosaccharomyces. Multilateral budding is the method of reproduction for yeasts of this genus, and the bean-shaped ascospores formed are generally free in asci. They are strong fermenters of sugars. *Z. bailli* is known as a fructophilic yeast (Jolly, 2007) and can be present in wine after bottling (Jolly et al., 2006).

2.2 Proteolytic enzymes of yeasts

Proteases hydrolyze proteins into smaller fragments, i.e., peptides or amino acids (Aehle, 2004). They are of widespread interest to the scientific community because they can be used as tools, and they play critical roles in biological systems. Proteases are very important in digestion as they break down the peptide bonds in the protein foods to liberate the amino acids needed by the organisms. Proteases or proteolytic enzymes differ in their ability to hydrolyze various peptide bonds. Each type of protease is specific to break a certain peptide bond. Proteases are involved in a multitude of important physiological processes ranging

from the functional activation of proteins by single proteolytic events, to the complete dissolution of protein to their constituent amino acids (Barrett et al., 2004).

Proteases in enology have been studied in various dimensions. The objectives of the studies were proteolytic activity screening (Bakalinsky & Boulton, 1985; Dizy & Bisson, 2000; Charoenchai et al, 1997; Sturley & Young, 1988; Iranzo, 1998; Rosi & Costamgna, 1987; Fernández et al., 2000), investigation on haze reduction (Lagace & Bisson 1990), autolysis (Moreno-Arribas et al, 1996; Martinez-Rodriguez & Polo, 2000; Rensburg & Pretorius, 2000; Alexandre & Guilloux-Benatier, 2001; Alexandre et al., 2001; Guilloux-Benatier & Chassagne, 2003; Perrot et al, 2002), and the ability to utilize protein in grape must (Conterno & Delfini, 1994, 1996). The scope of this study, however, is to explore the extracellular proteases of enological yeasts. Hence, the yeast exhibiting proteolytic activity related to winemaking will be summarized in this review.

Protease: definition, classification and mechanism

Enzymes, which hydrolyze peptide bonds, are commonly termed proteases, proteinases and peptidases, as well as proteolytic enzymes. Historically, these terms had slightly different meanings. Although the terms “Protease” and “proteinase” are often used interchangeably, some scientists (McDonald, 1985; Bilinski & Stewart, 1990; Koolman & Roehm, 2005) emphasized that the latter term specifies endopeptidases but not exopeptidases, whereas Nomenclature Committee of the International Union of Biochemistry Molecular and Biology (NC-IUBMB) recommends the term peptidase as the general term for all the enzymes that hydrolyze peptide bonds. Nevertheless, proteolytic enzymes are perhaps the most generally understood term in the current usage. The EC list recommends the term peptidase as the general term for all the enzymes that hydrolyze peptide bonds (subclass E.C.3.4). Most peptidases are either exopeptidases cleaving one or a few amino acids from the N- or C-terminus, or endopeptidases that act internally in polypeptide chain. The EC list also provides terms for subtype of exopeptidases and endopeptidases. The exopeptidases that act at a free N-terminus liberate a single amino acid residue (aminopeptidases) or a dipeptide or a tripeptide (dipeptidyl-peptidases and tripeptidyl-peptidases). Those acting at a free C-terminus liberate a single residue (carboxypeptidases) or a dipeptide (peptidyl-dipeptidases). Other exopeptidases are specific for dipeptides (dipeptidases) or remove terminal residues that are substituted, cyclized or linked by isopeptide bonds (peptide linkages other than those of α-carboxyl to α-amino groups) (omega peptidases). The endopeptidases are divided on the basis of catalytic mechanism into serine endopeptidases, cysteine endopeptidases, aspartic endopeptidases and metalloendopeptidases. The term

oligopeptidase is used to refer to endopeptidases that act optimally on substrates smaller than proteins.

Proteases can be classified in different ways, e.g. according to molecular size, charge or substrate specificity (Beynon & Bond, 1990). However, a more rational system is now based on a comparison of active sites, mechanism of action, and three – dimensional structure. Four mechanistic classes are recognized by the International Union of Biochemistry, and within these classes, six families of proteases are recognized to date. Each family has a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site (**Table 2-3**). The serine proteases include two distinct families: the mamalian serine proteases, for example chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), elastase (EC 3.4.21.11) and the bacterial serine proteases, for example subtilisin (EC 3.4.21.14). Analogously, the metallo – proteases include two families: the mammalian pancreatic carboxypeptidase (EC 3.4.17.1) which differ from bacterial thermolysin (EC 3.4.24.4) in chemical structure even though both are zinc mettallo enzymes and have similar active site configurations. The cystein proteases include several mammalian lysosomal cathepsins, the cytosolic calcium activated proteases (calpains) and the plant proteases papain and actinidin. The aspartic proteases include bacterial penicillopepsin (EC 3.4.23.6), which serves as the model, mammalian pepsin (EC 3.4.23.1), renin (EC 3.4.99.19), chymosin (EC 3.4.23.4) and certain fungal proteases. This classification by catalytic types has been suggested to be extended by a classification by families based on the evolutionary relationships of proteases.

In addition to these four mechanistic classes, there is a Section of the enzyme nomenclature which is allocated for proteases of unidentified catalytic mechanism. This indicates that the catalytic mechanism has not been identified but the possibility remains that novel types of proteases do exist. The EC List was last printed in full as Enzyme Nomenclature 1992 (NC – IUBMB, 2007), but the part dealing with peptidases has subsequently been amended by regular supplements and can be found in its revised form on the website (http://www.chem.qmul.ac.uk/iubmb/enzyme).

Table 2-3 Families of proteolytic enzymes

Family	Representative protease(s)	Characteristic active site residues
Serine protease I	Chymotrypsin Trypsin Elastase Pancreatic kallikrein	Asp^{102}, Ser^{195}, His^{57}
Serine protease II	Subtilisin	Asp^{32}, Ser^{221}, His^{64}
Cysteine proteases	Papain Actinidin Rat liver cathepsins B and H	Cys^{25}, His^{159}, Asp^{158}
Aspartic proteases	Penicillopepsin *Rhizopus chineses* and *Enkothia* *parasitica*, acid proteases Rennin	Asp^{11}, Asp^{213}
Metallo – proteases I	Bovine carboxypeptidase A	Zn,Glu^{270}, Try^{248}
Metallo – proteases II	Thermolysin	Zn, Glu^{143}, His^{231}

Source : Beynon and Bond (1990)

Role of proteases in vinification

Fungi are able to use nutrients by absorption of compounds from their environment. Most fungi abundantly secrete hydrolases that serve to degrade extracellular macromolecules to low molecular weight substrates. These hydrolysis products are then readily transported into the cell. Yeasts are unicellular fungi that also possess the ability to secrete extracellular enzymes. A number of different proteolytic enzymes are produced by yeasts (Klar & Halvorson, 1975; Barrett et al., 2004).

Members of the genus *Saccharomyces* do not normally secrete external hydrolases, although mutants releasing vacuolar hydrolases to the external environment have been isolated (Sturley & Youngm, 1988; Dizy & Bisson, 2000). The proteolytic system of the yeast *S. cerevisiae* is quite complex, consisting of carboxy peptidases, aminopeptidases and proteinases and of several specific inhibitors (Béhalová & Beran, 1979). However, protease A of *S. cerevisiae* (yeast) was detectable at low level in the extracellular fractions (Rothman & Stevens, 1986).

The ability of yeast species to produce extracellular proteases is not generally included in taxonomy. Furthermore, the predominance of *S. cerevisiae* in such researches, which secretes little or no extracellular protease activity, may be the reasons of the earlier assumption that yeasts secrete no proteases (Ogrydziak, 1993). Although various evaluation methods for proteolytic activity of yeasts have been proposed, plate assay using media supplemented with protein sources as enzyme substrates is a standard procedure for detecting protease production of yeasts. Surveys showed that the percentage of strains that are protease-positive varied greatly. For example, Lagace and Bisson (1990) evaluated a set of non-*Saccharomyces* yeasts and found that the greatest proteolytic activity was secreted by *K. apiculata* strains, whereas week activity was claimed by Charoenchai et al. (1997). This suggests strains influence the secretion of the enzymes. Some of the differences can probably be explained by differences in substrates, temperature and pH (Béhalová & Beran, 1979). A research carried out by Ganga and Martínez (2004) demnostrated that *Metschnikowia pulcherima and Candida* spp. showed high activity in media supplemented with casein under neutral and basic pH condition, while there was no activity at acid pH.

In most screening of *S. cerevisiae* for protease activity, negative results were found. In addition, yeasts may possibly not hydrolyze the substrate used in the first screening because proteases are produced at low levels. Thus the protease activity might not be detected by that substrate. Numerous yeasts were found to have caseinolytic acitivity, and the caseinolytic activity did not necessarily correlate with gelatine liquefaction (Strauss et al., 2001). Hence it was inferred that there was no convincing evidence for secretion of proteases by *S. cerevisiae*, or loosely related *Saccharomyces* strains, of proteases with broad enough specificity to be detected by assays based on hydrolysis of casein, BSA or haemoglobin. Further work is required to avoid the misleading conclusion that yeasts secrete actively proteases but in fact that release is due to cell lysis.

Studies demonstrated the lack of extracellular acid protease production among various species of *Saccharomyces* (Nelson & Young, 1986; Binlinski et al., 1987). However, the studies of Rosi and Costamagna (1987), Bilinski and Stewart (1990), Conterno and Delfini (1994, 1996), Moreno-Arribas et al. (1996), and Iranzo et al. (1998) revealed the existence of exocellular proteases in *Saccharomyces*. In contrast, the yeast *S. cerevsiiae* contains a large

number of intracellular proteases that are located in various compartments (cytosol, vacuole, mitochondria, endoplasmic reticulum, and Golgi complex) and membranes of the cell (Klar & Halvorson, 1975; Jones, 1991 a, 1991 b; Fukui et al., 1996; Barrett, 2004). *Saccharomyces* yeasts possess cytoplasmic proteases that serve to degrade cellular macromolecules (Ogrydziak, 1993). These proteases are confined to the vacuole. After cell lysis and death, these proteases can be released to the surrounding medium where they may retain activity.

Of the many cellular proteases, the vacuolar acid protease, endoproteinase A was studied widely since it has been considered to play an important role in enology (Moreno-Arribas et al., 1996; Alexandre et al., 2001). Protease A is classified to be an aspartic protease; endoproteinase and pepstatin is its inhibitor (Beynon & Bond, 1990). This vacuolar acid protease appears to be very active in degradation of grape proteins once released from the cells and its activity is detected for long periods of time during aging on the yeast lees (Carnevillier et al., 2000; Perrot et al., 2002). The study conducted by Alexandre et al. (2001) indicated that no extracellular protease A activity of *S. cerevisiae* was detected during the alcoholic fermentation, whereas a small but reproducible activity was measured in the autolysate from day 54. This is in agreement with the investigation of Moreno-Arribas et al. (1996). Nevertheless, the authors also raised the question whether the protease activity could diffuse outside the cell. Although during autolysis yeast cell wall becomes thinner, it remains unbroken and this could still act as an efficient barrier.

Besides *Saccharomyces*, extracellular protease activity was evaluated in many species of the yeast natural flora associated with grapes. Several genera of non-*Saccharomyces* yeasts were investigated for protease production. Up to date, it has been reported and accepted that non-*Saccharomyces* yeasts secrete significantly higher amounts of extracellular proteases than *Saccharomyces* yeasts. *Kloeckera apiculata* (perfect form: *Hanseniaspora uvarum*), *Hanseniaspora guilliermondii* (imperfect form: *Kloeckera apis*), *Candida pulcherrima* and its perfect form, *Metschnikowia pulcherrima*, *Torulaspora delbrueckii* (originally named *Torulaspora rosei*) *Pichia anomala, Candida stellata, Torulopsis magnoliae* (imperfect form: *Candida magnoliae*), and *Debaryomzces hansenii* can produce extracellular proteases in defined laboratory conditions (Rosi & Costamagna, 1987; Lagace & Bisson, 1990; Charoenchai et al., 1997 and Strauss et al., 2001). Some of these proteases have been shown to be active against wine proteins. Dizy and Bisson (2000) described that members of two genera, *Kloeckera* and *Hanseniaspora*, showed significant amounts of protease activity and reduced the protein concentration of the juice by approximately one-third. Rensburg and Pretorius (2000) confirmed some degradation of wine proteins in wines incubated with proteases from *K. apiculata*. Acid proteases secreted by *Kloeckera apiculata*, *Metschnikowia pulcherrima*, and *Torulaspora magnoliae* were found to be effective at the degradation of wine proteins in both wine and model solutions (Lagace & Bisson, 1990). On the contrary, it

has been suggested that grape and wine proteins are protease resistant (Water et al, 1996; Pocock et al., 2003).

Regarding the ability of yeasts to produce extracellular proteases, it is likely that they can generate small peptides and free amino acids. It will be interesting to determine whether the amino acids and peptides are generated from yeast proteases during wine fermentation. These proteolysis products may enhance flavour directly or by their conversion into volatile compounds. They also may be significant as nutritional factors that have an impact on microbial interactions during fermentation process (Bolumar et al., 2005). Although it is well established that non-*Saccharomyces* yeasts are dominant species during the early stages of fermentation, nothing is known about the impact of their proteases and autolytic behaviour. There should be a more extensive evaluation of proteolytic activity of yeasts and its application to industrial winemaking should be evaluated in a closer look. More specific information is required about its reaction in vinification. This may offer an opportunity for innovation and gainful exploitation in winemaking.

2.3 Nitrogen containing compounds in winemaking

Nitrogen containing compounds, e.g. amino acids, peptides and proteins, are commonly found in living organisms and important constituents of food. They supply the required building blocks for protein biosynthesis. In addition, they directly contribute to the flavour of food and are precursors for aroma compounds and colours formed during thermal or enzymatic reactions in production, processing and storage of food (Belitz et al. 2004).
Wine is composed of a complex mixture of organic molecules that present in an extremely wide range of concentrations. Of those molecules nitrogen containing compounds are great of interest. Like many other natural food products, wine contains varying amount of different nitrogenous substances (Valero et al., 2003). The nitrogenous components of must and wine play important roles in fermentation of winemaking since nitrogen is a necessary nutrient for yeast in winemaking. Lack of nitrogen is one of the principal factors limiting growth and sugar attenuation (Hernandez-Orte et al., 2006 a). These nitrogen-containing compounds also influence clarification and microbial instability. They may affect the development of wine aroma and flavour (Bell & Henschke, 2005) and foam characteristic in sparkling wines (Moreno-Arribas et al., 2000; Marchal et al., 2006). By degradation of nitrogen compounds, some metabolic byproducts considered detrimental to health, e.g. ethyl carbamate, biogenic amines, can be produced (Zoecklein et al., 1999; Bell & Henschke, 2005).

More recently, a research conducted by Osborne and Edwards (2006) indicated that many yeast strains inhibited the bacteria during fermentation under high nitrogen conditions. The effect of a low level of nitrogen-containing compound in grape juice on sluggish and stuck fermentation is perhaps the most widely studied (Siler & Morris, 1996, Mendes-Ferreira et al., 2007 a, 2007 b). However, some researchers link a low concentration of nitrogen-containing compound to low cellular activity and others consider this condition as a cause for low resultant biomass concentrations (Bisson, 1991; Cramer et al., 2002; Ribéreau-Gayon et al., 2006 a). Furthermore, numerous studies about the influence of nitrogen composition in must and wine on volatile and non-volatile metabolites have been conducted with significant interest (Moreira et al., 2002; Hernandez-Orte et al., 2006; Swiegers & Pretorius, 2007).

Nitrogen-containing compounds in grape juice and wine are made up of an ammonia component and a more complex amino-acid based nitrogen component, e.g. amino acids, oligopeptides, polypeptides, proteins, amide nitrogen, bioamines, nucleic acids, amino sugar nitrogen, pyrazines, vitamins and nitrate (Ough, et al., 1991; Henschke & Jiranek, 1993; Zoecklein et al., 1999; Cramer et al., 2002). In wine, concentrations of these compounds are found in a broad range. As shown in table 4 and 5 the total nitrogen content of grape must ranges from 60 to 2,400 mg N/l. Factors such as variety, root stock, environment, growing condition, and juice extraction method cause a variation of concentrations of nitrogen containing compounds in grape and juice (Monterio & Bisson, 1992; Swiegers et al., 2005). At an assimilable nitrogen level below about 140 mg N/l, growth and fermentation rates are retarded while above 400 mg N/l, growth and fermentation rates are strongly stimulated (Henschke & Jiranek, 1993; Zoecklein et al., 1999). An inadequacy of nitrogen-containing compounds of grape juices for wine fermentation has often been reported. Ribéreau-Gayon et al. (2006 b) described that analytical findings on the extent and frequency of nitrogen deficiencies in Bordeaux musts from 1996-2006 vintages were 22% in white must, 49% in red must, 60% in rose must, and 89% in botrytized musts of the samples. Hence, the assessment of the nitrogen requirement should be controlled because it can have an impact on fermentation kinetics. Regarding the variety of these nitrogenous compounds, only some compounds found in musts and wines are presented in this review.

Urea

Urea is a di-amino derivative of carbonic acid known as carbonic diamide (Francis, 2006). The concentration of urea in commercial wine is normally below 3 mg/l (Ough et al., 1992). Basically, urea is not detected in grape juice but it is often found in wine as a consequence of yeast metabolism. Tracer studies using radioactively labelled substrates have revealed arginine as the main precursor of urea in wines and suggested a minor contribution from the degradation of purines during the turnover of nucleic acid material late in fermentation

(Francis, 2006). Ethyl carbamate (urethane) is formed by the reaction of urea and ethanol. It is an undesirable component of wine since it is considered as carcinogen and mutagen (Valero et al., 1999; Bell & Henschke, 2005).

Ammonium

Ammonia serves as the primary form of available nitrogen for yeast metabolism (up to 40%) in grape juice (Beltran et al., 2004). Ammonia is usually considered to be yeast's preferred nitrogen source, but for many strains glutamine equally permits a maximal rate of growth (Dickinson, 2004). Adding ammonium salts to a nitrogen-deficient medium has a significant effect on cell population, fermentation time, the production of alcohol and volatile acidity, and pH (Mendes-Ferreira et al., 2004; Taillandier et al., 2007; Bely et al., 2008). As grapes mature, ammonia decreases with an increase in protein and peptide nitrogen. The concentration of ammonia ranges from 24 to 209 mg/l in grapes and from a few mg/l to 50 mg/l in wine. (Zoecklein et al., 1999)

Amino acids

Most of the 20 commonly occurring amino acids are found in must and wine (**Table 2-4**). Although the amount of each varies with grape variety, cultivation, region and processing techniques, arginine and proline are predominant amino acids found in must (Herbert et al., 2005; Linda, 1992; Moreira et al., 2002; Soufleros et al., 2003). Amino acids studies of must and wine have commonly presented the L-amino acids. D-amino acids have only been examined in a few studies as they have been considered as unnatural amino acids (Brueckner & Paetzold, 2006; Brueckner & Westhauser, 2003; Brueckner et al., 2007; Paetzold & Brueckner, 2007; Paetzold et al., 2007).

Boulton et al. (1999) described that the amino acids of grape juice are generally in the range of 1 to 3 g/l, while Radler (1993) stated amino acids in must and wine are in the range 1-6 g/l. The individual amino acids commonly found in the whole grape and grape juice vary significantly (**Table2-4**). In must, arginine is present in relatively high concentration at levels of 200-800 mg/l and also high concentration of proline at the 750-1500 mg/l are found in most cultivars. Arginine is quantitatively the most important amino acid utilizable by *Saccharomyces* in grapes and, subsequently unfermented juice. This amino acid is rapidly incorporated by yeast at the start of fermentation and subsequently released back into the wine during autolytic cycles (Fugelsang & Edwards, 2007).

Table 2-4 The identity and concentration of amino acids found in the whole grape and juice at harvest

Amino acid	3-Letter	1-Letter	Concentration (mg/l)
Alanine	Ala	A	10-227
Arginine	Arg	R	20-2322
Asparagine	Asn	N	1-171
Aspartic acid	Asp	D	10-138
Citrulline	Cit		0.1-83
Cysteine	Cys	C	1-8.2
Glutamine	Glu	E	9-4499
Glutamic acid	Gln	Q	27-454
Glycine	Gly	G	1-20
Histidine	His	H	5-197
Isoleucine	Ile	I	1-117
Leucine	Leu	L	2-160
Lysine	Lys	K	0.7-45
Methionine	Met	M	1-33
Ornithine	Orn		0.1-27.2
Phenylalanine	Phe	F	2.8-138
Proline	Pro	P	9-2257
Serine	Ser	S	13-330
Threonine	Thr	T	9-284
Tryptophan	Trp	W	0.2-11
Tyrosine	Tyr	Y	2-33
Valine	Val	V	7-116

Source: Bell and Henschke (2005)

Regarding the metabolism of yeast, a supplement of amino acids in grape juice could shorten fermentation time, lead to high alcohol production (Hermández-Orte et al., 2006 b) and favoured the formation of volatile compounds in wine (Garde-Cerdán & Ancín-Aypilicueta, 2007). The amino acid uptake of yeast influences the aroma generation during alcoholic fermentation (Swiegers et al., 2005). Wines obtained from musts supplemented amino acids have higher levels of γ-butyrolactone, isobutanol and isobutyric acid

(Hernández-Orte et al., 2005). Furthermore, an experiment carried out by Moreira et al. (2002) suggested that the addition of methionine to grape musts enhanced the production of sulphur compounds. In addition, the authors indicate that yeast strains influence the effect of amino addition.

Peptides

Peptides are formed by binding amino acids together through an amide linkage. On the other hand, peptide hydrolysis results in free amino acids. Peptides are denoted by the number of amino acid residues as di-, tri-, tetrapeptides, etc. The term “oligopeptides” is used for those with 10 or less amino acids. Higher molecular weight peptides are called polypeptides and referred to have molecular weight below 10 kDa (Fukui & Yototsuka, 2003).

Peptides exhibit interesting functional properties, e.g. as antioxidants, antimicrobial agents, and surfactants with foaming and emulsifying capabilities (Brueckner & Koza, 2003; Belitz et al., 2004). Besides this wide variability, peptides and their derivates are receiving immense attention for their bioactive properties, such as lowering the blood pressure and preventing the development of dental caries (Meisel, 2007). Recently, Titoria (2007) proposed the potential synergistic prebiotic effect. On the other hand, it is well established that peptides are responsible for bitter, sweet, and umami tastes, such as bitter peptides in cheese or aspartame a sweet peptide which is 180 times sweeter than sucrose (MacDonald, et al., 1980 ; Otagiri et al., 1985; Ishibashi et al., 1987; Ishibashi et al., 1988; Aso, 1989; Tamura et al., 1989; Kohmura et al., 1991; Kamei et al., 1992; Nakonieczna et al., 1995; Desportes, 2001).

Peptides are widespread in nature, wines included. Polypeptides constitute a significant proportion of the total nitrogen content in wine between 20 and 90% (Zoecklein, 1999). Analyses carried out by Monterio et al. (2001) revealed that wines contain a large number of distinct polypeptides. Peptides may have effects on some of the physico-chemical characteristics of wine, as they do in other foodstuffs (Aceo et al., 1994). Although the characteristics of peptides in wines have been studied to some extent, there is no evidence of the function of individual peptide fractions.

Among the great number of peptides found in wines, glutathione, a tripeptid ε, γ-glutamylcysteinylglycine is perhaps the most intensively studied. Glutathione is discussed as an anticarcinogenic molecule due to its ready oxidation (Haneklaus & Schnug, 2004; Robinson, 2007; Rousssis et al., 2007). It is involved in active transport of amino acids and many redox-type reactions (Eisenbrand et al., 2006). The peptide fraction with higher molecular weight has been studied in wide extent. Alcaide-Hidalgo et al. (2008) reported that the peptide fraction of the red wines is complex and is composed, at least partly, of glycopeptides from grapes and yeasts. The other study carried out by Osborne and Edwards

(2007) has shown that a polypeptide band of 5.9 kDa produced by *S. cerevisiae* inhibited the growth of *Oenococcus oeni*.

In sake, peptides contribute to an improvement of the taste (Yamada et al., 2005). On the contrary, it is difficult to quantify their impacts on wine taste because of the interaction with others wine compounds, whereas peptides had a sensory impact when tasted in water at high concentration (Desportes et al., 2001). Researches demonstrate that peptides could be released by yeast during base wine preparation and second fermentation of sparkling wine (Martínez- Rodríguez et al., 2002; Moreno-Arribas et al., 1996; Laguera et al., 1997). The enrichment of wine with short peptides and proteins can be made through addition of yeast autolysate which gives rounder and fuller wines (Feuillat, 2005). Furthermore, Oganesjanz and coworkers (2007) briefly note that an increase of nitrogen containing compounds, peptides included, through the addition of yeast lysate in wine improves the organoleptic quality, particularly its complexity and harmony is enhanced.

Recently, yeast derivative products have been introduced to the market and are claimed to have many properties, according to manufacturers. Of these, an interesting ability is to release nitrogenous compounds. Regarding these compounds, peptides and proteins are of great importance. These peptides and proteins are believed to have positive effect on modifying and ennobling the colloidal structure of wine thus, enhancing the mouth feel which makes the wines rounder. Application of these products is also suggested to improve wine aroma, protect wine from oxidation, and preserve colour and fruity flavours in red wine. However, there has been no clearly scientific evidence on this claim so far. In addition, particular problems are associated with attempts to measure peptide in yeasts. Unlike amino acids numerous sizes of peptides are in grape juice and wine. Furthermore, wine is a complex matrix and thus many methods for the determination of peptides have to be specifically developed for wine analysis. These methods and extraction techniques are necessary to remove interfering compounds. The applicability, advantages and disadvantages of these methods should be considerably appropriate for grape juice and wine. This may perhaps be an explanation of the challenging study of peptides as well as proteins in winemaking.

Proteins

Macromolecules containing more than ca. 100 amino acid residues, i.e. molecular weight about 10 kDa, are described as proteins (Kreutzig, 2001; Koolman & Roehm, 2005). Those amino acids are joined together by covalent peptide bond linkage. Covalently bound hetero constituents can also be incorporated into proteins. When the bonds are hydrolyzed, proteins yield polypeptides of various molecular sizes, peptides and amino acids.

In the last five decades protein studies in grape and wine have revealed better understanding of its role in winemaking (Bretthauer, 1959; Koch & Sajak, 1959; Diemair et al., 1961; Waters et al., 1996; Monteiro et al., 2001; Ferreira et al., 2002; Pocock et al., 2003; Waters et al. 2005). Surveys of the protein concentration of grape juice and wine conducted world wide have revealed a wide variability (**Table 2-5**). Proteins in must and wine could be derived from different origins of grape berry, yeasts, bacteria, and fungi as shown in **Figure 2-2** (Dambrock, 2003; Kwon, 2004; Cilindre et al., 2007; Claus, 2007). These various proteins in wine are mainly originated from must and others are brought in during fermentation (Gerland, 2001; Ferreira et al., 2002; Dambrock, 2003; Delfini et al., 2004).

Regarding yeasts, non-*Saccharomyces* species involved in winemaking can produce and secrete high levels of specific enzymes, e.g. esterase, glucosidase, proteases (Nelson & Young, 1986; Bilinski et al., 1987; Rosi & Costamagna, 1987; Sturley & Young, 1988; Lagace & Bisson, 1990; Chareoenchai et al., 1997; Iranzo et al., 1998; Dizy and Bisson, 2000). The species *S. cerevisiae* on the other hand is well known for invertase secretion (Toda, 1976; Lehle et al., 1979; Chan et al., 1991; Vitolo & Yassuda, 1991; Chan et al., 1992; Nam et al., 1993; Dynesen et al., 1998; Moine-Ledouxt & Dubourdieu, 1999; Ganeva et al., 2002; Kern et al., 2007). Besides the ability to secrete proteins, yeasts are able to exhibit killer phenomenon (Magliani et al., 1997; Ramon-Protugal, 1998; Zagorc et al., 2001; Comitini et al., 2004; Santos & Marquina, 2004; Golubev, 2006). These enzymes and some killer toxins are proteins and may remain in wine, although their conformations change according to the environmental matrix condition.

Table 2-5 Concentration of nitrogen containing compounds found in grape juice and wine

Components	Concentration	References
Soluble nitrogen compounds in must	100-1000 mg N/l	Ribéreau-Gayon et al.(2006 a)
Proteins in grape juice	1.5-100 mg/l	
Total nitrogen of Bordeaux wines	70-700 mg/l	Ribéreau-Gayon et al.(2006 b)
white wine	77-377 mg/l mg/l	
red wine	143-666 mg/l	
Amino acids in grape and wine	1000-4000 mg/l	
Urea in wine	< 1 mg/l	
Ethyl carbamate in wine	7.7 μg/l	
Histamine	ca. 10 mg/l	
Protein	10-300 mg/l	
Amides in must	10-40 mg/l	Carnevillier et al. (2000)
Amide in wine	8-35 mg/l	
Ammonium in must	10-120 mg/l	
Ammonium in wine	3-30 mg/l	
Amino acids in must	170-1120 mg/l	
Amino acids in wine 130-590	130-590 mg/l	
Peptides in Chardonnay must	7.6-20.3 mg/g	Nakopoulou et al. (2006)
Peptides in Chardonnay wine	104.8-139 mg/l	
Soluble proteins in must	93.5 mg/l	Bell and Henschke (2005)
Free amino acids in must	32.5 mg/l	
Ethyl carbamate in wine	1.2-4.3 μg/l	Zoecklein et al. (1999)
Histamine in wine	1.86-11.3 mg/l	
Free amino nitrogen (FAN)	15-230 mg N/l	
Histamine in wine	0-5 mg/l	Waters et al. (2005)
Ammonium in wine	24-209 mg/l	
Free amino nitrogen (FAN)	10-275 mg N/l	
Soluble proteins in un-fined white wine	118-800 mg/l	Herbert et al. (2005)
Assimilable amino acids in musts	331-1375 mg/l	Fukui and Yokotsuka (2003)
Proteins in Japanese wine	29.8-107.1 mg/l	Farkas (1988)
Stable soluble proteins in wine	13.6-36.4 mg/l	
Nitrogen compounds in grape	600-2400 mg/kg	Radler (1992)
Nitrogen compounds in wine	200-1400 mg/l	
Amino acids in must and wine	1-6 g/l	Bouton (1999)
Amino acids in grape juice	1-3 g/l	Marchal et al. (1997) and Vincenzi et al.(2004)
Protein in wine	< 1 to > 1 g/l	Murphey et al. (1989)
Soluble protein in grape juice	118-800 mg/l	Wigan and Decker (2007)
Protein in white wine	8-500 mg/l	Bisson (1991)
Amino acids in must	65-1130 mg/l	Ferreira et al.(2002)

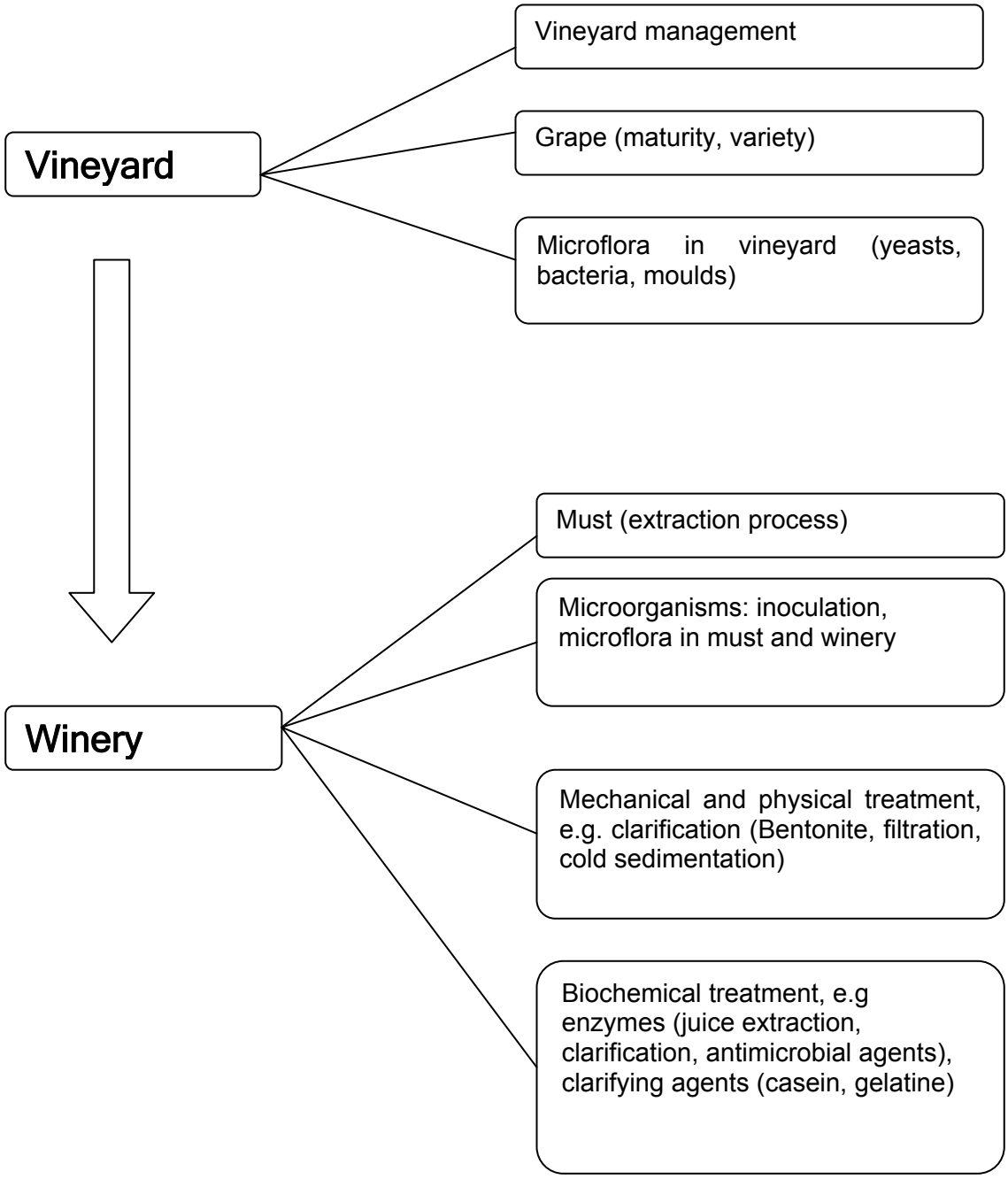


Figure 2-2 Factors affecting the concentration of peptides and proteins in must and wine

In addition to intrinsic proteins mentioned above, extrinsic proteins are in practical applied for certain purposes in winemaking. For instance, lysozyme is added as antimicrobial agent (Tirelli & Noni, 2007), pectinase as aid of juice extraction (Vine et al., 2002), gelatine and egg white as stabilizing and clarifying agents (Steidl, 2004; Wigand & Decker, 2007). Other potential utilization of extrinsic protein include the addition of enzymes to enhance the composition of flavour compounds and flavour impression (Cabaroglu et al., 2003; Genovés et al., 2005), additive enzyme to increase polysaccharides (Palomero et al., 2007). Yeast hulls and inactive yeasts are added to grape must as nutritional source for yeast growth and some compounds of the products may remain in the wine. These added proteins can change the composition of nitrogen-containing compounds in wine products. The presence of some extrinsic proteins in wine can cause allergies to consumers (Stein-Hammer, 2004) but there is no research about the impact of these proteins on organoleptic quality of wine.

Apart from addition of proteins, other steps in the winemaking process can also affect nitrogen-containing compounds of wine (Farkas, 1988, Wigand & Decker, 2007). Koch and Sajak (1959), Ribéreau-Gayon et al. (2006) described that grape proteins increases relatively fast during ripening process of grapes and the variety influences the protein concentration (Henschke & Jiranek, 1992). When grape berries are transferred to the winery, the level of protein extracted from the fruit is influenced by initial grape processing methodology (Zoecklein et al., 1999). Alcoholic fermentation results in a decrease of protein content (Koch & Bretthauer, 1957; Bayly & Berg, 1967), whereas an increase of protein content is observed at the end of fermentation (Nakopoulou et al., 2006). Furthermore, Charpentier (1998) notes that protein concentration of wine was higher than that in the original grape juice. Growth of yeasts and lactic acid bacteria during wine fermentation was mentioned as a cause of this increase.

Due to the interaction of many factors, e.g. grape variety, must preparation, and the differences of measurement methods, estimates of protein concentration in must and wine range between a few milligrams to greater than 1 g/l as documented in **Table 2-5**. The protein nitrogen content of juice ranges from about 1 to 13% of the total nitrogen content. In wine, the levels are higher, approaching 38%. However, it has to be realized that estimates of soluble protein concentrations in wines can vary depending on the analysis method (Zoecklein et al, 1999). Although proteins are usually present in wines in low concentrations, they greatly affect the clarity and stability of wines. Therefore, many studies have been concerned with proteins in grape juice and wine because they may become insoluble and precipitate in wine products (Hsu & Heatherbell, 1987; Monteiro et al., 2001).

The solubility of wine proteins depends primarily on temperature, alcohol level, ionic strength, and pH. Therefore changes in any parameter may affect the potential for protein precipitation (Zoecklein et al., 1999). As proteins affect clarity and stability of wines, studies on of proteins have been focused on the reduction of proteins to avoid turbidity in wine (Sarmento et al., 2000; Pocock et al., 2003; Pocock et al., 2007). In these studies proteins are reported to be the most common cause of haze or cloudy amorphous precipitates in white wines.

The precipitation of soluble proteins in bottled wines creates an amorphous haze or deposit formed most frequently in white wines or wines of low polyphenol content. (Zoecklein et al., 1999). The so-called pathogenesis related (PR) proteins are believed to be the principal haze protein. PR proteins are likely a complex of proteins, polysaccharides and polyphenols with minor amounts of inorganic ash (Waters et al., 1996 and 1998; Hayasaka et al., 2001). In general, PR proteins are considered as plant defending proteins, functioning in prevention or limiting pathogens (Monteiro et al., 2007). These proteins have been identified as thaumatin-like proteins and chitinase. They are ubiquitous, acid-stable, resistant to proteolysis and derived from grapes. The PR proteins, are the major soluble protein components of grapes from five cultivars of *Vitis vinifera* (Pocock et al., 2000). Chitinases have been found ca. 50% of the soluble proteins in grape berries. Thaumatin-like proteins are also included in this type of proteins (Waters et al., 1998). There is no information about the influence of these proteins as well as of other proteins in relation to sensorial quality of wine.

On the other hand, a remarkable number of researches report that proteins have sweet taste, e.g. thaumatin, monellin, mabinlin, brazzein, egg lysozyme and neoculin (Ota & Ariyoshi, 1998; Kaneko & Kitabatake, 2001; Masuda and Kitabatake, 2006). The potential of these proteins to elicit a sweet-taste response on the human palate are different. Among these proteins, thaumatin is used commercially for its sweetness, tasted masking, flavour enhancement, and synergistic properties to produce dramatic effects in food products (Kaneko & Kitabatake, 2001). Thaumatin is an intensely sweet protein of 100,000 times sweeter than sucrose (Kaneko & Kitabatake, 2001; Masuda & Kitabatake, 2006). The threshold value of sweetness of thaumatin is about 50 nM, whereas egg lysozyme is 10 μM (Masuda & Kitabatake, 2006).

Great interest has been dedicated to mannoproteins in enological protein research. Mannoproteins comprised between 25% and 34% of cell walls of yeasts (Nguyen et al., 1998). Mannoproteins released by *S. cerevisiae* are found in significant amounts in the wine (Goncalves et al., 2002; Comuzzo et al., 2006). Mannoproteins have interesting enological ability, e.g. inhibit tannin aggregation in wine (Poncet-Legrand et al., 2007), improve tartaric stability (Comuzzo et al., 2006), enhance the complexity and balance of aromas in wine (Bautista et al., 2007), protect wine from protein haze spoilage (Waters, 1994), and adsorb of ochratoxin A (Caridi, 2006). Another study carried out by Moine-Ledoux and Dubourdieu

(1999) reports that an N-glycosylated mannoprotein (31.8 kDa) which corresponds to a parietal invertase fragment of *S. cerevisiae* improves the protein stability of white wines aged on their lees, sur lies (Moine-Ledoux & Dubourdieu, 1999). In addition, it is described that mannoproteins adsorb ochratoxin, increase growth of malolactic bacteria and play a role in yeast flocculation and autolysis (Caridi, 2005). On the contrary, mannoproteins related to indigenous yeasts and their influence to winemaking has not been well studied.

In conclusion, finding reliable methods of assessing proteins in grape juice and wine remains a great challenge since there is a tremendous diversity of proteins and a high complexity of the matrix. Several direct and indirect methods have been adopted to investigate the protein concentration in grape juices and wines. For example, protein concentration in must and wine is considered as the difference between total nitrogen and free amino nitrogen (Martínez-Rodriguez, 2001 b), protein can also be examined by dye-binding assays (Vincenzi, et al., 2005), and protein concentration can be determined by the Kjeldahl method (Fukui and Yokosuka, 2003).

Other nitrogen-containing components

Nitrates (NO_3^-) and Nitrites (NO_2^-) are present in wines at low levels, usually less than 0.3% of the total nitrogen. Nitrate levels of less than 7 mg/l, have been found in German wines and lower values with an average of 1.65 mg/l, in Italian white wines (Amerine & Ough, 1980).

Another group of nitrogen compounds present in wines are biogenic amines, e.g. putrescine, phenylethylamine, spermidine, spermine, histamine, tyramine, cadaverine, mercaptoethylamine, ethanolamine, and serotonin (Leitao et al.,2000; Caruso et al., 2002; Ansorge, 2007). Biogenic amines are derived from microbial decarboxylation of the corresponding amino acids or by transamination of aldehydes by amino acid transaminases (Zhijun et al., 2007). Bogenic amines in wines may come from two different sources, i.e. raw materials and fermentation processes (Zhijun et al., 2007; Marques et al., 2008). In general, low levels of biogenic amines were found in musts and wines in comparison to other foodstuffs, where biogenic amines can occur in much higher concentrations (Herbert et al., 2005).

Nitrogen-containing flavour compounds are also important in enology. For example, methyl anthranilate and *o*-aminoacetophenone are related to the “foxy” taste of labrusca grapes and related hybrids (Rapp, 1998). Additionally, 2-methoxypyrazines are reported to be responsible for the vegetative, herbaceous aromas frequently noted in wines produced from Cabernet Sauvignon and Sauvignon blanc (Sala et al., 2002; Bell & Henschke, 2005). The nitrogen-containing flavour compounds formed during fermentation may influence the fermentation bouquet (Amerine & Ough, 1980).

Utilization of nitrogen containing compounds by yeasts

A broad range of nitrogen compounds, e.g. amino acids, ammonium, amines, amides, S-adenosylmethionine, nucleotides and nucleic acid derivatives, vitamins and peptides found in grape juice can be metabolized by yeasts (Henschke & Jiranek, 1993). Within the complexity of nitrogenous components, amino acids and ammonium ions are the most important for yeast growth (Valero et al., 2003). Metabolism of organic nitrogen compounds by yeast cells may have three possible fates (Large, 1986): (i) they may be taken up by the cells and incorporated without modification into cellular constituents, e.g. the incorporation of amino acids into protein; (ii) they may be degraded by the cells and the nitrogen that they contain may be liberated (usually, but not always, as ammonia) and may be used for the synthesis of other nitrogenous cell constituents; (iii) the carbon of the organic nitrogen compound may also be used by the cell for synthetic purposes and in this case the compound is acting as a carbon source.

In general, yeasts utilize ammonium and amino acids of the fermentation substrate for their growth. Yeasts use a mechanism called nitrogen catabolite repression (NCR), which mediates the selection of good nitrogen sources by the expression of appropriate transport system (permeases) and the degradation of non appropriate permeases (Bell & Henschke, 2005). The wine yeast, *S. cerevisiae* is able to assimilate various nitrogen sources. However, preference of nitrogen source is different such as glutamine, asparagine or ammonium are classified as good nitrogen source (Henschke & Jiranek, 2002; Beltran et al., 2004). Nitrogen sources that favour high growth rates are preferentially assimilated because their metabolism readily yields ammonia, glutamate or glutaminewhich play a central role in nitrogen metabolism (Dickinson, 2004).

Ammonia serves as the primary form of available nitrogen in yeast metabolism in grape juice (Fugelsang & Edwards, 2007). This inorganic nitrogen is fixed into organic forms through reaction with α-keto-glutarate to yield glutamate by glutamate dehydrogenase (**Figure 2-3**). Glutamate can be further used by the cell to produce other amino acids important for metabolism. As part of the metabolism, degradation of nitrogen-containing leads to the two end-products, ammonium or glutamate as summarized by Large (1986). These end products are interconverted in the catabolic pathways of yeasts.

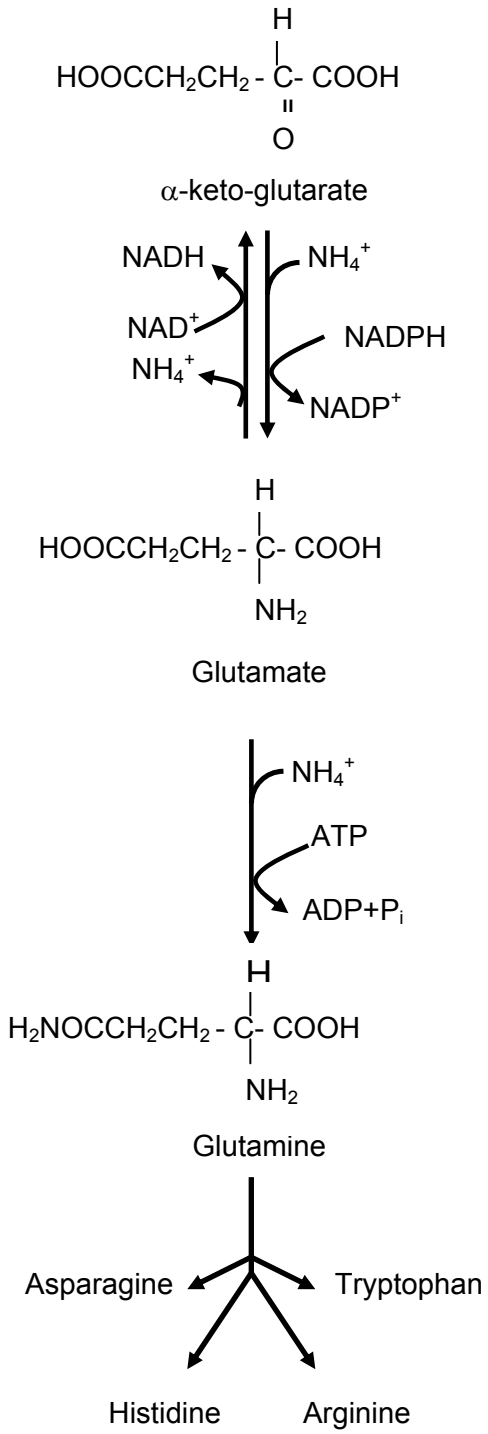


Figure 2-3 Reaction of ammonia with α-keto-gutarate to incorporate inorganic forms of nitrogen by *Saccharomyces*. Source: Fugelsang and Edwards (2007)

It is well established that sulphur compounds in wine can be formed metabolically by yeasts from organic and inorganic compounds. Concerning nitrogen-containing compounds, hydrogen sulphide is usually formed in response to biosynthesis of cysteine and methionine and glutathione (Rauhut, 1993). Other sulphur compounds are also synthesized by yeasts. For instance, dimethyl sulphide is derived from degradation of cysteine, and cystine and glutathione and methanethiol is derived from methionine (Rauhut, 1993). Nitrogen compounds in the media can influence the formation of sulphur compounds (Moreira et al., 2002) and a carcinogenic compound, ethyl carbamate (Valero et al., 2003), in wine.

The metabolism of nitrogen-containing compounds can also make up a major group of wine aroma compounds such as higher alcohols. They can be formed by catabolism of amino acids via the Ehrlich pathway (Bell & Henschke, 2005). However, nitrogen metabolism is depending on many factors. For example, temperature influences the quantity and the quality of yeast nitrogen requirements (Beltran et al., 2006, 2007, 2008). Good nitrogen sources such as glutamine, asparagine or ammonium decrease the level of enzymes required for utilization of poorer nitrogen sources (Beltran et al., 2004). In addition, utilization of nitrogen-containing compound by yeasts is dependent on both yeast strain and the fermentation conditions (Valero et al., 1999), e.g. yeasts consume less nitrogen at low temperature (Beltran et al., 2006) and ethanol inhibits the uptake of most amino acids (Bisson, 1991).

Although it is well established that yeasts preferentially utilize ammonium and amino acids, peptides of appropriate amino-acid composition can also be consumed by yeasts (Marder et al., 1977; Payne and Smith, 1994; Yamada et al., 2005). Peptide transport and utilization is known to occur not only in *S. cerevisiae* but also in non-*Sacchararomyces* species (Milewski et al 1988; Shallow et al., 1991). In complex nitrogen mixtures (three amino acids and three dipeptides), *S. cerevisiae* NCYC 1324 simultaneously used both amino acids and peptides as sources of nitrogen (Patterson & Ingledew, 1999). This research demonstrates that the dipeptides are definitely used as additional sources of nitrogen for continued yeast growth in a defined medium. In addition, it appears that the presence of ammonium ions in a defined culture medium inhibited peptide utilization inside the yeast cells, whereas leucine enhanced the ability of the yeast to utilize peptides.

3. MATERIALS AND METHODS

Yeast strains, media, chemical reagents, devices and equipments applied in this study are listed in this Section. Yeast cultivation and enumeration, analysis methods, and experimental designs are described in the following text.

3.1 Yeast strains

44 non-*Saccharomyces* yeast strains, 6 *Saccharomyces* strains, and 1 mixed yeast product were used in this study (**Table 3-1**). They were obtained from; Section of Microbiology and Biochemistry, Geisenheim Research Center, Geisenheim, Germany; Agroscope Changins-Waedenswil ACW, Waedenswil, Switzerland; Chr. Hansen Inc. Hoersholm, Denmark.

Table 3-1 List of the yeasts used in the study

Strains	Code*
Metschnikowia pulcherrima	N-1
Hanseniaspora uvarum	N-2
Dekkera bruxellensis	N-4
Dekkera bruxellensis	N-5
Dekkera bruxellensis	N-6
Dekkera bruxellensis	N-7
Dekkera bruxellensis	N-8
Zygosaccharomyces mellis	N-9
Zygosaccharomyces mellis	N-10
Zygosaccharomyces bailii	N-11
Zygosaccharomyces bailii	N-12
Saccharomycodes ludwigii	N-13
Saccharomycodes ludwigii	N-14
Saccharomycodes ludwigii	N-15
Hansenula saturnus	N-16
Hansenula sp.	N-17
Pichia farinosa	N-18

* Character abbreviation used throughout text.

Table 3-1 (continued) Listt of the yeasts used in the study

Strains	Code*
Debaromyces hansenii	N-19
Debaromyces nicotianae	N-20
Rhodotorula glutinis	N-21
Mycoderma bispora	N-22
Mycoderma bispora	N-23
Hansenula anomala	N-24
Metschnikowia pulcherrima	N-25
Kloeckera apiculata	N-26
Hanseniaspora uvarum	N-27
Zygosaccharomyces bailii	N-28
Zygosaccharomyces bailii	N-29
Brettanomyces sp.	N-30
Zygosaccharomyces bailii	Z-CM
Kluyveromyces thermotolerans	K-MB
Torulaspora delbrueckii	T-MB
Hanseniaspora uvarum	H 045
Hanseniaspora uvarum	H 097
Hanseniaspora uvarum	H 182
Hanseniaspora uvarum	H 155
Hanseniaspora uvarum	H 030
Hanseniaspora uvarum	H 210
Hanseniaspora uvarum	H 146
Metschnikowia pulcherrima	M 004
Rhodotorula sp.	R-1
Rhodotorula sp.	R-2
Rhodotorula sp.	R-3
Rhodotorula sp.	R-4
Saccharomyces cerevisiae	S-CM
Saccharomyces cerevisiae	S-CEG
Saccharomyces cerevisiae	S-CY
Saccharomyces cerevisiae	S-EC
Saccharomyces cerevisiae	S-S6U
Saccharomyces cerevisiae	S-Rb
Mixed yeasts- Harmony	Hmy

* Character abbreviation used throughout text.

3.2 Chemical reagents

Ammonium peroxodisulfate, ammonium sulfate, bromophenol blue, bromocresol green, Coomassie brilliant blue R 250, ethanol, galactose, haemoglobin, trichloroacetic acid (TCA), *N,N,N',N'*-tetramethylethylenediamine, peptone were obtained from Fluka, Buch, CH. Acrylaminde, Folin-Ciocalteu's phenol reagent, glucose, lysine monohydrate, methylene blue, methyl red were from Merck, Darmstadt, Germany. Acrylamide: N,N'-methylenebisacrylamide, bovine serum albumin, Citric acid monohydrate, glycine, dimethyl dicarbonate (DMDC), tyrosine were from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. Protein dye reagent was obtained from Bio-Rad, Munich, Germany. Methionine was from Carl Roth GmbH and Co.KG, Karlsruhe, Germany. Low molecular weight protein markers were obtained from GE Healthcare Biosciences, Buckinghamshire, UK. Lysozyme was from Erbsloeh, Geisenheim, Germany. Agar was obtained from Difco Laboratories, Sparks, MD, USA. Yeast nitrogen base (YNB) was from Beckton Dickinson and Company, Sparks, MD, USA.

3.3 Media

BSA-medium (a) was prepared according to Bilinske et al. (1987) and Chareoenchai et al. (1997). YEPD (b) and Lysine (c) media were prepared as described by Sturm et al. (2006). HDM-medium (d) was prepared according to Grossmann & Begerow GmbH (1990).

(a) BSA-medium; Glucose, 10 g/l; yeast nitrogen base (YNB) without amino acid and ammonium sulfate, 1 g/l; and ammonium sulphate, 0.66 g/l (b) YEPD-agar; glucose, 20 g/l; yeast extract, 10 g/l; peptone, 20 g/l; and agar, 15 g/l (c) Lysine agar; glucose 50 g/l; YNB w/o amino acid and ammonium sulfate, 6.7 g/l; lysine monohydrate, 0.8 g/l and agar, 15 g/l (d) HDM; glucose, 60.0 g/l; YNB without amino acid and ammonium sulfate, 5.0 g/l; ammonium sulfate 2.3 g/l; bromocresol green, 125 mg/l; methyl red 125 mg/l; methionine, 7.5 g/l; galactose, 9.0 g/l; ammonium sulfate, 22.5 g/l; and agar, 15.0 g/l These media were adjusted pH 6.5.

3.4 Yeast maintenance and enumeration

Stock cultures were maintained on YPD agar which contained glucose 20 g/l; yeast extract 10 g/l; peptone 20 g/l; and agar 15 g/l. The medium was incubated for 48 h at 25 ^{O}C, and subsequently stored at 4 ^{O}C. The cultures were maintained by periodical transfer onto fresh agar medium in every 3 months. Enumeration of total yeast count was performed on YPD medium. Non-*Saccharomyces* population was examined by Lysine medium. Plating samples onto HDM medium was used to differentiate yeast strains. Total and viable cell numbers of yeasts were estimated microscopically by using a counting chamber slide. Cells (450 μl) were added to 50 μl of methylene blue solution (0.4% methylene blue, 10% ethanol and 0.4 M KH_2PO_4) and mixed. Blue cells were counted as dead cells, while cells without obvious color were counted as live cells.

3.5 Measurement of yeast cell density in the medium with spectrophotometric method

Cell density of yeasts was evaluated by measurement of the optical density at 600 nm (OD_{600}). Dilutions were made as necessary in order to keep the optical density below 0.5 AU. OD values were corrected for the initial OD reading obtained from the medium.

3.6 Fermentation kinetics

Fermentation kinetic was obtained by monitoring carbon dioxide production during yeast growth. The amount of carbon dioxide released was determined by weight loss everyday.

3.7 Proteolytic activity assay

The modified assay procedure of Lowry et al. (1951) and Charoenchai et al.(1997) was used in order to analyze cell-free supernatants for acid protease production. Volumes (1ml) of cell-free supernatant were added to 2-ml volumes of a haemoglobin substrate solution. After 1 h of incubation in a water bath at 37 ^{O}C, 5 ml of 5% trichloro acetic acid was put into each assay tube. The precipitates were removed by filtration through MicroScience no.595 ½ filter paper, and 1-ml samples of filtrate were assayed. A 5-ml volume of Lowry reagent was added to each sample. After 10 min at room temperature, 1 ml of Folin-Ciocalteu's phenol reagent, diluted 1:1 with deionized water was added. Tubes were immediately vortexed, stored in the dark for 30 min, and then read against blanks at 750 nm in spectrophotometer. One unit of enzyme is defined as the amount of enzyme which releases the colour equivalent

of 1 μg of tyrosine in 1min. All assays were performed in duplicate, and data given represent an average of the two determinations.

3.8 Analyses

The analyses which were used in the study are described as follows. Modified dye-binding procedure Bradford assay was used for total soluble protein determination (Bradford, 1976; Bio-Rad, 2007). A modified procedure from Wylie and Johnson (1961) was used to quantify free alpha amino nitrogen (FAN). Ethanol, residual sugars, glycerol, tartaric acid, malic acid, lactic acid, total acidity, volatile acidity and pH were analyzed by FTIR spectrometry as described in Baumgartner et al. (2001) and Patz et al. (1999). Free and total sulphur dioxide in wine was determined by FIAstar^{TM} 5000 following instructions of the manufacturer. Amino acids were determined by Amino Acid Analysator A200, Knauer, Germany according to Prior (1997). Esters, higher alcohols, fatty acids, and terpenes were detected by Gas chromatography-mass spectrometry GC-MS according to Rauhut et al. (2005) and Irmler et al. (2008). Low volatile sulphur compounds were detected by gas chromatography (Rauhut et al., 1997). Acetaldehyde, pyruvate, and α-ketoglutarate were investigated according to Boehringer Mannheim GmbH, Germany (1984). Ammonium was determined according to Erbsloeh Geisenheim (1997).

3.9 Evaluation of yeast proteolytic enzyme production

To study the proteolytic enzyme production of yeasts, the experiments were carried out with 3 conditions as follows.

3.9.1 Enzyme production in shaken flask

Yeasts were examined for extracellular protease production. The sources are listed in **Table 3-1**. Culture grown in 50 ml YPD broth overnight was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of liquid medium, which consisted of the following ingredients (g/l of distilled water): bovine serum albumin (BSA), 10; Glucose, 10; yeast nitrogen base (YNB), 1; and ammonium sulphate, 0.66. Cultures with an initial yeast population of 2 x 10^6 cells/ml were incubated at 30 ^oC in a rotary shaker operating at 120 rpm for three days. The crude supernatant was used for proteolytic activity assay. Viable cells and death cell were observed under microscope at the beginning and the end of incubation. The experiments were carried out in duplicate.

3.9.2 Assessment of extracellular protease production in grape juice

Fermentation was conducted in 250-ml Erlenmeyer flasks with a grape juice volume of 50 ml. The properties of the juice employed in this study were 18 ^{o}Brix, pH 3.22 and total acidity of 8.46 g/l. *H. uvarum* (H 045), *H. uvarum* (H 097), *M. pulcherrima* (M 004), *K. thermotolerans* (K-MB), *Rhodotorula sp.* (R-3), *Rhodotorula sp.* (R-4), *S. ludwigii* (N-13), *T. delbrueckii* (T-MB), and *Z. bailii* (Z-CM) were chosen to examine their proteolytic activity in grape juice. Yeast cultures prepared in grape juice was inoculated into grape juice at a concentration of 2 x 10^6 cells/ml as viable cells. Cultures were incubated at 25 ^{o}C 120 rpm for 3 days in a water bath shaker. Replication of fermentation was done. After 3 days cells were pelleted by centrifugation. The resultant cell-free supernatants were assayed for proteolytic activity.

3.9.3 Effect of proteins isolated from must on yeast growth.

Protein precipitation was performed at 8 ^{o}C by adding 2 volumes of ethanol to grape juice. The pellet of protein was obtained by centrifugation under the conditions of 14000 *g*, 15 min, and 4 ^{o}C. This isolated proteins were resuspended in distilled water. 1.0 ml of suspension was then added to synthetic grape juice (SGJ) consisting of glucose 180 g/l; tartaric acid 3 g/l ; L(+) malic acid 2 g/l ; YNB 1 g/l. pH was adjusted to be 3.00 with potassium hydroxide. Medium with a nitrogen level of 1050 mg N/l was prepared by supplementation of ammonium sulphate. Yeasts were inoculated at a concentration of 5 x 10^6 cells/ml to the medium. Species applied for this study were *M. pulcherrima* (M 004), *H. uvarum* (H 097), *T. delbrueckii* (T-MB), and *K. thermotolerans* (K-MB). Volume of fermentation was 50 ml in Erlenmeyer flasks. Replication was performed. Yeast growth was monitored turbidimetrically at 600 nm wavelength using a spectrophotometer.

3.10 Fermentative characteristics of non-*Saccharomyces* yeasts in grape juice

Fermentation was carried out in 1-liter bottles containing 700 ml pasteurized grape juice. The pasteurization was carried out at 80 ^{o}C for 10 min. Properties of initial grape juice were total sugar content 169 g/l, pH 3.2, and total acidity 4.3 g/l. No yeast nutrient was added to the grape juice prior to the alcoholic fermentation. The fermentation was carried out at 20 ^{o} C. 11 non-*Saccharomyces* strains were used in this trial; *K. thermotolerans* (K-MB), *T. delbrueckii* (T-MB), *Z. bailii* (N-29), *S. ludwigii* (N-15), *R. glutinis* (N-21), *Z. bailii* (N-11), *S. ludwigii* (N-13), *M. pulcherrima* (M 004), *H. uvarum* (H 045), *H. uvarum* (H 097), and *Z. bailii* (Z-CM).

3.11 Mixed yeast cultures of *Saccharomyces* and non-*Saccharomyces* yeasts in grape juice fermentation

Mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts were performed in Mueller-Thurgau and Riesling grape juices.

3.11.1 Fermentation of grape juice with *Saccharomyces* yeasts and non-*Saccharomyces* yeasts exhibiting proteolytic activity

The *Saccharomyces* and non-*Saccharomyces* yeast strains were used in this study. Fermentation was carried out in filtrated Mueller-Thurgau juice. The grape juice composition was sugar 210.3 g/l and pH 3.3. Duplicate experiments were performed in 1-l bottles filled with 650 ml of grape juice and fitted with closures that enable the carbon dioxide to escape. DMDC 500 mg/l and lysozyme 250 mg/l were added to grape juice. Fermentation took place at 20 ^{o}C. Yeast starter cultures were added to give ca. 2 x 10^6 and 4 x 10^6 cells/ml inoculum for the inoculated fermentations of non-*Saccharomyces* and *Saccharomyces* strains respectively. *Saccharomyces* yeasts in sequential treatments were inoculated when sugar consumption reached the determined point of ca. 2.5 % of total carbon dioxide evolution. Fermentation was terminated after 50 days. The experimental scheme is shown in **Table 3-2**.

Table 3-2 Experimental plan of yeast inoculation

Strain and code	Treatments	
	First inoculation	Second inoculation
S-EC	*S. cerevisiae* (S-EC)	-
S-CM	*S. cerevisiae* (S-CM)	-
H 097/S-EC	*H. uvarum* (H 097)	*S. cerevisiae* (S-EC)
H 045/S-EC	*H. uvarum* (H 045)	*S. cerevisiae* (S-EC)
H 045/S-CM	*H. uvarum* (H 045)	*S. cerevisiae* (S-CM)
M 004/S-EC	*M. pulcherrima* (M 004)	*S. cerevisiae* (S-EC)

3.11.2 Fermenation of Riesling grape juice with *Saccharomyces* and non-*Saccharomyces* yeasts

Saccharomyces and non-*Saccharomyces* yeast strains were used in this study. Fermentation was carried out using Riesling grape musts with different turbidity. Triplicate experiments were performed in 1.5-l bottles filled with 1 l of grape juice and fitted with closures that enable the carbon dioxide to escape. The grape juices were supplemented with thiamine 0.6 mg/l and lysozyme 250 mg/l. Fermentation took place at 20 ^{o}C. A population of certain non-*Saccharomyces* species *averaging* 5 x 10^{6} cells/ml taken from preculture was inoculated into grape juice. *S. cerevisiae* EC 1118 and the mixed yeast Harmony (Hmy) were inoculated following the manufacturer's recommendations. The trials were run in pure, mixed and sequential fermentation mode (**Table 3-3**). Sequential fermentation was done with the addition of the *S. cerevisiae* EC 1118 after 4 days.

Table 3-3 Experimental plan of the inoculation of yeasts

Codes	**Treatments**
S-EC	*S. cerevisiae* EC 1118 monoculture
H 097/S-EC	*H. uvarum* 097 and *S. cerevisiae* EC 1118 sequential inoculation
M 004/S-EC	*M. pulcherrima* 004 and *S. cerevisiae* EC 1118 sequential inoculation
T-MB/S-EC	*T. delbrueckii* and *S. cerevisiae* EC 1118 sequential inoculation
H 097,M 004/S-EC	*H. uvarum* 097, *M. pulcherrima* 004 coinoculation and *S. cerevisiae* EC 1118 sequential inoculation
H 097,M 004,T-MB/S-EC	*H. uvarum 097*, *M. pulcherrima* 004, *T.delbrueckii* coinoculation and *S. cerevisiae* EC 1118 sequential inoculation
Hmy	Mixed yeast Harmony (applied in preclarified must only)

3.13 Production of yeast proteins in synthetic medium

The experiment was designed for a replication. Yeasts were analyzed for extracellular protein production in synthetic medium containing 20 g/l glucose and 1 g/l yeast nitrogen base (YNB). The medium containing a nitrogen level of 1050 mg N/l was supplied with 5 g/l ammonium sulfate. Yeasts, *M. pulcherrima* (M 004), *H. uvarum* (H 097), *S. cerevisiae* (S-EC), *T. delbrueckii* (T-MB), *K. thermotolerans* (K-MB), *S. cerevisiae* (S-Rb), and mixed yeasts (Hmy), were inoculated into 20-ml volume of medium in 50-ml flasks. After 3 days of incubation at 25 ^{o}C with agitation of 50 rpm, viable cell count was examined by microscopic method. Cells were pelleted by centrifugation, and the resultant cell-free supernatants were determined for protein by Bradford assay.

3.14 Investigation of peptides and proteins in fermented grape juice and wine

Description of samples used to study peptides and proteins is presented in Table 3.4. Nanosep devices (Pall Corporation, Ann Arbor, USA) with a 3 kDa or 10 kDa molecular weight cutoff (MWCO) membrane were selected, depending on the size of the molecules. Membrane of Nanosep devices was an omega membrane containing modified polyethersulfone on polyethylene substrate. The Nanosep centrifugal devices with 10 kDa MWCO, was applied to prepare ≥10 kDa proteins. The sample was placed into the upper sample reservoir and the Nanosep device was capped and centrifuged 10,000 g for 10 minutes. The step was repeated to achieve the desired concentration of retentate. After centrifugation, 500 μl of deionized water was put into the sample reservoir and centrifuged 10,000 *g* for 5 minutes. This step with the deionized water was repeated 2 times. At the end of centrifugation, the retentate from the bottom filtrate receiver were collected and determined for protein concentration. The filtrate that had passed the membrane with a 10 kDa MWCO was collected and further subjected in a 3 kDa MWCO of Nanosep device. The procedure was performed as described in the preparation of 10 kDa proteins. Polypeptides (MW 3-10 kDa) and proteins (≥10 kDa) were determined quantitatively by Bradford assay.

3.15 Application of electrophoresis to study polypeptide and protein profiles

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) coupled with the simple technique of ethanol precipitation for protein separation from grape juice and wine was applied in this study. The samples from **Table 3-4** were selected for the study of peptide and protein profiles. Proteins were isolated from samples by addition of 5 volume ethanol to the sample. Proteins were precipitated at 5000 *g* for 20 min. Protein pellet was washed 3 times with deinonized water and resuspended in 10 mM Tris-HCl pH 8.0. Crude proteins of

the resuspension were subjected on the gels. SDS-PAGE was conducted according to Laemmli (1970) using a Mini-Protean 3 slab gel apparatus, Bio-Rad, Hercules, CA, USA. A vertical slab gel unit was employed for protein separation. 12% gels were prepared to separate the proteins and the standard, respectively. Crude protein samples were dissovled in Laemmli sample buffer. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250.

Table 3-4 Description of samples for the investigation of polypeptides and proteins and SDS-PAGE analysis

No.	Description
1	Fermented grape juice - *K. thermotolerans* (K-MB)
2	Fermented grape juice - *T. delbrueckii* (T-MB)
3	Fermented grape juice - *S. ludwigii* (N-15)
4	Fermented grape juice - *S. ludwigii* (N-13)
5	Fermented grape juice - *M. pulcherrima* (M 004)
6	Fermented grape juice - *H. uvarum* (H 097)
7	Fermented grape juice by *Z. bailii (*Z-CM)
8	Clarified Riesling grape juice of the vintage 2006
9	Unclarified Riesling grape juice of the vintage 2006
10	Riesling grape juice of the vintage 2006
11	Riesling grape juice of the vintage 2005
12	Weissburgunder grape juice of the vintage 2006
13 [a]	Wine fermented by single strain inoculation of *S. cerevisiae*
14 [a]	Wine fermented by mixed culture fermentation: *M. pulcherrima* (M 004) and *H. uvarum* (H 097) were inoculated prior to *S. cerevisiae.*
15 [a]	Wine produced by spontaneous fermentation (Ansteller/Pied de Cuve)

The same letters of a, b, c, d and e denote samples from the same fermentation.

Table 3-4 (continued) Description of samples for the investigation of polypeptides and proteins and SDS-PAGE analysis

No.	Description
16 [a]	Fermented must at day 9 of fermentation course of wine W1
17 [a]	Fermented must at day 9 of fermentation course of wine W4
18 [a]	Fermented must at day 9 of fermentation course of wine W5
19 [a]	Fermented must at day 12 of fermentation course of wine W1
20 [a]	Fermented must at day 12 of fermentation course of wine W4
21 [a]	Fermented must at day 12 of fermentation course of wine W5
22 [b]	Wine fermented by an inoculation of *S. cerevisiae* (TM 14-RH)
23 [b]	Wine produced by spontaneous fermentation (TM 15-SPF)
24 [c]	Wine fermented by an inoculation of *S. cerevisiae* (CT 12-RH)
25 [c]	Wine produced by spontaneous fermentation (CT 12-SPF1)
26 [c]	Wine fermented by an inoculation of *S. cerevisiae* (CT 14-RH)
27 [c]	Wine produced by spontaneous fermentation (CT 14-SPF2)
28 [d]	Fermented grape juice; must added lysozyme and inoculated with M. pulcherrima (M004).
29 [d]	Fermented grape juice; must added lysozyme and inoculated with *T. delbrueckii*
30 [e]	Wine made from must added lysozyme and inoculated with *S. cerevisiae*
31 [e]	Wine made from must added lysozyme and inoculated with mixed cultures of yeasts (sequential inoculation)
32 [e]	Wine made from must added lysozyme and inoculated with mixed cultures of yeasts (co-inoculation)

The same letters of a, b, c, d and e denote samples from the same fermentation.

4 RESULT

The following chapter will give an overview on the conducted research trials and the obtained results. Investigations on the proteolytic activity of *Saccharomyces* and non-*Saccharomyces* yeasts cultivated in different media are demonstrated in Section 4.1. Results of evaluation of the fermentation behaviour of selected yeasts as single and mixed culture inoculation are presented in Section 4.2, 4.3 and 4.4. Finally, the results of the production of polypeptides and proteins by yeasts and the investigation of these molecules in grape juice and wine are reported in Section 4.4. Each experiment was carried out in duplicate. In the following average values of the measurements are presented in Tables and Figures except for Section 4.2 and Section 4.3.1, in which only the data of the variants without sampling are presented. Results of the analysis of the different variants and their replications are listed in the Appendix.

4.1 Screening of wine yeasts that secrete extracellular proteases for the use in grape juice fermentation

The experiments which are described in the following Sections deal with extracellular protease production by yeast strains isolated from grapes and wines. These yeasts were grown in various circumstances to screen for their proteolytic activity.

4.1.1 Protease production in synthetic medium

The ability of yeasts to secrete proteolytic enzymes during growth in synthetic medium was investigated. Fifty strains of yeasts were examined for their extracellular protease production. *Saccharomyces* strains showed no detectable proteolytic activity while non-*Saccharomyces* strains under the determined conditions defined in this study showed activities in the range of non-detectable to 21.11 units (**Table 4-1**).

The protease release of *R. glutinis* (N-21) and *M. pulcherrima* (N-1) with a higher cell density of inocula was examined in parallel in the medium. As expected, the quantity of yeast cells affected proteolytic activity. The increase of population was related to higher proteolytic activity. *M. pulcherrima* formed clusters when it reached high population.

Table 4-1 Proteolytic activity of yeasts grown in synthetic medium

Yeast species	Strain/Code*	Proteolytic activity (unit)
Metschnikowia pulcherrima	N-1	0.46
Hanseniaspora uvarum	N-2	0.35
Dekkera bruxellensis	N-4	0.03
Dekkera bruxellensis	N-5	nd
Dekkera bruxellensis	N-6	0.05
Dekkera bruxellensis	N-7	0.19
Dekkera bruxellensis	N-8	0.19
Zygosaccharomyces mellis	N-9	0.35
Zygosaccharomyces mellis	N-10	0.57
Zygosaccharomyces bailii	N-11	0.93
Zygosaccharomyces bailii	N-12	0.44
Saccharomycodes ludwigii	N-13	1.01
Saccharomycodes ludwigii	N-14	nd
Saccharomycodes ludwigii	N-15	1.00
Hansenula saturnus	N-16	0.38
Hansenula sp.	N-17	nd
Pichia farinosa	N-18	0.11
Debaromyces hansenii	N-19	nd
Debaromyces nicotianae	N-20	0.16
Rhodotorula glutinis	N-21	7.63
Mycoderma bispora	N-22	0.49
Mycoderma bispora	N-23	nd
Hansenula anomala	N-24	nd
Metschnikowia pulcherrima	N-25	0.16
Kloeckera apiculata	N-26	nd
Hanseniaspora uvarum	N-27	0.19
Zygosaccharomyces bailii	N-28	nd
Zygosaccharomyces bailii	N-29	1
Brettanomyces sp.	N-30	nd
Brettanomyces sp.	N-31	nd

One unit of enzyme is defined as that amount of enzyme which releases the colour equivalent of 1 μg of tyrosine in 1min.

nd denotes not detectable.

Table 4-1 (continued) Proteolytic activity of yeasts grown in synthetic medium

Yeast species	Strain/Code*	Proteolytic activity (unit)
Zygosaccharomyces bailii	Z-CM	1.01
Kluyveromyces thermotolerans	K-MB	nd
Torulaspora delbrueckii	T-MB	0.08
Hanseniaspora uvarum	H 045	1.22
Hanseniaspora uvarum	H 097	1.36
Hanseniaspora uvarum	H 182	1.36
Hanseniaspora uvarum	H 155	0.87
Hanseniaspora uvarum	H 030	1.11
Hanseniaspora uvarum	H 210	0.25
Hanseniaspora uvarum	H 146	1.08
Metschnikowia pulcherrima	M 004	1.05
Rhodotorula sp.	R-1	1.37
Rhodotorula sp.	R-2	0.37
Rhodotorula sp.	R-3	21.11
Rhodotorula sp.	R-4	2.44
Saccharomyces cerevisiae	S-CM	nd
Saccharomyces cerevisiae	S-CEG	nd
Saccharomyces cerevisiae	S-CY	nd
Saccharomyces cerevisiae	S-EC	nd
Saccharomyces cerevisiae	S-S6U	nd

One unit of enzyme is defined as that amount of enzyme which releases the colour equivalent of 1 μg of tyrosine in 1min.

nd denotes not detectable.

4.1.2 Protease production in grape juice

To investigate protease production in grape juice, yeasts as shown in **Table 4-2** were selected based on their ability to exhibit proteolytic activity. They were grown in pasteurized grape juice. The properties of the juice employed in this study were 18 ^{o}Brix, pH 3.22 and total acidity of 8.46 g/l. The distinct sugar consumption of yeasts measured as total soluble solids (TSS) is noted in **Table 4-2**. *S. ludwigii* (N-13) utilized the highest amount of sugars whereas *Rhodotorula* spp.(R3, R4) consumed only little amount of sugars.

Table 4-2 Effect of protease production of yeasts on concentration of total soluble solids (TSS) of fermented grape juice and on their viable population after fermentation

Yeasts	TSS (^{O}Brix)	Yeast cells/ml
H. uvarum (H 045)	16.0	1.25 x10^{8}
H. uvarum (H 097)	13.5	3.78 x10^{7}
K. thermotolerans (K-MB)	6.0	3.44 x10^{8}
M. pulcherrima (M 004)	8.0	4.47 x10^{8}
Rhodotorula sp. (R-3)	17.5	2.59 x10^{8}
Rhodotorula sp. (R-4)	17.5	1.33 x10^{8}
S. ludwigii (N-13)	5.5	8.44 x10^{7}
T. delbrueckii (T-MB)	6.3	3.28 x10^{8}
Z. bailii (Z-CM)	7.0	1.44 x10^{8}

Figure 4-1 shows the protease activity of yeasts after 3-day growth in pasteurized grape juice. It was found that *M. pulcherrima* (M 004) showed protease activity as high as *Rhodotorula* sp. (R 3), whereas very low activity was found in *H. uvarum* (H 045). Other investigated strains demonstrated undetectable protease activity.

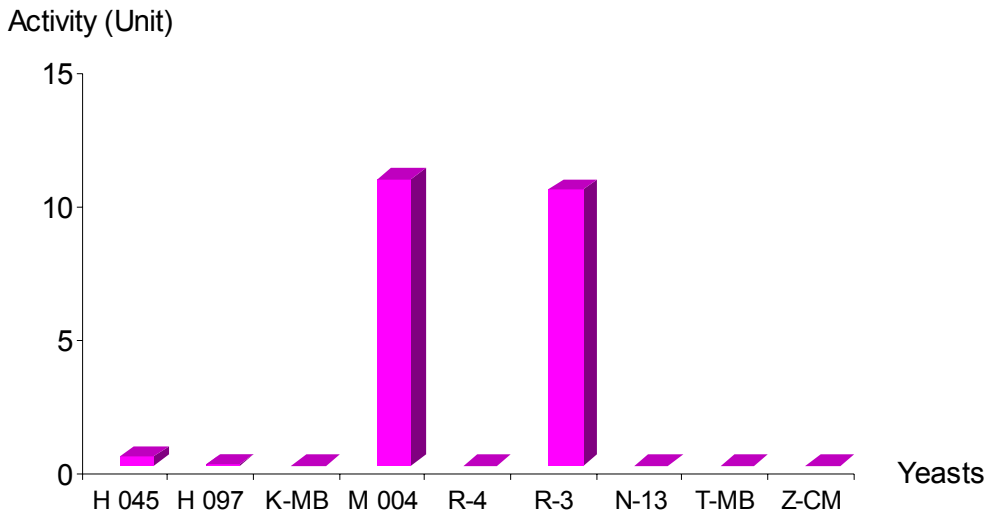


Figure 4-1 Proteolytic activity in supernatants obtained from yeasts grown in grape juice; systematic names of the yeasts are noted in **Table 4-1**.

4.1.3 Effect of proteins isolated from must on yeast growth

Crude proteins were isolated from must by precipitation with alcohol as described in the Section 3. These crude proteins were called in this study isolated proteins (IP). In order to examine the potential of yeasts to use grape proteins, synthetic grape juice (SGJ) with different sources of nitrogen were used; WON, without nitrogen source; IP, with protein isolated; WN, with ammonium sulphate. Non-*Saccharomyces* yeasts showing protease activity; *M. pulcherrima* (M 004) and *H. uvarum* (H 097) and non-*Saccharomyces* yeasts showing undetectable protease activity; *T. delbruekii* (T-MB) and *K. thermotolerans* (K-MB), in the previous studies were applied in this experiment.

The concentrations of proteins and ammonium of the IP are shown in **Table 4-3**. The investigation of amino acids revealed that, the concentration of the total amino acids in the IP was 981 mg/l. The addition of the IP to SGJ resulted in a total concentration of amino acids of 17 mg/l in the SGJ supplemented with the IP.

Table 4-3 Nitrogen-containing components in the isolated proteins (IP) from must and in the synthetic grape juice (SGJ) supplemented with the IP

Component	IP	SGJ supplemented with the IP
Protein (mg/l)	11400	200
Total amino acids (mg/l)	981	17
Ammonium (mg/l)	Trace	Trace

The use of alcohol to isolate proteins from must resulted in a protein fraction that contained also free amino acids as shown in **Table 4-3**. These amino acids were determined and their composition is demonstrated in the chromatogram (**Figure 4-2**).

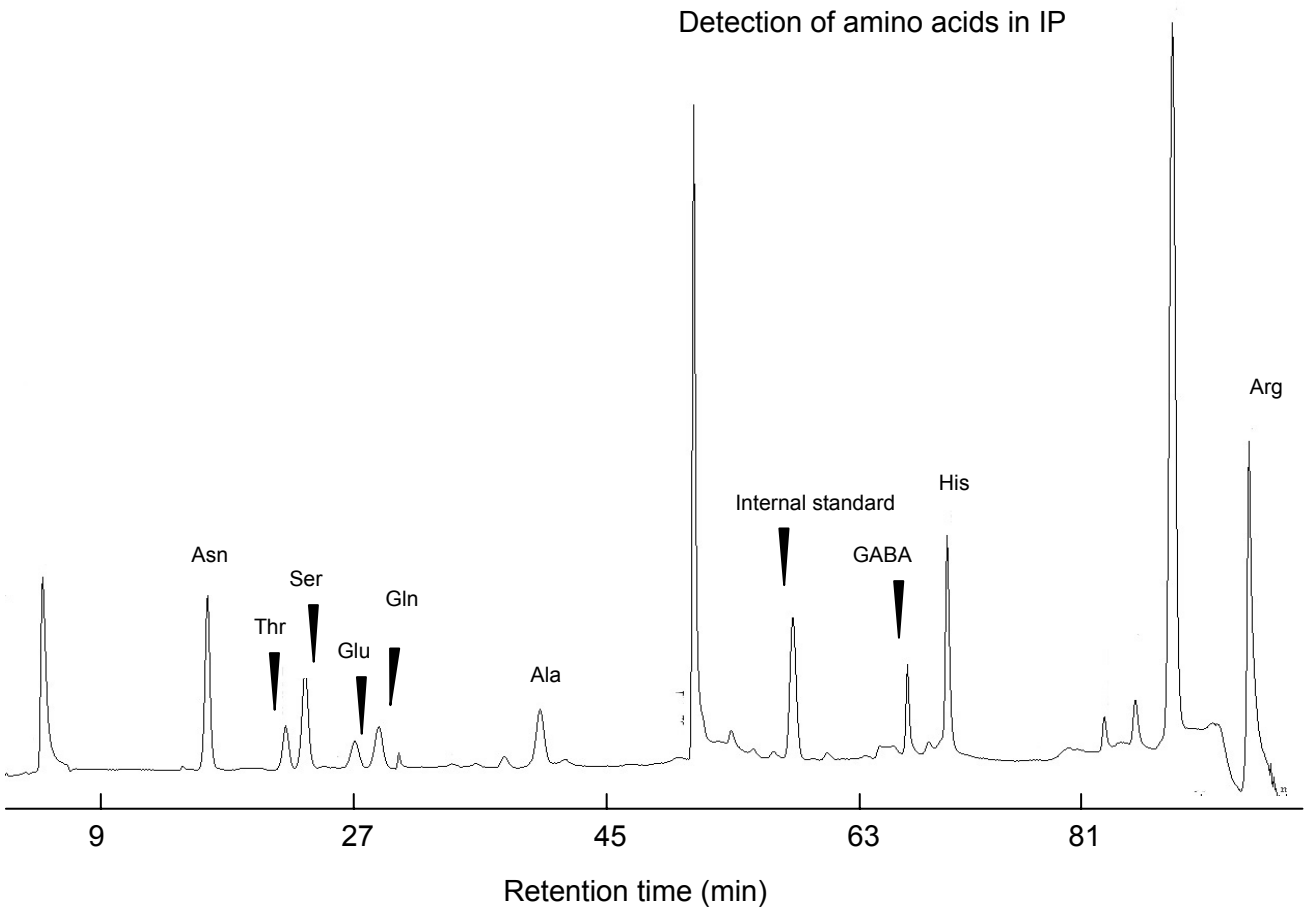


Figure 4-2 Chromatogram of amino acids found in isolated proteins (IP) from grape juice

The IP was added to SGJ to obtain the final concentration of proteins of 200 mg/l. This SGJ supplementd the IP was determined for amino acids. It was found that amino acids were not detected in the medium supplemented protein isolated as shown in the chromatogram (**Figure 4-3**).

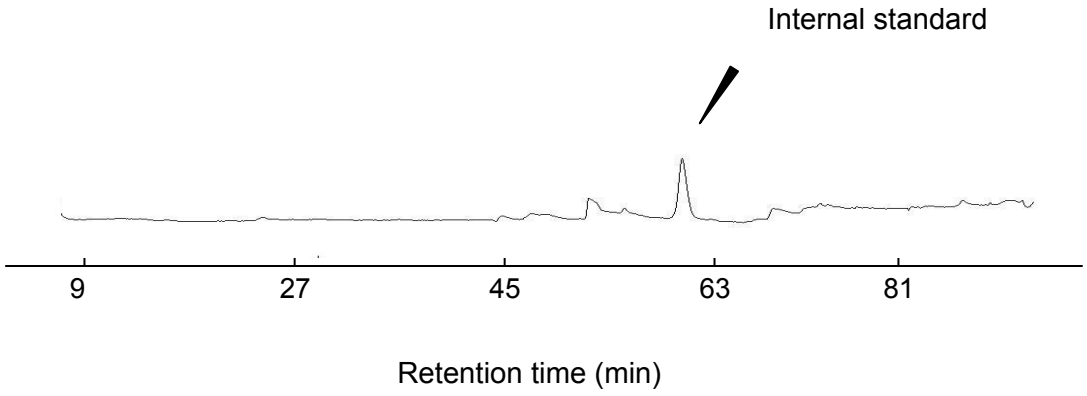


Figure 4-3 Chromatogram of amino acids found in the synthetic grape juice (SGJ) supplemented with the isolated proteins (IP)

The SGJ supplemented the IP was inoculated with yeasts and the growth kinetics of the yeasts were evaluated by optical density at wavelength 600 nm (OD_{600}). Monitoring OD_{600} displayed that yeasts could grow in the SGJ supplemented with the IP. The kinetics of yeast growths are illustrated in **Figure 4-4**.

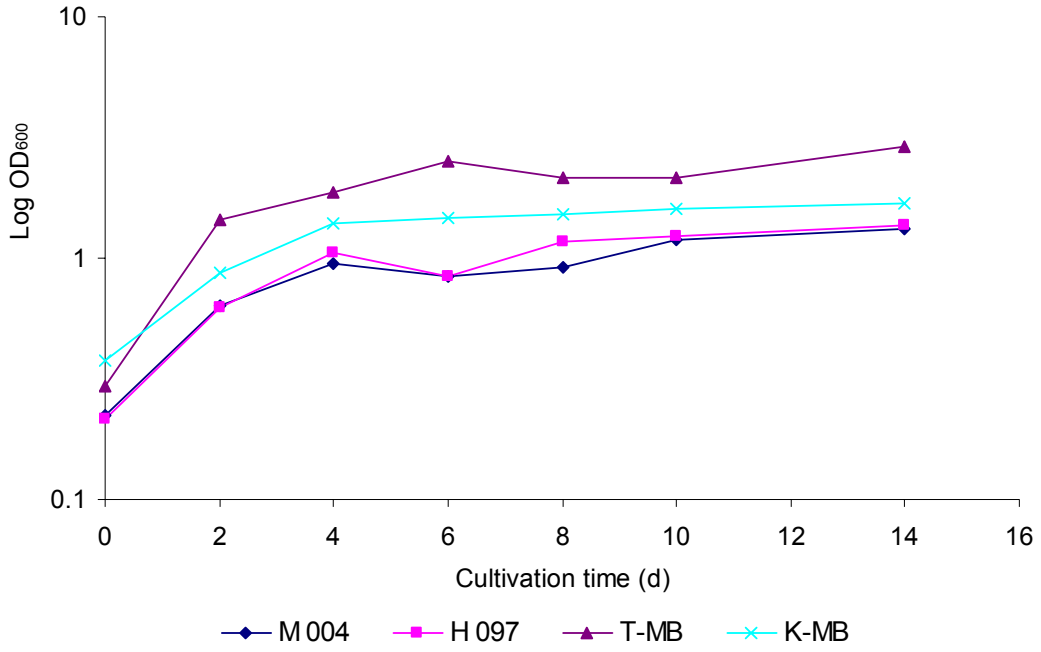


Figure 4.4 Yeast growth in the synthetic grape juice (SGJ) containing isolated proteins (IP), systematic names of yeasts are noted in **Table 4-1**

An addition of the isolated proteins to synthetic grape juice (SGJ) resulted in an increase of cell concentration, while using ammonium sulphate as nitrogen source yielded the highest cell density (**Figure 4-5**). In the SGI supplemented with isolated proteins, the multiplication of *M. pulcherrima* (M 004), *H. uvarum* (H 045), and *K. thermotolerans* (K-MB) was slightly higher than without nitrogen source. Compared with other yeasts, *Torulaspora delbrueckii* (T-MB) had better growth. The higher growth of yeasts was obviously observed in the SGJ supplemented with ammonium sulphate.

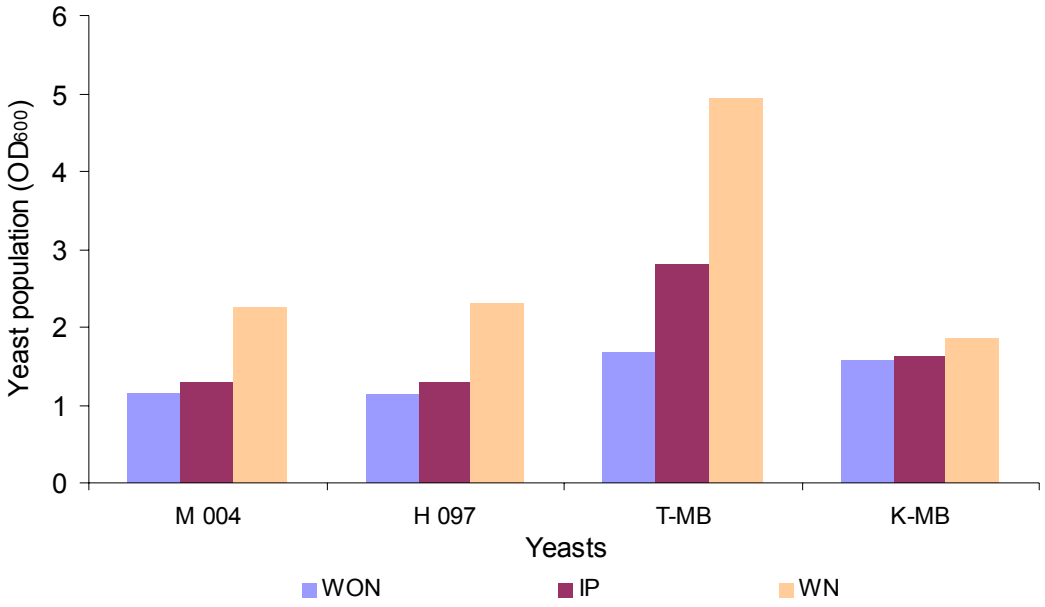


Figure 4-5 Optical density at wavelength 600 nm of the synthetic grape juice (SGJ) containing different sources of nitrogen; systematic names of yeasts are noted in **Table 4-1**

4.2 Fermentative characteristics of non-*Saccharomyces* yeasts exhibiting extracellular proteases in fermentation of grape juice

Fermentation was performed in pasteurized grape juice. Eleven yeast strains were selected from the previous study to ferment grape juice. The fermentation kinetics and some metabolites in final products were determined. Plating of samples that were taken during fermentation on HDM medium was used to control homogeneity of growth during fermentation. The diagnosis was based on the colonial characteristic. It was found that contaminated colonies were not distinguished on the medium throughout the investigation of the fermentation course. This can be expressed that only inoculated strains grew in the must during fermentation.

Fermentation kinetics and concentrations of fermentation products are shown in **Figure 4-6** and **Table 4-4**, **4-5**, **4-6**, **4-7**, **and 4-8**. The fermentation kinetics, concentrations of residual sugar and ethanol at the end of fermentation were substantially different even in the same species. The factors of genera, species, and strains affected fermentation kinetics. Within the

11 examined yeasts, *Z. bailii* (N-29) showed the highest of growth rate. The results also showed that certain yeasts could ferment sugar as rapid as *Saccharomycodes ludwigii* (N-13). The variability of growth of individual strains was examined within the strains of *Zygosaccharomyces bailii* and *S. ludwigii*. The inability to ferment sugar of *Rhodotorula glutinis* (N-21) was evident by no development of the fermentation kinetic.

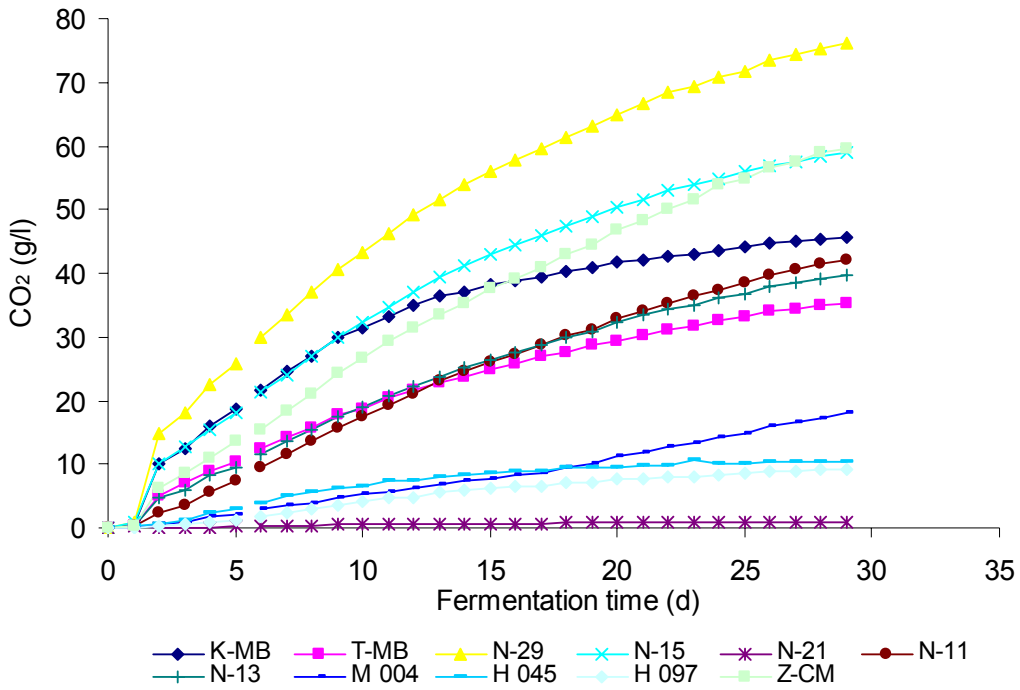


Figure 4-6 Fermentation kinetics of non-*Saccharomyces* yeasts during fermentation of grape juice; systematic names of yeasts are noted in **Table 4-1**

The analysis of the concentrations of residual sugars in the final products showed differences between the different strains in their capability of fermentation. *Z. bailii* (N-29) consumed the highest amount of sugars and produced the highest amount of ethanol (**Table 4-4**). *Z. bailii* strain Z-CM and N-29 had similar rate of sugar consumption but produced different amounts of ethanol. Species of *Metschnikowia pulcherrima* (M 004) and *Hanseniaspora uvarum* (H 045 and H 097) poorly fermented sugars as high concentrations of the residual sugars were left in final products.

Concerning the concentration of acids, malic acid did not vastly vary in any fermentation of the yeasts (**Table 4-4**). High concentration of volatile acids in fermented must was found for species *Z. bailli* (N-11) and *H. uvarum* (H 045), whereas the concentration of *K. thermotolerans* was the lowest. *K. thermotolerans* was the only yeast which produced an extremely high amount of lactic acid.

Table 4-4 Composition of end products obtained from grape juice fermentation with non-*Saccharomyces* yeasts

	Yeasts*										
Composition	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
Reducing Sugars (g/l)	91.8	95.6	55.4	73	177.9	86.4	87.7	96.1	136.4	120.4	57.6
Alcohol (% v/v)	4.2	3.1	8.2	6.1	0.2	3.7	3.3	1.4	1.2	0.9	4.8
Glycerol (g/l)	2.8	2.1	5.1	4.9	1.3	3.6	2.9	2.0	2.2	1.8	3.5
Volatile acidity (g/l)	0.1	0.3	0.3	0.4	0.3	0.8	0.3	0.3	0.6	0.5	0.2
pH	3.2	3.2	3.0	3.1	3.2	3.4	3.2	3.2	3.2	3.2	3.3
Malate (g/l)	3.2	3.0	3.0	2.8	3.0	3.0	3.1	3.3	3.0	3.1	2.7
Lactate (g/l)	2.1	0.2	0.2	0.2	0.2	0.6	0.2	0.5	0.4	Trace	0.7
Total Acidity (g/l)	5.3	4.5	5.1	4.9	5.1	3.9	4.5	3.5	4.7	4.1	4.0

* Systematic names of yeasts are noted in **Table 4-1**.

The concentrations of metabolites binding sulphur dioxide, acetaldehyde, pyruvate, and α-ketoglutaric acid were found in the end products at different quantities (**Table 4-5**). *Rhodotorula glutinis* (N-21) and *Zygosaccharomyces bailii* (Z-CM) produced lower pyruvate than other yeast strains.

Table 4-5 Concentrations of metabolites binding sulphur dioxide in end products obtained from grape juice fermentation with non-*Sccharomyces* yeasts

	Yeasts*										
Products	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
Acetaldehyde (mg/l)	8	5	20	7	10	20	10	14	35	8	24
Pyruvate (mg/l)	177	180	74	119	10	112	143	174	111	116	13
α-Ketoglutarate (mg/l)	100	29	49	37	9	23	16	47	13	17	25

* Systematic names of yeasts are noted in **Table 4-1.**

The production of sulphur-containing compounds by the 11 strains of non-*Saccharomyces* yeasts is shown in **Table 4-6**. Different quantities of the concentrations of sulphur-containing compounds were found in the end products. *Saccharomycodes ludwigii* (N-15) produced the highest concentration of hydrogen sulphide (7.2 μg/l), whereas it was not detectable in juice fermented with the strain *R. glutinis* (N-21) and the 2 strains of *H. uvarum* (H 045, H 097). Dimethyl disulphide was only detected in the final products fermented with *Rhodotorula glutinis* (N-21), *S. ludwigii* (N-13), *Metschnikowia pulcherrima* (M 004), *H. uvarum* (H 045, H 097), and *Zygosaccharomyces bailii* (Z-CM) Other investigated sulphur-containing compounds, methanethiol, ethanethiol, carbon disulphide, thioacetic-S-methyl ester, thioacetic-S-ethyl ester, diethyl disulphide, dimethyl trisulphide were not detected in the end products of all treatments.

Table 4-6 Concentrations of sulphur-containing compounds in end products obtained from grape juice fermentation with non-*Saccharomyces* yeasts

	Yeasts*									
Products	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
H_2S (μg/l)	2.2	1.6	2.3	7.2	n.d.	2.0	2.0	2.3	nd	nd	2.1
DMS (μg/l)	1.1	1.1	1.1	1.0	1.4	0.9	1.0	0.9	1.3	1.2	0.7
DMDS (μg/l)	nd	nd	nd	nd	7.5	nd	5.4	5.5	6.8	5.9	6.0

nd denotes not detectable. *Systematic names of yeasts are noted in **Table 4-1**.
H_2S, hydrogen sulphide; DMS dimethyl sulphide; DMDS, dimethyl disulphide

Figure 4-7 shows the concentrations of free alpha amino nitrogen (FAN) estimated by ninhydrin method varied among species and strains. Differences between strains can be observed in the species *Z. bailii*. *Z. bailii* (N-11) displayed the highest concentration of FAN (60 mg/l), whereas *Z. bailii* (N-29) displayed the lowest concentration of FAN (18 mg/l).

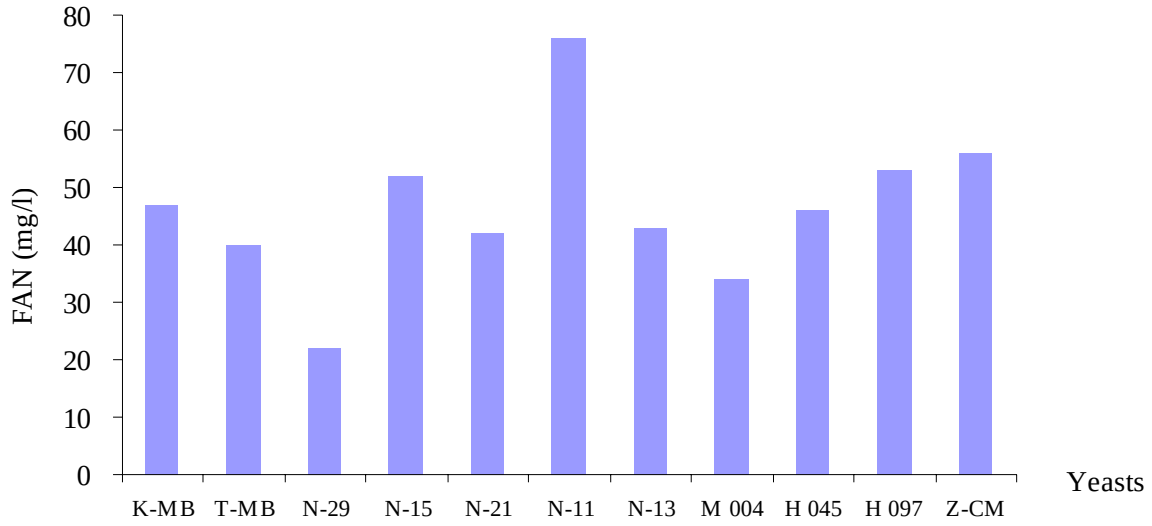


Figure 4-7 Concentrations of assimilable nitrogen in fermented grape juice at the end of fermentation expressed as free α-amino nitrogen (FAN); systematic names of yeasts are noted in **Table 4-1**

Amino acids in fermented juice were determined and their concentrations are presented in **Table 4-7**. The production of odouriferous compounds was evaluated as shown in **Table 4-8**. The amounts of components found in fermented grape juice of non-*Saccharomyces* species were different. The concentrations of esters vastly varied in the final products. Acetic acid ethyl ester was the principal odouriferous compound produced by all yeast strains except *Rhodotorula glutinis* (N-21). Species *Saccharomycodes ludwigii* (N-15, N-13) extremely produced this compound. The concentrations of acetic acid 3-methylbutyl ester found in the final products were different depending on both species and strains of yeasts, whereas another acetic acid ester, acetic acid phenylethyl ester was not detected. Genus and species of yeasts were also the factors that affected the concentrations of higher alcohols, i.e. 2 methyl propanol, 3-methyl butanol, 2-methyl butanol, and 2 phenylethanol, in the final products. *S. ludwigii*, for example, had the potential to produce greater concentrations of higher alcohols than *Z. bailii*, although *S. ludwigii* consumed a lower amount of sugars. *Kluyveromyces thermotolerans* (K-MB) produced remarkably higher concentration of 2-pheynylethanol than other yeast species.

Table 4-7 Concentration of amino acids in fermented grape juice at the end of fermentation

Concentration (mg/l)	Yeast*											
	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM	Must
Alanine	93	103	59	107	111	100	132	153	110	100	107	93
Arginine	176	217	155	161	278	209	210	301	276	261	220	260
Asparagine	5	5	1	21	6	8	5	7	5	4	5	4
Aspartic acid	21	28	1	39	55	46	28	51	48	39	34	42
Citrulline	9	10	11	13	10	13	14	13	9	11	11	10
Cystine	1	1	1	1	1	1	Trace	1	1	1	1	6
Glutamine	30	47	12	48	51	52	43	46	44	42	49	45
Glutamic acid	38	49	12	51	57	57	53	60	53	53	50	56
Glycine	8	5	7	7	5	5	6	5	3	4	8	3
Histidine	7	18	3	8	21	15	14	15	19	20	11	19
Isoleucine	2	17	Trace	18	30	26	21	10	22	23	15	26
Leucine	1	19	Trace	19	36	27	23	7	27	29	18	33
Lysine	5	1	1	1	5	1	1	1	Trace	1	1	5
Methionine	1	Trace	Trace	3	7	4	4	3	2	3	3	6
Ornithine	1	3	7	2	2	2	3	2	1	2	3	2
Phenylalanine	1	19	1	21	39	27	27	17	31	33	24	38
Serine	35	42	1	58	66	61	52	61	62	56	39	56
Threonine	27	43	Trace	65	73	73	55	65	68	64	46	67
Tyrosine	5	9	Trace	9	12	10	10	10	10	11	10	11
Valine	5	6	Trace	19	16	Trace	27	9	7	16	13	16
GABA	89	91	16	95	101	92	107	116	97	84	97	82

*Systematic names of yeasts are noted in **Table 4-1**

GABA denotes γ-aminobutyric acid

Table 4-8 Concentration of odouriferous compounds found in fermented grape juice at the end of fermentation

Compounds (μg/l)	*Yeast										
	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
2-methyl propanol	14300	10400	24800	35200	nd	11700	20300	22500	12500	10300	22700
3-Methyl butanol	17300	15400	48600	87700	nd	17800	47400	30200	7000	4700	57500
2-Methyl butanol	12100	3900	7900	19100	nd	2300	8400	11000	5300	3800	13500
2-Phenylethanol	21500	7600	13200	16600	nd	18100	10400	15600	4900	3300	14400
Hexanol	1555	1655	1475	1714	1731	1960	1747	1263	2790	2659	1809
Acetic acid ethyl ester	20100	54300	35500	589100	nd	37000	699300	34500	141900	75300	88200
Acetic acid 3-methylbutyl ester	100	98	598	299	nd	99	204	294	98	100	505
Acetic acid 2-methylbutyl ester	nd	nd	51	23	nd	7	16	44	18	8	49
Acetic acid hexyl ester	nd	5	147	8	nd	16	9.5	83	7	6	149
Acetic acid phenylethyl ester	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
Acetic acid 2-phenylethyl ester	nq	27	179	13	nd	124	6	69	nq	nq	77
Propionic acid ethyl ester	318	141	38	560	nd	37	376	16	53	41	28
Isobutanoic acid ethyl ester	21	14	20	55	nd	16	25	nd	nd	nd	nd
Butanoic acid ethyl ester	11800	nq	170200	9900	nd	nq	4600	63300	nq	nq	114900

nd and nq are abbreviated for not detectable and not quantifiable respectively. *Systematic names of yeasts are noted in **Table 4-1**

Table 4-8 (continued) Concentration of odour compounds found in fermented grape juice at the end of fermentation

Compounds (μg/l)	*Yeast										
	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
Lactic acid ethyl ester	14200	1400	4600	2600	nd	1200	1900	900	900	800	1900
Hexanoic acid ethylester	113	83	544	113	nd	69	101	220	nd	nq	411
Succinic acid diethyl ester	nd	nd	651	nd	nd	nd	nd	nd	nd	nd	nd
Octanoic acid ethylester	18	20	428	21	nd	12	16	60	nq	nd	239
Decanoic acid ethylester	12	25	98	11	nd	n.q.	n.q.	17	nq	nq	81
Hexanoic acid	700	600	3500	800	nd	600	700	1900	nd	nd	3400
Octanoic acid	600	600	3200	600	nd	600	600	1500	500	500	2500
Decanoic acid	300	300	1100	200	nd	200	200	400	n.d.	n.d.	700
trans-Linalool oxide	18	16	18	22	18	16	17	16	16	14	20
cis-Linalool oxide	9	6	7	9	8	8	9	8	7	6	10
Linalool	86	81	89	73	74	92	69	66	71	73	85
α-Terpineol	56	52	55	53	49	54	53	49	48	43	57
Geraniol	12.4	19.5	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd and nq are abbreviated for not detectable and not quantifiable respectively. *Systematic names of yeasts are noted in **Table 4-1**

4.3 Effect of yeast producing proteases in mixed cultures for winemaking

A number of *Saccharomyces* and non-*Saccharomyces* yeasts were used to ferment grape juice. Yeast cultures were inoculated to must with different strategies; 1) single strain inoculation 2) co-strains inoculation and 3) sequential-strains inoculation. According to experimental design, results are separately presented in Section 4.3.1 and 4.3.2.

4.3.1 Influence of yeasts expressing protease activity on fermentation activity

To find out whether yeast exhibiting protease activity could support *Saccharomyces* yeasts growth, the sequential inoculation was performed in grape juice fermentation. Yeasts described in the previous Sections were selected to study their influences on fermentation activity. 3 of the 5 chosen strains were non-*Saccharomyces* yeasts which were inoculated to begin the fermentation and later cells of *S. cerevisiae* were added. The single strain fermentations of *S. cerevisiae* (S-EC) and *S. cerevisiae* (S-CM) were performed simultaneously.

Dimethyl dicarbonate (DMDC) and lysozyme were added into grape juice in order to suppress microflora. The study of the use of DMDC and lysozyme in must consisting of yeast cells below 300 CFU revealed that the treatment with DMDC 500 mg/l and lysozyme 250 mg/l could suppress growth of microorganisms for at least 30 days. Furthermore, it was found that the application of DMDC 250 μg/l and lysozyme 250 mg/l could retard growth of microorganism in grape juice with an initial yeast population of 4.48 $x10^3$ CFU for approximately 2 weeks. Thus the amounts of 500 μg/l DMDC and 250 mg/l lysozyme were applied in this study.

The fermentation kinetics monitored by carbon dioxide production are shown in **Figure 4-8**. The fermentation characteristics varied according to different strains and inoculation treatments. The growth development of the sequential inoculation of *H. uvarum* and *S. cerevisiae* (H 045/S-EC) reached a maximum peak faster than the single strain inoculation of S-EC. *S. cerevisiae* (S-CM) had a lower rate of the conversion of sugar to alcohol and carbon dioxide than *S. cerevisiae* (S-EC).

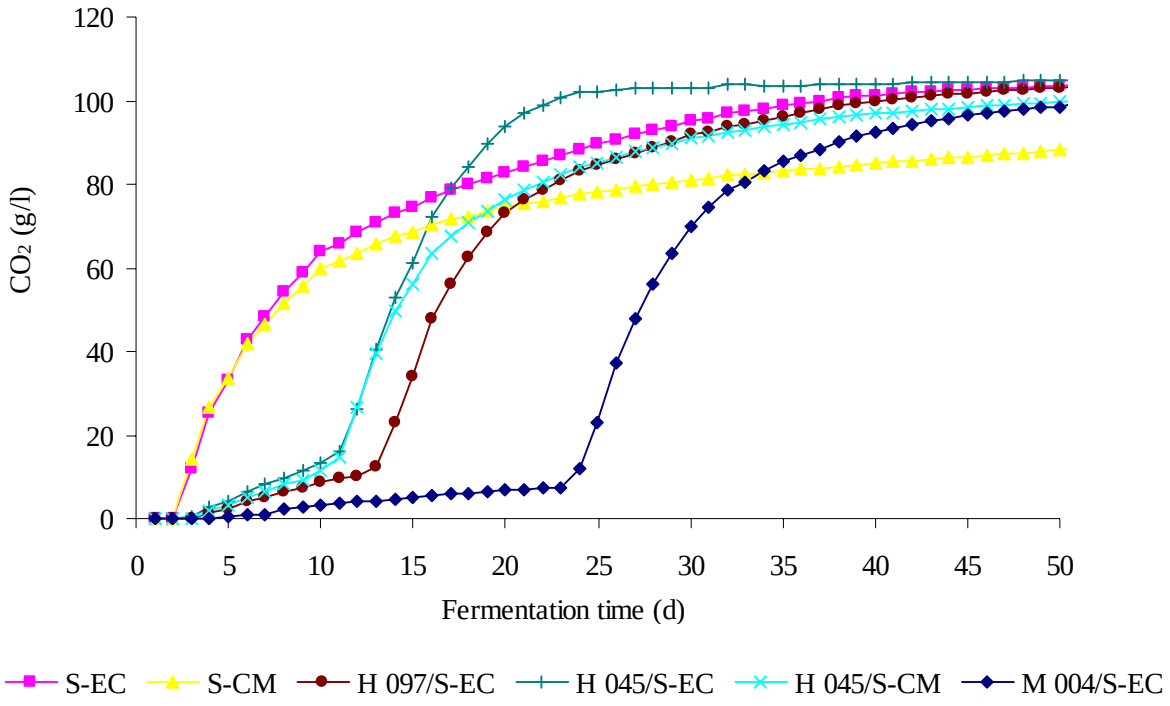


Figure 4-8 Growth kinetics of grape juice fermentation with single and sequential Inoculation; systematic names of yeasts and inoculation protocols are noted in **Table 3-2**

The highest concentration of alcohol with the lowest of residual sugar was found in wine treated with a sequential inoculation of H 045/S-EC. The concentrations of residual sugar of the sequential inoculation, H 045/S-EC (0.5 g/l) and H 045/S-CM (6.9 g/l) were substantially lower at the end of fermentation in comparison to pure strain fermentation of *S. cerevisiae*. Compared with pure strain inoculation of *S. cerevisiae* (S-CM), the yield of alcohol was also improved when sequential inoculation (H 045/S-CM) was applied to fermentation as shown in **Table 4-9**.

Table 4-9 Composition of wines from fermentation of single and sequential inoculation

	*Yeast					
Composition	S-EC	S-CM	H097/S-EC	H045/S-EC	H045/S-CM	M004/S-EC
Reducing sugars (g/l)	2.1	35.1	2.9	0.5	6.9	4.7
Alcohol (%v/v)	13.3	11.7	13.2	13.3	13.0	13.1
Glycerol (g/l)	5.2	5.7	6.5	6.4	5.8	6.3
Volatile acidity (g/l)	0.5	0.5	0.6	0.6	0.6	0.5
pH	3.6	3.5	3.7	3.7	3.7	3.6
Total acidity (g/l)	4.7	5.6	5.4	5.6	5.4	5.5

*Systematic names of yeasts and inoculation protocols are noted in **Table 3-2**

Figure 4-9 shows concentrations of free alpha amino nitrogen (FAN) in wines. The trend of FAN values of wines initially inoculated with *Hanseniaspora* species was slightly higher than that of wines inoculated without these species. Despite the low concentration of FAN (68 mg/l) and the high sugar concentration (210 g/l) in the must applied in this trial, the fermentation proceeded. Although the fermentation retarded, the target concentration of residual sugar (less than 2 g/l) was achieved in the wine inoculated with the sequential protocol of *H. uvarum* and *S. cerevisiae* (H 045/S-EC). The concentration of free alpha amino nitrogen (FAN) in this wine also remained higher than in wines with other inoculation treatments. The tendency of FAN concentration corresponded to the concentrations of amino acids (**Table 4-10**). This effect seemed to reflect in the wine inoculated with *H. uvarum* (H 097) as well.

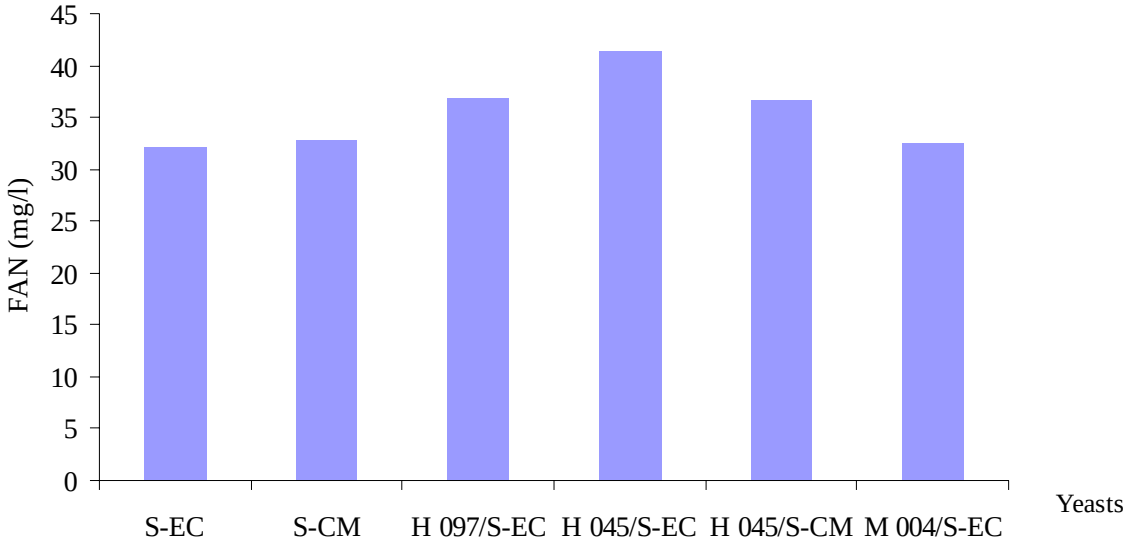


Figure 4-9 Assimilable nitrogen expressed as free α-amino nitrogen (FAN) in wines from fermentation of single and sequential inoculation; systematic names of yeasts and inoculation protocols are noted in **Table 3-2**

As shown in **Table 4-10**, within inoculation protocols, the concentrations of amino acid in wines fermented with S. cerevisiae (S-EC) remained lower in comparison to wine fermented with mixed cultures, i.e. H 097/S-EC, H 045/S-EC, and H 045/S-CM. Alanine, arginine, glutamine, glutamic acid, serine, threonine, and γ-aminobutyric acid (GABA) were largely absorbed by yeasts during fermentation. In contrast, the increase of the concentration of proline was detected in all wines.

Table 4-10 Concentration of amino acids in wines from fermentation of single and sequential inoculation

Amino acid		*Yeast					
(mg/l)	Must	S-EC	S-CM	H097/S-EC	M004/S-EC	H045/S-EC	H045/S-CM
Alanine	67	15	23	23	18	21	25
Arginine	218	16	21	19	18	27	33
Asparagine	6	9	10	12	13	9	12
Aspartic acid	24	7	11	11	8	13	11
Citrulline	3	Trace	Trace	Trace	Trace	Trace	Trace
Cystine	3	7	5	6	6	6	5
Glutamine	52	4	4	5	4	2	6
Glutamic acid	79	13	20	20	14	19	23
Glycine	3	8	9	10	7	7	10
Histidine	16	10	9	8	6	8	7
Isoleucine	12	3	6	5	4	7	7
Leucine	17	13	22	18	16	23	22
Lysine	4	22	28	31	20	34	29
Methionine	18	19	20	21	22	23	24
Ornithine	1	1	1	1	1	Trace	1
Phenylalanine	14	10	15	10	9	18	13
Proline	405	473	435	480	424	459	468
Serine	37	5	8	8	7	10	8
Threonine	56	5	8	7	6	8	6
Tyrosine	5	9	11	6	7	8	7
Valine	29	15	19	19	16	19	11
GABA	93	12	10	11	11	14	9
Total	1162	676	695	731	637	735	737
Total (no proline)	757	203	260	251	213	276	269
Total Nitrogen	195	90	94	98	85	100	102
Total Nitrogen (no proline)	145	32	41	40	33	44	45

*Systematic names of yeasts and inoculation protocols are noted in **Table 3-2**

4.3.2 Influence of inoculation treatment on fermentation of Riesling grape juice

This experiment purposed to investigate the effect of the inoculation protocols of *Saccharomyces* and non-*Saccharomyces* yeasts on metabolite formation of Riesling juice fermentation. Yeasts were inoculated into clarified and unclarified musts. The influence of the inoculation protocols on wine composition was examined. The strains *M. pulcherrima* (M-004), *H. uvarum* (H-097), *T. delbrueckii* (T-MB), *S. cerevisiae* (S-EC) and a mixed yeast product Harmony (Hmy) were used. The musts were obtained from grapes harvested in Geisenheim, Germany in the year 2006. The composition of the musts is shown in **Table 4-11**.

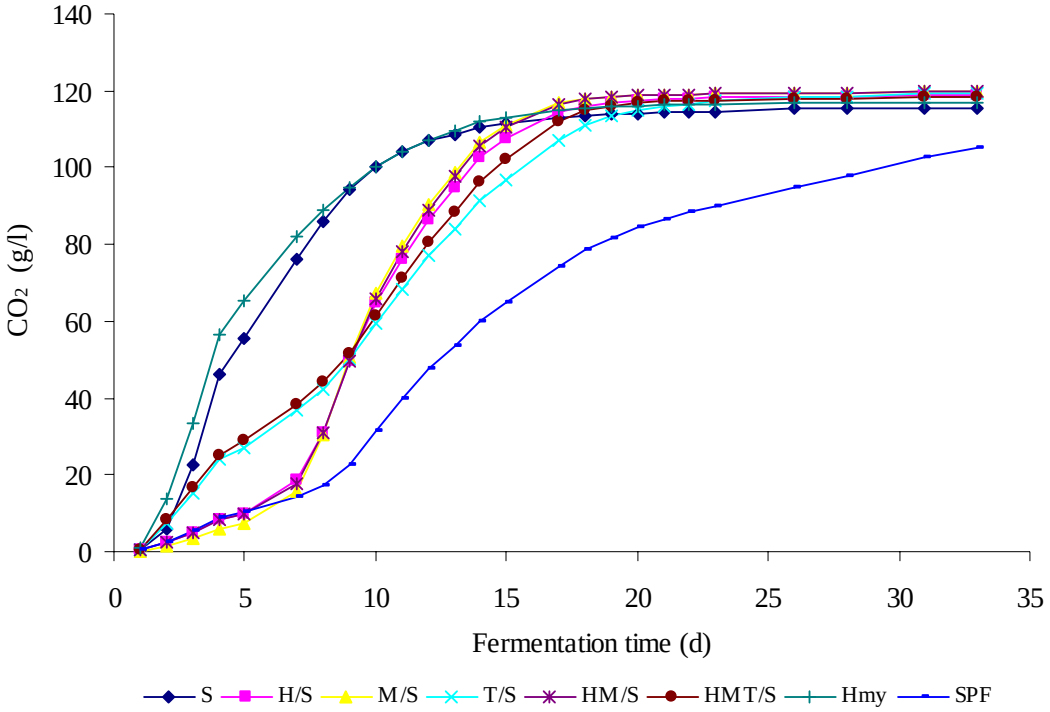
Table 4-11 Composition of clarified and unclarified musts

Must	pH	Total soluble solid (^{o}Brix)	Total acidity (g/l)	FAN (mg N/l)	Turbidity (NTU)
Clarified must	3.2	26.0	4.2	71.1	205
Unclarified must	3.2	26.0	4.1	84.0	586

FAN and NTU denote free alpha amino nitrogen and nephelometric turbidity units, respectively.

The profiles of fermentation kinetics in the clarified and unclarified musts of each inoculation protocol are shown in **Figure 4-10**. As expected, the fastest exponential phase was observed in wine where *S. cerevisiae* was used as an initial inoculation, whereas sequential culture fermentations of *S. cerevisiae* displayed an intermediate rapid rate. The inoculation treatments initiated with non-*Saccharomyces* yeasts (H 097, M004, and mixed culture of H 097 and M 004) displayed a slow rate of fermentation in the first 5 days but the fermentation rate drastically increased after the inoculation with *S. cerevisiae*. These effects were obviously observed in fermentation of the clarified must rather than the unclarified must. The yeast populations counted on lysine medium also confirmed the dominant presence of non-*Saccharomyces* species until *S. cerevisiae* was sequentially inoculated. As the fermentation progressed, the viable population of non-*Saccharomyces* yeasts slowly declined. The fermentation showed a relatively constant rate after 15 days until the point where fermentation ceased approximately after 34 days as determined by carbon dioxide production.

a



b

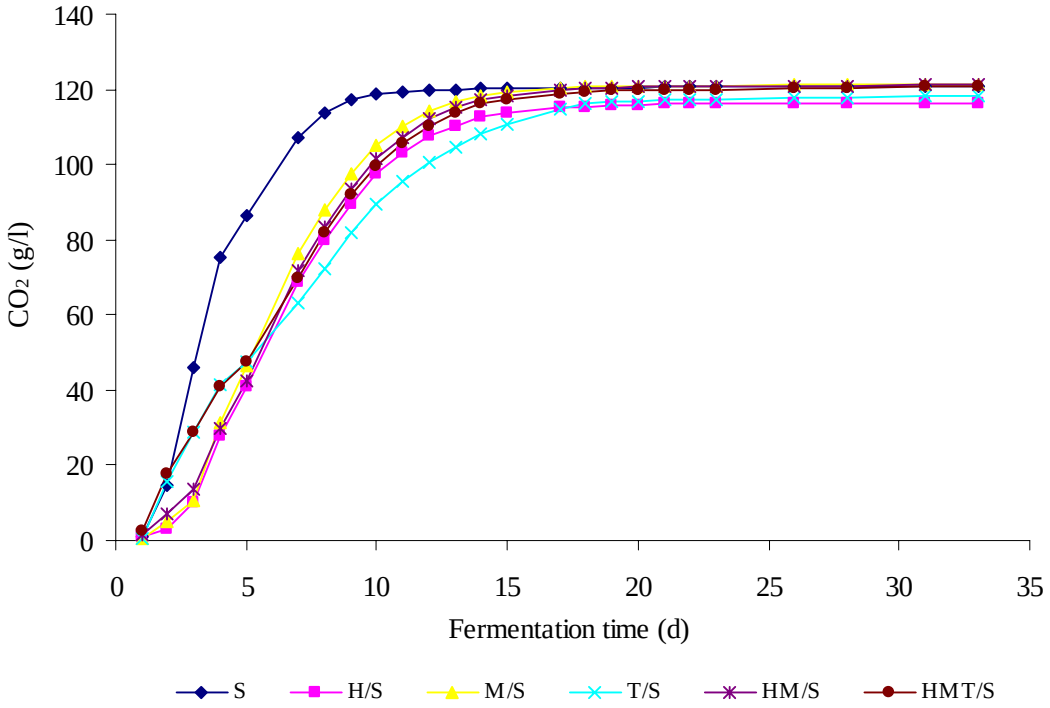


Figure 4-10 Effect of inoculation protocols on fermentation kinetics during fermentation of Riesling musts; a, clarified; b, unclarified; systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Concentrations of reducing sugars, alcohol, glycerol, volatile acidity, total acidity and pH value were evaluated in the wines after fermentation was finished. **Table 4-12** shows the composition of wines fermented from grape juice with the turbidity of 205 NTU. In monoculture of *S. cerevisiae* (S-EC), the concentration of remaining sugar after completion of the fermentation was 11.0 g/l and *S. cerevisiae* (Hmy) had the same concentration at the moment. It is interesting to note that when *S. cerevisiae* was used to initiate the fermentation, the remaining fructose concentration in wine was higher in comparison to wines made by sequential inoculation. Low concentrations of residual sugars in wine (3.5-4.1 g/l) were obtained from grape juice fermentation where sequential inoculation was applied. The concentrations of alcohol in wines were nearly at the same levels. Nevertheless the values were slightly higher when clarified juice was fermented with a sequential inoculation.

Table 4-12 Composition of the wines produced by fermentation of clarified Riesling grape juice fermented with different inoculation protocols

Composition	Yeast†						
	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Reducing sugars (g/l)	11.0	4.0	3.6	4.1	3.5	3.6	11.0
Alcohol (%v/v)	15.6	16.0	15.9	16.0	16.0	16.0	15.8
Glycerol (g/l)	12.8	13.3	13.6	13.0	13.4	13.4	12.1
Volatile acidity (g/l)	0.7	0.6	0.7	0.5	0.6	0.6	0.5
pH	3.6	3.6	3.6	3.6	3.7	3.6	3.5
Total acidity (g/l)	7.3	7.3	7.3	7.2	7.3	7.2	7.2

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table 4-13 shows the composition of wines fermented from grape juice with the turbidity of 586 NTU. No apparent differences of the wine composition could be observed between wines made by unclarified grape juice.

Table 4-13 Composition of the wines produced by fermentation of unclarified Riesling grape juice fermented with different inoculation protocols

Composition	Yeast†					
	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
Reducing sugar (g/l)	4.0	2.9	3.1	3.6	3.0	3.2
Alcohol (%v/v)	16.0	16.0	16.0	15.9	16.0	15.9
Glycerol (g/l)	14.0	14.6	14.6	14.3	14.5	15.0
Volatile acidity (g/l)	0.5	0.6	0.6	0.6	0.6	0.6
pH	3.5	3.6	3.6	3.6	3.6	3.6
Total acidity (g/l)	7.1	7.0	7.1	6.9	7.0	7.0

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

The concentrations of other primary fermentation metabolites in the wines are presented in **Table 4-14** and **4-15**. It seems like sequential inoculation of *S. cerevisiae* produced lower amounts of acetaldehyde, but higher of α-ketoglutarate than grape juice was only inoculated with *S. cerevisiae*.

Table 4-14 Concentrations of metabolites binding sulphur dioxide in wines from clarified Riesling grape juice fermented with different inoculation protocols

Composition	Yeast†						
	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Acetaldehyde (mg/l)	38	32	31	26	32	25	43
Pyruvate (mg/l)	22	18	19	27	18	28	20
α-Ketoglutarate (mg/l)	34	41	43	50	39	46	46

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table 4-15 shows the concentration of sulphur dioxide binding compounds in wines fermented from grape juice with the turbidity of 586 NTU. In comparison to wines made from grape juce with the turbidity 205 NTU, these wines had higher concentrations of α-ketoglutarate but lower concentrations of pyruvate.

Table 4-15 Concentrations of metabolites binding sulphur dioxide in wines from unclarified Riesling grape juice fermented with different inoculation protocols

Composition	Yeast†					
	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
Acetaldehyde (mg/l)	36	28	28	23	28	30
Pyruvate (mg/l)	16	14	12	29	16	26
α-Ketoglutarate (mg/l)	63	62	58	51	60	52

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

The concentrations of free alpha amino nitrogen (FAN) of wines where non-*Saccharomyces* yeasts were inoculated into their musts showed a tendency of higher concentrations than in wines obtained from fermentation without inoculation of non-*Saccharomyces* yeasts. In deed, the concentrations of FAN in wines from clarified juice were higher than unclarified juice (**Figure 4-11**). The result agrees with the values of the concentrations of total nitrogen without proline (**Table 4-16**, **Table 4-17**).

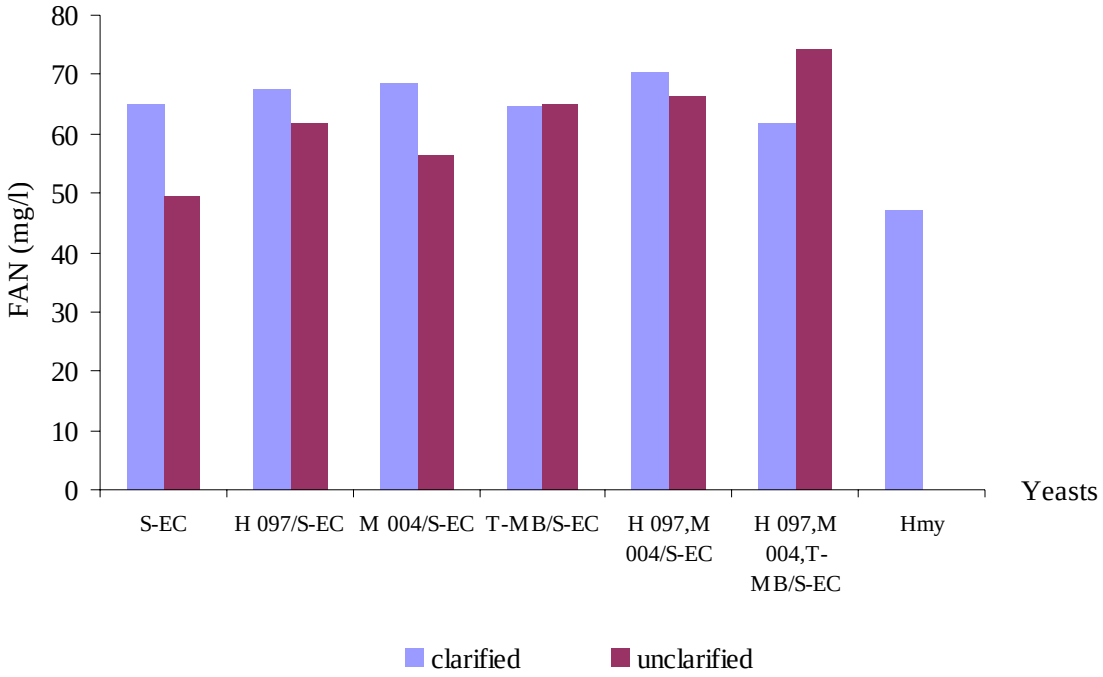


Figure 4-11 Assimilable nitrogen expressed as free α-amino nitrogen (FAN) in wines from clarified and unclarified musts fermented with different inoculation protocols; systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

The composition of amino acids was determined in grape juices and wines. The results are shown in **Table 4-16** and **Table 4-17**. It was found that, alanine, arginine, glutamine, γ-aminobutyric acid, and proline were predominant amino acids in both grape musts. Concentrations of arginine in wines were decreased but proline was increased from the original amount determined in musts. The concentrations of glutamine in clarified and unclarified musts were reduced from 111 and 108 mg/l, respectively, to trace amounts in wines. Considering the amino acid concentrations in wines made from clarified must, approximately 80% of arginine was taken up by

yeasts in single inoculation of *S. cerevisiae* and sequential inoculation, but 93% of arginine was comsumed by mixed culture product Harmony (Hmy). Besides serine and threonine, γ-aminobutyric acid was taken up in great amounts. By comparison, γ-aminobutyric acid was drastically consumed (92%, others 50-60%) in the use of mixed culture product (Hmy) as inoculant.

Table 4-16 Concentration of amino acids in clarified must and wines fermented with different inoculation protocols

Amino acids (mg/l)	Must	Yeast†						
		S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Alanine	102	36	47	37	29	39	25	51
Arginine	486	115	151	110	204	127	125	38
Asparagine	9	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Aspartic acid	26	33	35	33	29	34	29	33
Citrulline	11	8	8	8	8	9	7	8
Cystine	0	5	6	4	5	5	4	5
Glutamine	111	Trace	2	3	2	2	2	2
Glutamic acid	45	31	37	33	28	32	26	40
Glycine	7	16	20	17	21	18	21	17
Histidine	30	14	14	13	21	12	19	15
Isoleucine	8	7	8	6	5	8	6	8
Leucine	4	23	23	24	15	27	19	29
Lysine	19	35	33	32	24	31	20	41
Methionine	Trace	5	6	5	4	4	4	7
Ornithine	7	12	13	12	10	11	6	7
Phenylalanine	13	16	18	13	11	14	16	19
Proline	276	391	377	410	346	375	356	414
Serine	57	15	13	14	11	13	12	16
Threonine	45	8	9	9	6	8	7	10
Tyrosine	12	15	17	11	13	15	9	19
Valine	19	17	19	11	12	14	13	19
GABA	171	114	135	104	116	112	106	13
Total	1458	916	991	909	920	910	832	811
Total (no proline)	1182	525	614	499	574	535	476	397
Total Nitrogen	289	144	162	143	163	146	135	116
Total Nitrogen (no proline)	255	97	116	93	121	100	92	65

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

GABA denotes γ-aminobutyric acid

Table 4-17 Concentration of amino acids in unclarified must and wines fermented with different inoculation protocols

Amino acids (mg/l)	Must	Yeast†					
		S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
Alanine	102	31	42	38	37	45	43
Arginine	445	37	35	33	31	34	36
Asparagine	9	Trace	Trace	Trace	Trace	Trace	Trace
Aspartic acid	27	33	32	31	31	32	34
Citrulline	11	1	4	4	4	4	3
Cystine	Trace	6	6	7	8	8	8
Glutamine	108	2	2	2	1	1	2
Glutamic acid	45	36	39	42	37	45	43
Glycine	8	13	20	17	24	22	25
Histidine	26	16	15	17	24	14	22
Isoleucine	7	10	8	9	8	8	9
Leucine	3	34	29	30	26	26	30
Lysine	21	48	45	45	40	40	40
Methionine	Trace	8	8	9	9	9	11
Ornithine	7	1	3	4	7	7	7
Phenylalanine	10	22	21	20	18	19	21
Proline	251	418	421	413	420	390	402
Serine	58	16	17	16	17	17	18
Threonine	46	12	10	12	11	11	11
Tyrosine	10	16	15	14	16	18	17
Valine	21	20	19	19	19	22	21
GABA	163	98	49	123	122	93	16
Total	1378	878	840	905	910	865	819
Total (no proline)	1127	460	419	492	490	475	417
Total Nitrogen	270	123	119	128	129	123	117
Total Nitrogen (no proline)	240	72	67	77	77	75	68

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

GABA denotes γ-aminobutyric acid

The influence of different yeast species and inoculation protocols on the composition of odouriferous substances of wine was assessed as shown in **Table 4-18** and **4-19**. Inoculated yeasts in clarified and unclarified grape juice produced different odouriferous profiles. The composition of odouriferous compounds of wine fermented by the inoculation of a yeast mixture were different to the composition of wine fermented with *S. cerevisiae* inoculation alone. The treatment of grape juices of turbidities (205 and 586 NTU) with the same protocol of inoculation also yielded different odouriferous compounds in the wines. Wines inoculated with non-*Saccharomyces* yeasts had lower concentrations of hexanol. In fermentation of clarified juice, wines produced with sequential inoculation of *T. delbrueckii* had notably different profiles compared to those wines fermented with mixed cultures without this species. This was due to an increased production of higher alcohols. The different profile was obtained when this species was co-inoculated with *S. cerevisiae* species (Hmy).

Table 4-18 Concentrations of odouriferous compounds in wines from clarified Riesling grape juice fermented with different inoculation protocols

	Yeast†						
Compounds (μg/l)	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
2-methyl propanol	43800	43300	45500	47700	44800	41900	78000
3-Methyl butanol	85600	87800	91900	116600	104200	98000	71600
2-Methyl butanol	24600	21100	25000	32000	28200	24300	17200
2-Phenylethanol	18000	19100	18800	24700	19800	23100	15000
Hexanol	601	616	441	459	369	377	538
Acetic acid ethyl ester	178000	198600	155400	260400	186600	201700	125200
Acetic acid 3-methylbutyl ester	747	756	728	2960	806	2804	553
Acetic acid 2-methylbutyl ester	32	22	33	139	54	108	15
Acetic acid hexyl ester	32	35	24	79	27	61	37
Acetic acid phenylethyl ester	4	4	4	4	4	4	4
Acetic acid 2-phenylethyl ester	54	70	62	195	62	200	39
Propionic acid ethyl ester	60	44	45	93	51	74	29
Isobutanoic acid ethyl ester	50	42	49	66	57	58	78
Butanoic acid ethyl ester	254	253	242	281	252	220	271
Lactic acid ethyl ester	nq	nq	nq	nq	nq	nq	8
Hexanoic acid ethylester	956	835	841	530	801	462	929
Succinic acid diethyl ester	345	321	331	522	299	583	784
Octanoic acid ethylester	1136	1016	969	493	907	449	1175
Decanoic acid ethylester	482	401	358	215	307	154	432
Hexanoic acid	6500	5900	5900	4400	5600	3800	6300
Octanoic acid	6400	5400	5300	3500	4800	2800	5900
Decanoic acid	2600	2200	2100	1200	1600	800	2100
trans-Linalool oxide	nq	nq	nq	nq	nq	nq	nq
cis-Linalool oxide	nq	nq	nq	nq	nq	nq	nq
Linalool	98	106	103	106	100	100	99
α-Terpineol	36	36	33	32	33	31	34
Geraniol	nq	nq	nq	nq	nq	nq	nq

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

nq denotes not quantifiable.

Table 4-19 Concentrations of odouriferous compounds in wines from unclarified Riesling grape juice fermented with different inoculation protocols

Compounds (μg/l)	Yeast†					
	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
2-methyl propanol	58400	55200	55000	58000	59600	57800
3-Methyl butanol	132000	110300	108100	133100	119100	124900
2-Methyl butanol	33600	32900	31900	36500	36700	39000
2-Phenylethanol	34500	20400	24400	32500	24300	30400
Hexanol	614	524	442	499	479	354
Acetic acid ethyl ester	203400	230800	181800	223200	241700	198200
Acetic acid 3-methylbutyl ester	730	556	498	681	649	864
Acetic acid 2-methylbutyl ester	37	61	38	42	45	55
Acetic acid hexyl ester	10	7	4	7	11	8
Acetic acid phenylethyl ester	5	4	6	5	4	5
Acetic acid 2-phenylethyl ester	50	32	36	75	43	76
Propionic acid ethyl ester	75	44	38	72	41	64
Isobutanoic acid ethyl ester	70	52	58	78	49	90
Butanoic acid ethyl ester	252	361	349	240	367	263
Lactic acid ethyl ester	nq	nq	nq	nq	nq	nq
Hexanoic acid ethylester	897	821	917	397	929	473
Succinic acid diethyl ester	872	683	978	1145	661	1292
Octanoic acid ethylester	1101	813	1036	407	1007	491
Decanoic acid ethylester	327	215	293	168	303	149
Hexanoic acid	6100	5500	6200	4000	6200	4100
Octanoic acid	5300	4500	5500	2600	4900	2900
Decanoic acid	1700	1200	1600	800	1400	800
trans-Linalool oxide	nq	nq	nq	nq	nq	nq
cis-Linalool oxide	nq	nq	nq	nq	nq	nq
Linalool	107	106	112	108	108	108
α-Terpineol	34	34	37	35	33	33
Geraniol	nq	nq	nq	nq	nq	nq

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nq denotes not quantifiable.

Table 4-20 and **4-21** show concentrations of sulphur-containing compounds in wines. 5 of 11 examined sulphur-containing compounds were detected in wine made from clarified and unclarified musts. The influence of grape juice turbidity and inoculation protocols on the concentrations of sulphur-containing compounds was observed. The concentrations of sulphur-containing compounds of wines made from clarified grape juice were not much varied like wines made from unclarified grape juice. Wines made from clarified must had higher concentration of suphur dioxide but lower concentration of hydrogen sulphide, methanethiol, carbon disulphide than wines made from unclarified must. The concentration of hydrogen sulphide in wine made by unclarified must increased by two times of concentrations compared to the concentrations found in wines made by clarified grape juice. The concentration of dimethyl sulphide of both wine showed no difference, even they were treated with different inoculation protocols.

Table 4-20 Concentrations of sulphur-containing compounds in wines from clarified Riesling grape juice fermented with different inoculation protocols

Composition		Yeast†					
	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Total SO_2 (mg/l)	18	14	12	6	12	4	21
H_2S (µg/l)	22	16	19	12	21	17	26
MeSH (µg/l)	5	5	6	5	5	5	7
DMS (µg/l)	3	3	3	3	3	3	3
CS_2 (µg/l)	5	8	nd	4	7	nd	2

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

nd denotes not detectable.

SO_2 sulphur dioxide; H_2S, hydrogen sulphide; MeSH, methanethiol; DMS dimethyl sulphide; CS_2; carbon disulphide

Table 4-21 Concentrations of sulphur-containing compounds in wines from unclarified Riesling grape juice fermented with different inoculation protocols

Composition	S-EC	Yeast†				
		H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
Total SO_2 (mg/l)	13	7	8	5	7	6
H_2S (µg/l)	55	41	52	25	47	42
MeSH (µg/l)	11	9	10	6	9	8
DMS (µg/l)	3	3	3	3	3	3
CS_2 (µg/l)	12	9	47	49	16	16

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

SO_2 sulphur dioxide; H_2S, hydrogen sulphide; MeSH, methanethiol; DMS dimethyl sulphide; CS_2; carbon disulphide

4.4 Influence of yeasts on polypeptides and proteins in winemaking

In the experiments of this Section, peptides and proteins were studied. Investigation of peptides and proteins in must and wine, and their impact on wine making were focused.

4.4.1 Production of yeast proteins in synthetic medium

Seven strains of yeasts which have been reported as widely used or found in winemaking were cultivated in synthetic medium. *S. cerevisiae* (S-Rb) and mixed yeasts (Hmy) were prepared following the instruction of the manufacturer and other yeasts were prepared on agar medium. The proliferation after 3-day cultivation is shown in **Figure 4-12**. Pure strains of *S. cerevisiae* (S-EC and S-Rb) and mixed yeasts consisting *S. cerevisiae* could proliferate better than non-*Saccharomyces* yeasts. Among non-*Saccharomyces* yeasts, *M. pulcherrima* (M 004) showed the highest proliferation.

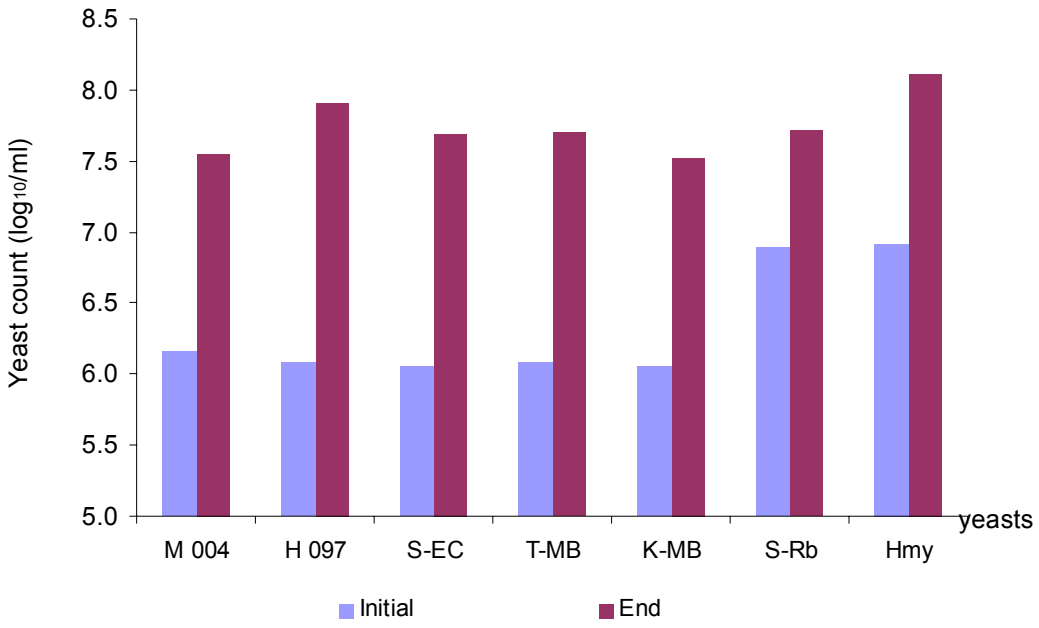


Figure 4-12 Total number of yeast cells at inoculation and after three days of cultivation in synthetic medium; systematic name of yeasts are shown in **Table 4-1**

The yeast species produced soluble extracellular peptides and proteins between 3600-12100 μg/l in culture medium (**Figure 4-13**).

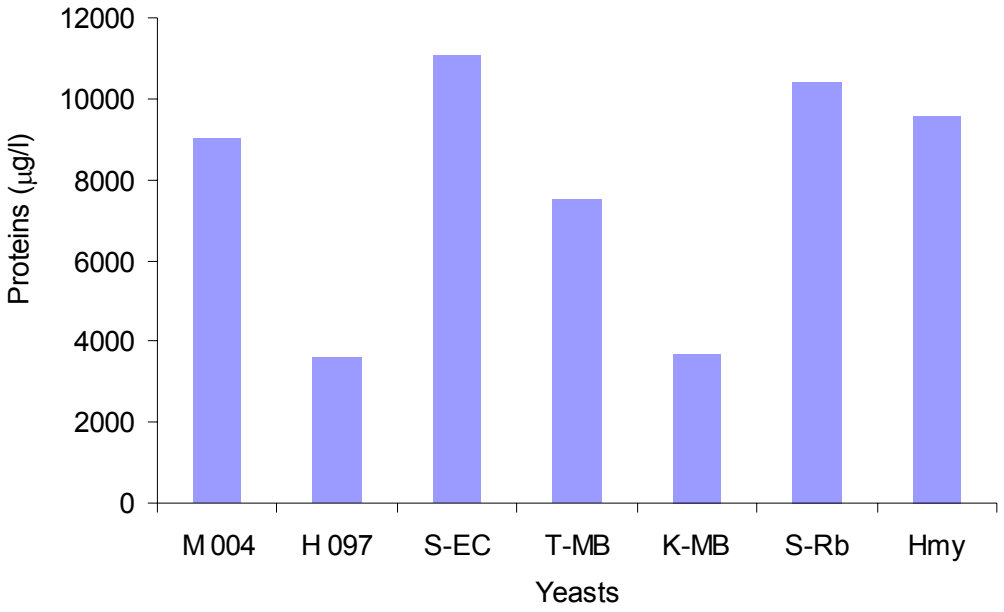


Figure 4-13 Concentrations of extracellular proteins released by species of non-*Saccharomyces* into synthetic defined medium; systematic name of yeasts are shown in **Table 4-1**

4.4.2 Investigation of polypeptides and proteins in fermented grape juice and wine

The separation of polypeptides and proteins was carried out by using ultrafiltration techniques. The Nanosep devices having a membrane with a molecular weight cutoff of 3 and 10 kDa were chosen to study polypeptides and proteins. Proteins were isolated by ultrafiltration with 10 kDa devices and polypeptides were separated by ultrafiltration of the permeate of the 10 kDa membrane on a 3 kDa membrane. The grape juices fermented by non-*Saccharomyces* yeasts were examined for soluble

peptides (≥ 3-10 kDa) and proteins (≥ 10 kDa). The description of these fermented juices is shown in Section 3.10. It was found that the concentrations of polypeptides and proteins in fermented must were between 14-1500 μg/l and 1490-2301 μg/l, respectively (**Figure 4-14** and **20**). The concentrations of proteins were slightly different in the fermented products inoculated with different yeast species. The maximum concentration of proteins found in the juice fermented with *Saccharomycodes ludwigii* (N-13). The concentrations of polypeptides were relatively different in fermented must inoculated with different yeast species. The maximum concentration of polypeptides was detected in the grape juice fermented with *Hanseniaspora uvarum* (H097).

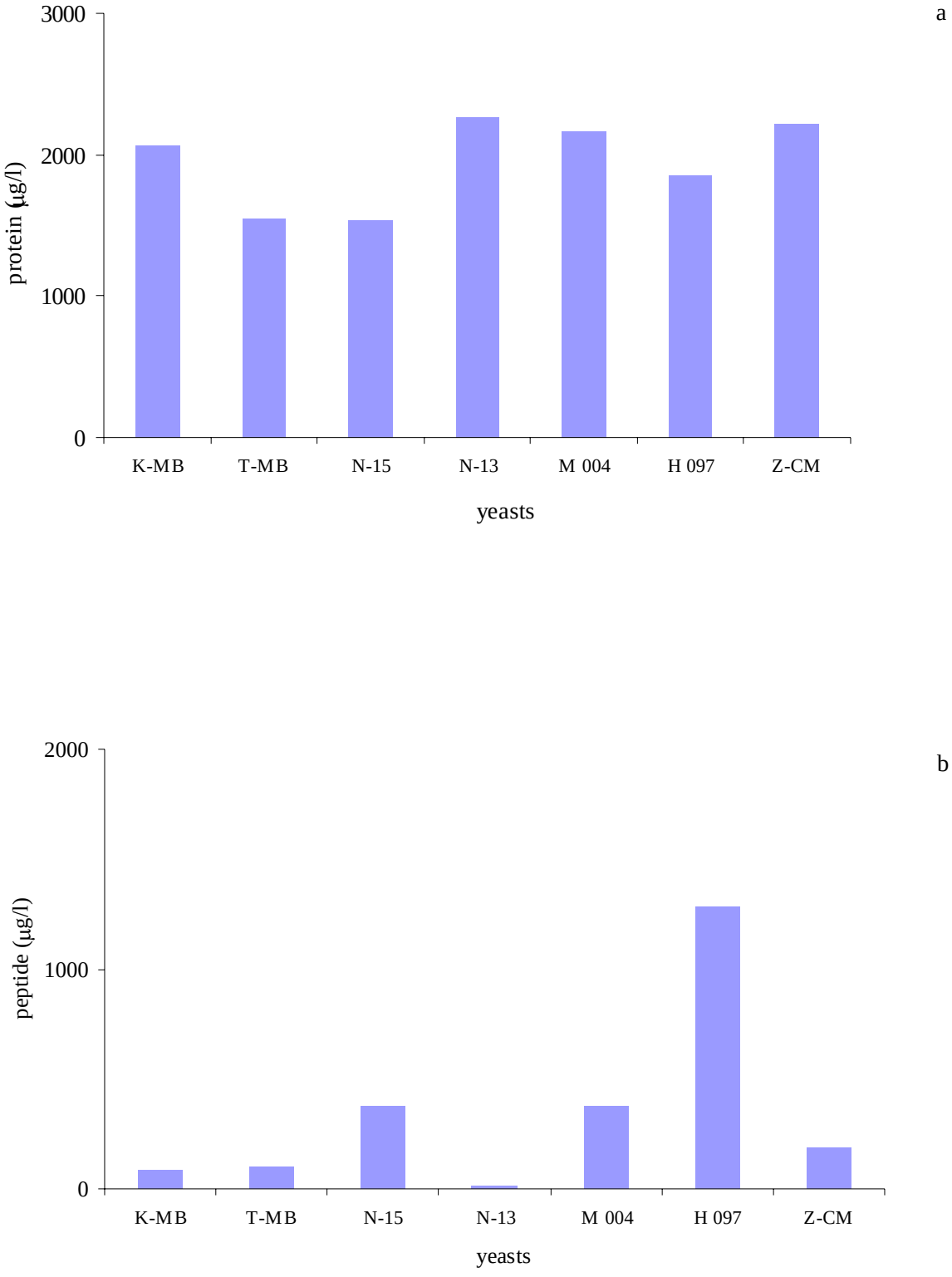


Figure 4-14 Estimation of the concentrations of polypeptides (a) and proteins in grape juice fermented with different yeast strains; systematic names of yeasts are noted in **Table 4-1**

Wines obtained from spontaneous fermentation and wines inoculated with S. cerevisiae (S-EC) were selected to study the composition of polypeptides and proteins. These wines were made from Riesling grape juices of vintage 2005 and 2006. The composition of their yeast population and the pattern of development were different. The data are not shown in this work but available in the studies of Rueck (2006) and Stoelben (2007). The results showed the variability of the concentrations of proteins (**Figure 4-15 a**). Wines obtained from spontaneous fermentation had higher concentrations of polypeptides than wines inoculated with *S. cerevisiae* (S-EC).

4.4.3 Application of electrophoresis to study polypeptide and protein profiles

Grape juices fermented must and wines as described in **Table 3-4** were selected to study the polypeptide and protein profiles. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of crude proteins from grape juices, fermented musts and wines showed variability of protein profiles. The range of molecular weights of the proteins of the samples was estimated to be between lesser than 14.4 to greater than 99 kDa.

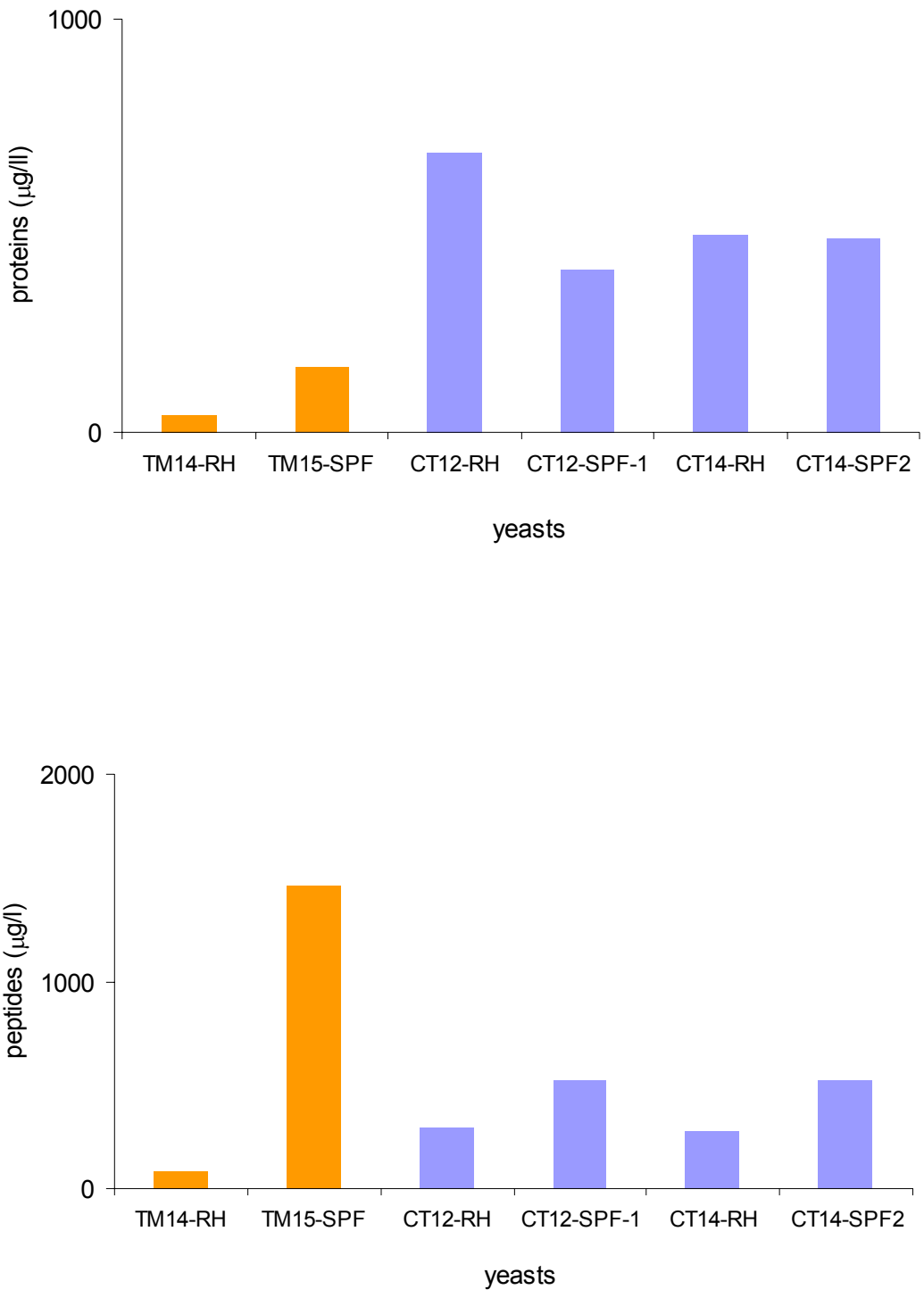


Figure 4-15 Estimation of the concentrations of polypeptides (a) and proteins (b) in wines from different conditions(**Table 3-4**) of fermentation; the same colour of bars denote samples from the same fermentation

Figure 4-16 compares the results obtained from the SDS-PAGE analyses of several samples of grape juice. Crude proteins of the grape juices showed molecular weights between lesser than 14.4 to greater than 99 kDa on the gels. Crude proteins of clarified and unclarified Riesling grape juices had relatively similar protein profiles (24 to ≥99kDa) but the latter had protein bands with strong intensity indicating higher concentrations in unclarified grape juice than in clarified grape juice. Similar sizes of proteins were also found in juices of the vintages 2005 and 2006. In contrast, crude proteins of Weissburgunder juice showed only one visual band on gel (lane h).

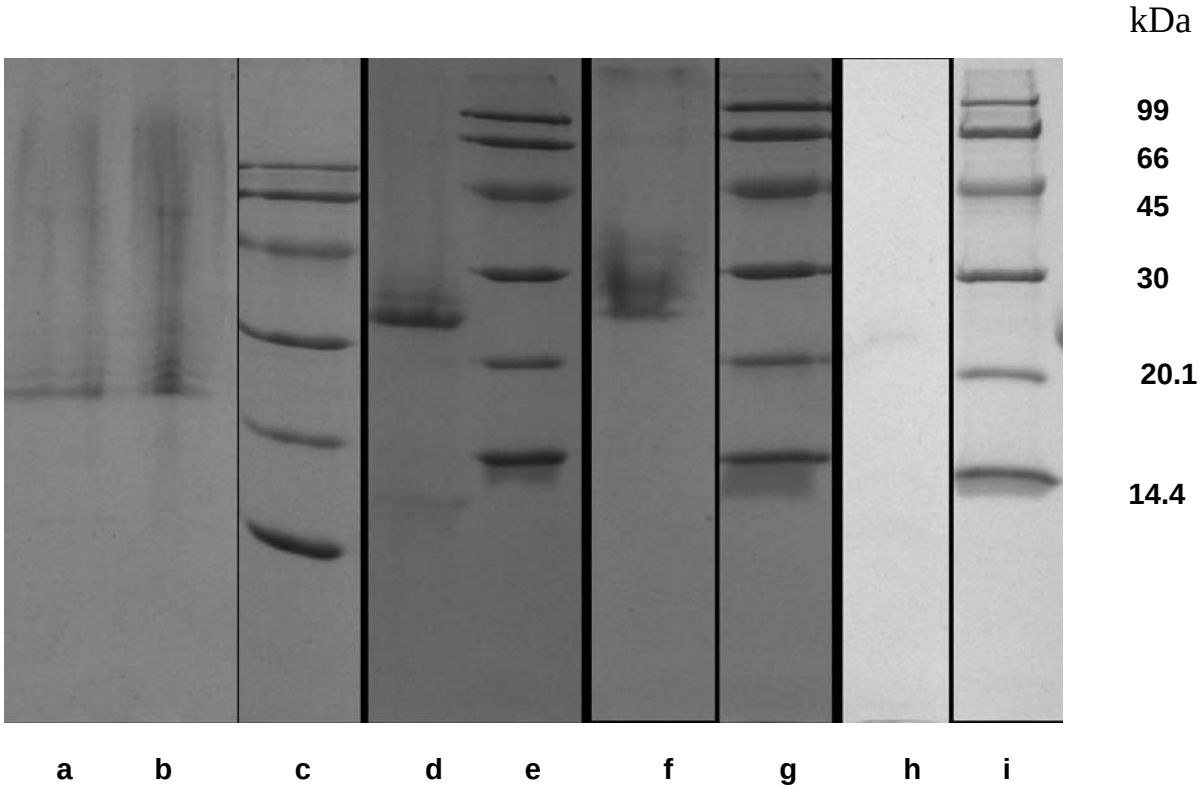


Figure 4-16 SDS-PAGE patterns of grape juice proteins; (a) 2006 clarified Riesling grape juice; (b) 2006 unclarified grape Riesling juice; (d) 2005 Riesling grape juice; (f) 2006 Riesling grape juice; (h) 2006 Weissburgunder grape juice; and (c, e, g, i) low molecular weight marker

To study the persistence of lysozyme addition in grape juice and during fermentation, samples which were undergone lysozyme were investigated. It was found that the utilization of lysozyme could be tracked during fermentation and in wine products. Lysozyme added to must fermented with *Metschnikowia pulcherrima* and *Torulaspora delbrueckii* seemed to remain in final products as shown in lane f and g of **Figure 4-17**. This protein with a molecular weight of 14.6 kDa could correspond to lysozyme since the original lysozyme preparation showed a band of the same size. Similar observations were also found in wines (lane b,c,d).

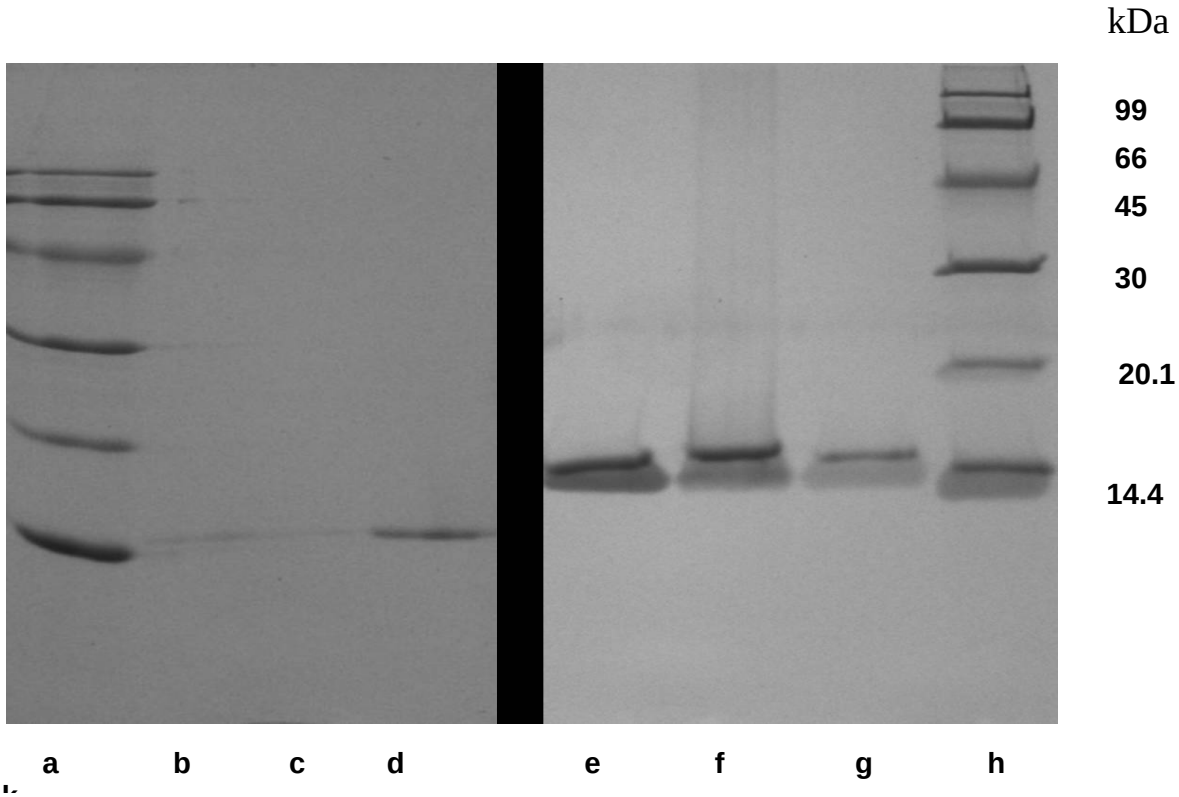


Figure 4-17 SDS-PAGE patterns of proteins isolated from fermented musts and wines; (b, c, d) 3 wines obtained from must added lysozyme; (f, g) fermented must obtained from must added lysozyme; (e) original lysozyme; and (a, h) low molecular weight marker

Wines from spontaneous fermentations and inoculated fermentations were taken to study the influence of microbial diversity on protein composition. Crude proteins of these wines showed different protein profiles (**Figure 4-18**). The patterns of wine proteins from fermentation of the vintage 2006 showed a similar profile. Besides the band intensity, a difference of protein patterns between wines obtained from spontaneous fermentation and wines inoculated with *S. cerevisiae* was not observed in fermentations of the vintage 2006. On the other hand, protein with molecular weight of 27 kDa appeared in wines of the vintage 2006 but not in the must of the vintage 2006. In fermentation of the vintage 2005, an additional protein (99 kDa) was only found in wine from spontaneous fermentation (lane h). It is interesting to note that proteins with the molecular weight of 66 and greater than 99 kDa found in fermentations of the vintage 2005 were not detected in must of the vintage 2005.

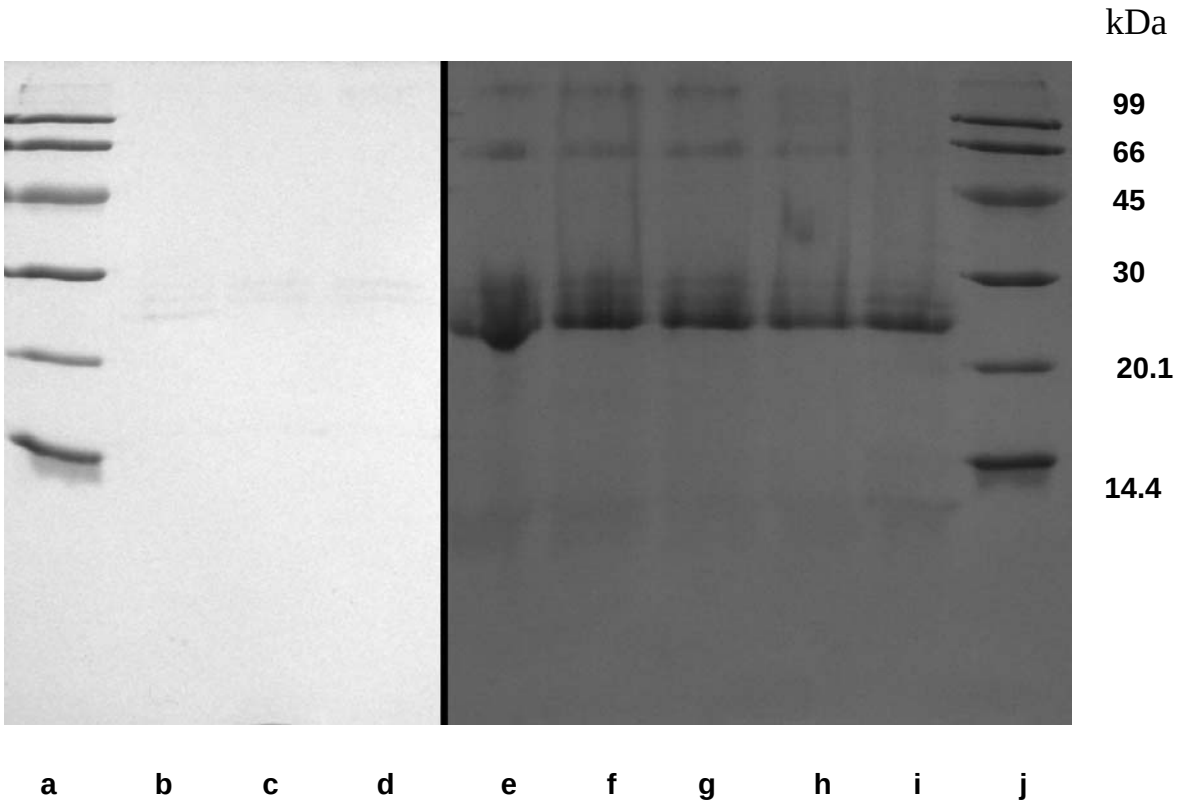


Figure 4-18 SDS-PAGE patterns of proteins of Riesling grape juices and wines: (b, i) Riesling grape juices of 2006 and 2005, respectively; (d, f, h) wines from spontaneous fermentation; (c, e, g) wines from inoculation with *S. cerevisiae* and (a, j) low molecular weight marker; (b, c, d) vintage 2006; (e, f, g, h, i) vintage 2005

In order to make comparisons, the crude proteins of grape must, must inoculated with non-*Saccharomyces*, must inoculated with non-*Saccharomyces* and *S. cerevisiae*, and wine from the fermentation of Weissburgunder grape juice were analysed by SDS-PAGE. The crude proteins had a different protein composition (**Figure 4-19**). Besides a major protein band of 28 kDa, crude proteins from fermented musts after the inoculation of *S. cerevisiae* had an additional band of 29 kDa on the gel. There was a variability of the molecular weights of proteins. These proteins remained in wine with a dramatic increase in concentration as shown in lane h, i, j.

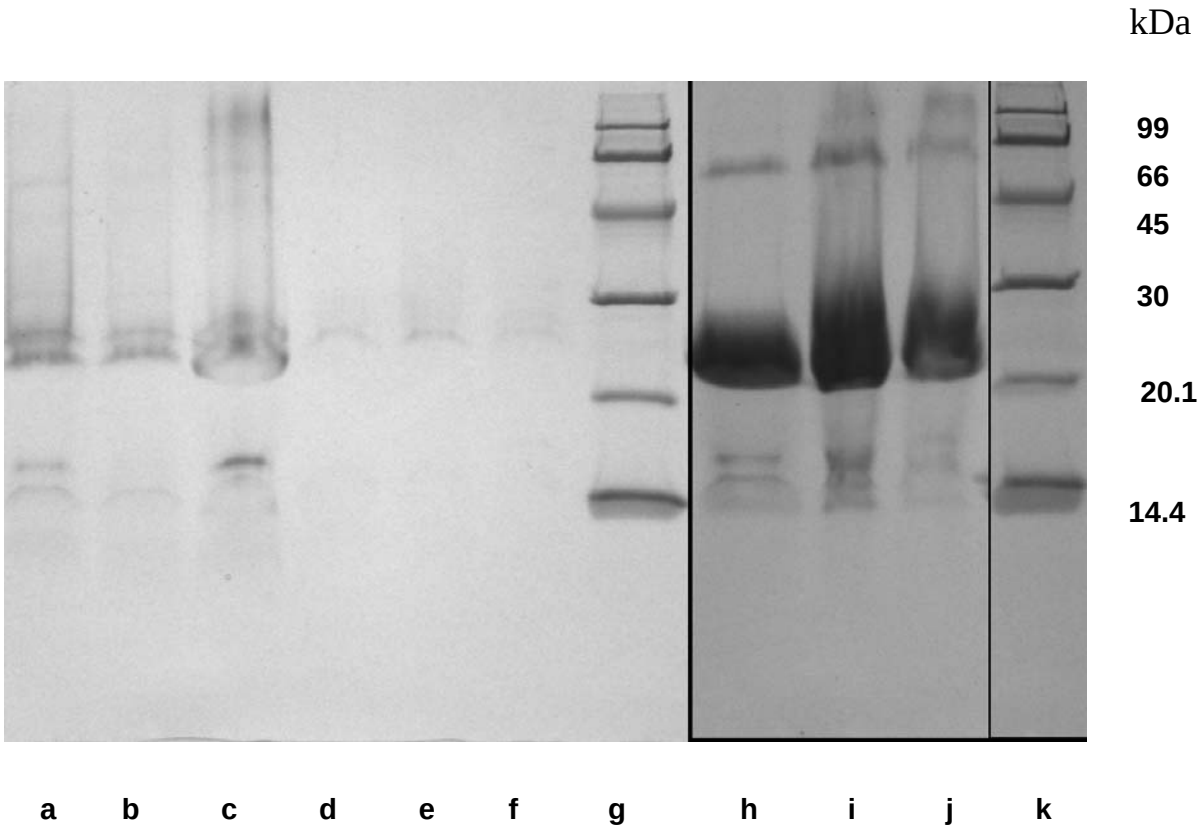


Figure 4-19 SDS-PAGE patterns of Weissburgunder grape juice fermentation: (h) Wine-W4 from spontaneous fermentation (Anstellhefe); (i) Wine-W1 from initial inoculation of *M. pulcherrima* (M 004) and *H. uvarum* (H 097) followed with a sequential inoculation of *S. cerevisiae*; (j) Wine from inoculated *S. cerevisiae* (single strain inoculation); (a) W5 in fermentation at day 9; (b) W4 in fermentation at day 9; (c) W1 in fermentation at day 9; (d) W5 in fermentation at day 5; (e) W4 in fermentation at day 5; (f) W1 in fermentation at day 5 and (g, k) low molecular weight marker

5 DISCUSSION

The production of proteolytic enzymes and the proteolytic activity of yeasts isolated from grapes and wines are discussed in this Section. The discussion is extended to the fermentation behaviour of the non-*Saccharomyces* yeasts and the influence of these yeasts and *Saccharomyces* yeasts in mixed cultures on composition of fermented musts and wines.

5.1 Screening of wine yeasts that secrete extracellular proteases for the use in grape juice fermentation

Some strains of non-*Saccharomyces* yeasts were proteolytic (Charoenchai, 1997; Lagace & Bisson, 1990). Thus, there is a good possibility that proteolytic enzymes of yeasts can degrade grape juice proteins and the proteolytic products can serve as a nutrient source for microorganisms. The results of experiments dealing with proteolytic activity of yeasts will be considered in this Section.

S. cerevisiae strains used in this study either produced no extracellular proteases or exhibited a very low proteolytic activity which was undetectable. Bilinski et al. (1987) also found no protease activity in this species, while a weak activity was detectable in the studies of Rosi and Costamagna (1987), Lagace and Bisson (1990), and Dizy and Bisson (2000). On the contrary, proteolytic activity of non-*Saccharomyces* yeasts varied between genera, species, and strains. For instance, *Rhodotorula* spp. expressed a proteolytic activity from 0.37-26.11 units (**Table 4-1**). Besides the influence of yeast strains, this variability can possibly be explained by an influence of growth factors and assay conditions, e.g. pH, temperature, substrates (Ogrydziak, 1993). As the result of this study, it should not be concluded that some yeast species which showed no proteolytic activity, had no extracellular protease production (Section 4.1.1). The activity of these yeasts may be too low to be detected by the method used for the activity assay.

Cultivation of yeasts in pasteurised grape juice gave a notable difference of proteolytic activity in comparison to cultivation in synthetic medium (Section 4.1.2**)**. In grape juice with aerobic condition, the ability of sugar consumption substantially improved in the species *M. pulcherrima and Hanseniaspora* spp*.* in comparison to grape juice with microaerobic condition. A reason for that may be that oxygen can be used to generate unsaturated fatty acids that are important for maintaining membrane fluidity (Ribéreau-Gayon et al., 2006 a).

Grape sugars were slightly utilized by *Rhodotorula* spp. because of the inability to ferment sugars of this genus (Longo et al., 1991; Barnett et al., 2000; Dittrich & Grossmann, 2005; Jolly et al., 2006), whereas it inversely grew well in the aerobic condition of this study. Cell concentrations of *Rhodotorula* spp. had increased approximately 100 times at three days after inoculation. *M. pulcherrima* (M 004) showed the highest protease activity in grape juice. Proteolytic activity was also detected in strains of *Metschnikowia pulcherrima* by Ganga & Martinez (2004).

Charoenchai et al. (1997) examined extracellular proteases of twenty-six yeasts. The authors detected the highest protease activity (65.4 units) in *Candida pulcherrima*, the anamorphic form of *Metschnikowia pulcherrima*. The degree of protease activity of *M. pulcherrima* (M 004) was linked to the level of growth therefore high activity was investigated in the present study. A similar behaviour was also found for the strains *R. glutinis* (N-21) and *M. pulcherrima* (N-1) grown in the BSA-medium. The results showed that the amount of yeast cells and the growth conditions could affect the protease production of yeasts.

Forgarty and Kelly (1990) reported that extracellular proteases from microorganisms hydrolyse protein into peptides and amino acids. These low molecular weight substrates can be readily transported into cells and used to support growth and metabolism. The investigation of this thesis showed that yeasts had a potential to produce extracellular proteases. It is therefore not unreasonable to propose that these extracellular proteases may break down proteins in musts and that the proteolytic products may serve as nitrogen sources for the growth of the microorganisms. In order to prove this assumption, yeasts exhibiting proteases were selected to grow in synthetic grape juice (SGJ) supplemented with the isolated proteins (IP) or ammonium sulphate or without nitrogen source. Although cell proliferation of all yeast species occurred in all media, yeasts showed slightly better growth in the SGJ supplemented with the IP than in the SGJ without nitrogen source. Yeasts were able to grow in medium supplemented with IP. This perhaps implies the ability of the examined yeasts to secrete proteases.

Although no protease activity was detectable for *T. delbrueckii* (T-MB), it showed almost double proliferation in the SGJ supplemented with the IP in comparison to the SGJ without nitrogen source. Compared with *M. pulcherrima* (M 004) and *H. uvarum* (H 097) which showed proteolytic activity, low nitrogen requirement and the fermentative behaviour of *T. delbrueckii* (T-MB) may be the reasons. The highest yeast cell density of all strains was found in the medium supplemented with ammonium sulfate 5 g/l. This agrees with the fact that ammonium is preferentially

metabolized as nitrogen source by yeasts (Bisson 1991, Henschke & Jiranek, 1993; Dickinson, 2004). On the other hand, no nutrient limitation due to a lack of nitrogen compounds was observed in the medium without nitrogen source. A possible explanation is that yeasts can carry their own nitrogen reserve (Gafner., 2002). In addition, the concentration of isolated proteins may be insufficient to provide a suitable source of assimilable nitrogen for yeasts under the conditions of this study. Although the isolated proteins of must contained amino acids (**Table 4-3**) the amount of protein added to the medium provided only 17 mg/l of amino acids. This can be considered to be a tiny quantity as grape must contains 200 to 4000 mg/l amino acids (**Table 2-5**). It is also possible that proteolytic activity of the yeasts may be negligible to accelerate the growth and that proteolytic products like oligopeptides and polypeptides could not be absorbed by yeasts. In this regard these products may remain in wines and influence the composition of wines. Furthermore, the extracellular proteases, while active against BSA, may not degrade wine proteins as some studied revealed that grape and wine proteins are protease resistant (Waters et al, 1992, 1995 a, 1995 b).

5.2 Fermentative characteristics of non-*Saccharomyces* yeasts exhibiting extracellular proteases in enological fermentation

The results of the previous experiments conducted to examine the production of proteolytic enzymes of yeasts showed that extracellular proteases of yeasts are probably relevant to enological fermentations. The extracellular proteases of yeasts may be active or partly active during winemaking. This means that extracellular proteases of these yeasts may degrade proteins present in must and thereby provide nitrogen compounds of low molecular weight like amino acids and peptides that may be utilized as substrates by microorganisms present during fermentation. It is also possible that these low molecular weight nitrogen-containing compounds can impact wine quality. Non-*Saccharomyces* yeasts are also responsible for the production of other metabolites which influence the chemical composition of the wines (Ciani & Maccarelli, 1998; Jolly et al., 2003 a). In order to understand the effect of these metabolites on fermentation and wines, the fermentative characteristics of the selected yeasts were examined.

The examined yeasts can be divided into 3 groups according to their ability to ferment sugars. The species of *Zygosaccharomyces bailii*, *Saccharomycodes ludwigii*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* were in the group of high fermentation rate. The species of *Hanseniaspora uvarum* and *Metschnikowia pulcherrima* showed low fermentation activity when the reducing sugar content was considered as the indicator. Such a behaviour was also described in other works (Ciani & Maccarelli, 1998; Ciani et al., 2006). Therefore, *Metschnikowia pulcherrima* and *H. uvarum* species were in the second group which displayed a low rate of fermentation whereas *Rhodotorula glutinis* was in the third group of non-fermentative yeasts. The inability of *Rhodotorula* spp. to ferment has been described by Longo et al. (1991), Dittrich and Grossmann (2005) and it is also observable in this study. A similar description of non-*Saccharomyces* yeasts has been established by Jolly et al. (2006). In this review, *K. thermotolerans*, *T. delbrueckii* and *Z. bailii* species were grouped in non-*Saccharomyces* species with fermentative metabolism, while *H. uvarum* was classified to be an apiculate yeast with a low fermentative activity.
Volatile acidity and glycerol were produced in different degree depending on species and strains. The concentrations were in a range found in wines (Jakob 1995; Scanes et al., 1998; Gawel et al., 2007). A high concentration of volatile acidity is often used as an indicator of the spoilage of wine while a high concentration of glycerol improves the sensory characteristics of wines, particularly of wines lacking in body (Remize et al., 2000; Godden & Gishen, 2005; Yanniotis et al., 2007). Therefore, metabolites of the non-*Saccharomyces* yeasts should be regarded when these yeasts will be applied as inoculum in wine fermentation.

As shown in the results, the chemical composition of fermented musts inoculated with different non-*Saccharomyces* yeasts was either slightly or considerably different. Therefore, if these yeasts can grow during alcoholic fermentation of grape must they can influence the sensory characteristics of wine due to the production of fermentation products. There were differences in the production of volatile and non-volatile metabolites within the genera, species and strains as described in the results. Furthermore, non-*Saccharomyces* species could produce metabolites that were not typical of other species. A marked impact on glycerol and acid production was observed. It is reasonable to expect that these compounds could also influence wine sensory properties (Yalcin & Öezbas, 2005; Ferreira et al., 2006; Gawel et al., 2007). These compounds could be considered as desirable aroma attributes depending upon the wine style. The diversity of the metabolite production of non-*Saccharomyces* has also been reported by many researchers (Sponholz & Dittrich, 1974; Heard, 1999;

Lambrechts & Pretorius, 2000; Garcia et al., 2002; Jolly et al., 2003a,b; Rojas et al. 2001; Clemente-Jimenez et al., 2005; Hernández-Orte et al., 2008; Torrens et al., 2008). A study conducted by Kapsopoulou et al. (2005, 2006) demonstrated that *K. thermotolerans* produced high concentrations of lactic acid in grape juice fermentation. High lactic acid production in grape juice fermentation is likely the specific characteristic of *K. thermotolerans*. This behaviour was also confirmed in this study.

The concentrations of esters, higher alcohols, and volatile fatty acids produced by different strains of yeasts varied in the experiments of this thesis (Section 4.2). For some strains these variations were not sufficient to produce odouriferous profiles that are noticeably different. Esters, higher alcohols, and volatile fatty acids principally arise from yeasts' primary metabolism of sugars and from the metabolism of amino acids (Pretorius, 2000; Swiegers & Pretorius, 2007). Therefore, the production of these flavour-active compounds during fermentation can be expected to be influenced by yeast species and the composition of must. For instance, fermentation of must with *Z. bailii* strains resulted in higher concentration of butanoic acid ethyl ester in final products than fermentation of must with other strains. Another example, extremely high concentration of acetic acid ethyl ester was noticed in must fermented by *S. ludwigii* (N-15, N13).

Monoterpenes are typical aroma compounds of floral grape varieties, e.g. Riesling and Gewuerztraminer (Clarke & Bakker, 2004; Palomo et al., 2007). They exist in the berry principally as glycoconjugate with only a small proportion present in the free form and are liberated during fermentation (Genovés et al., 2005; Maicas & Mateo, 2005). Monoterpenes can be released from glycosides by glycosidases (Sponholz & Rauhut, 1992; Cabaroglu et al., 2003; Genovés et al., 2005). Non-*Saccharomyces* are capable of producing glycosidases (Charoenchai et al., 1997 & Garcia et al., 2002). A potential impact of non-*Saccharomyces* yeasts on the transformation of free terpenes has been reported (Fernández-González et al., 2003). These yeasts are responsible for an increased concentration of monoterpenes in wine (Grossmann et al., 1987; Hernández-Orte et al., 2008). However, no clear differences were observed when non-*Saccharomyces* yeasts were used in a pure culture fermentation of grape juice in this study.

Since sulphur containing compounds have a significant role on wine flavour (Rauhut, 1996, 2003, 2006), the musts fermented with the non-*Saccharomyces* yeasts (11 strains) were investigated for the concentration of the sulphur containing compounds. The fermented musts had relatively low concentrations of the sulphur containing compounds and those concentrations were below sensory thresholds according to

Rauhut (1993). The concentrations of hydrogen sulphide, dimethyl sulphide (DMS) dimethyl disulphide (DMDS) were affected by genera, species, and strains. The concentrations of the other investigated sulphur containing compounds were undetectable. However it could not be clearly concluded that the 11 yeasts either produced no differences in the concentrations or low concentrations of sulphur containing compounds because their concentrations can be influenced by other effects. Many factors have been reported to affect the production of sulphur-containing compounds, e.g. sulphur source, nitrogen composition (Rauhut, 1993, 1996; Henick-Kling & Park, 1994). Furthermore, the authors noted that the production of these compounds by yeasts is related to the metabolism of nitrogen containing compounds.

The concentrations of FAN seemed to remain high in most fermented musts obtained from fermentation of 11 yeasts in comparison to original must (47 mg/l). A low concentration of FAN was found in must fermented by *Z. bailii* (N-29). This may be linked to its ability to ferment in the conditions of this study because in the fermented juice the highest concentration of alcohol and the lowest concentration of reducing sugars was found compared with musts fermented by other yeasts. The similar tendency was also found in the concentration of amino acids.

High consumption of alanine and arginine was observed for *Z. bailii* (N-29) being able to ferment a lot of sugar. γ-aminobutyric acid was also substantially more consumed by species of this yeast compared to other yeasts. Different pattern of the consumption of amino acids could be affected by many factors, e.g. the quantity of amino acids present in must, micro and macro nutrients necessary for yeast growth, yeast preference of amino acids, transport systems of yeasts, growth conditions (Bisson, 1991). It should also be taken into account that yeasts could take up and release amino acids during fermentation as well as they could release amino acids during autolytic cycle (Monteiro & Bisson, 1992; Fugelsangs & Edwards, 2007). Since a secretion of protease was investigated in several yeasts in this study (Section 4.1), it is possible that the degradation products of those proteases, i.e. amino acids, peptides can affect concentration of yeast assimilable nitrogen including amino acids. The ability of the genera *Kloeckera* & *Hanseniaspora* to produce significant amounts of protease activity and to reduce protein concentration of grape juice was reported by Dizy and Bisson (2000). The authors proposed that the products of proteolysis would be available for yeast nutrition during the non-proliferative phase. Thus, the behaviour of yeasts to take up amino acids may also be influenced by their ability to produce proteases besides the factors mentioned above.

5.3 Effect of yeasts producing protease in mixed cultures on winemaking

Wines of a high sensory complexity can be produced by spontaneous fermentation where natural yeasts develop in an optimal manner. These wines attract and appeal consumers and connoisseurs. Unfortunately, undesirable sensory characters can be also found in wines from spontaneous fermentation depending on the presence of yeast species and their development (Soden et al., 1998; Ciani et al., 2006; Grossmann, 2006; Maro et al., 2007; Nisiotou et al., 2007). Winemaking with the spontaneous fermentation is always associated high risks because excellent results are only achieved in seldom cases (Grossmann, 2007). This is why the inoculated fermentation of *S. cerevisiae* is used to induce and conduct the fermentation (Heard and Fleet, 1986, Grossmann et al., 1996).

To mimic the process of spontaneous fermentation which produces wine with desirable sensory characters, the use of the non-*Saccharomyces* yeasts to begin the fermentation has been studied (Soden et al., 1999; Garcia et al., 2002; Nissen et al., 2004; Clemente-Jimenez et al., 2005; Heidkamp, 2005; Ciani et al., 2006; Bergdolt, 2007; Bely et al., 2008). Apart from that, various techniques such as pied-de-cuve (inoculation of indigenous yeasts) (Bergdolt, 2007), and co-inoculation (Soden et al., 2000; Ciani & Comitini, 2006; Sommer et al., 2007) have been applied in wine fermentation. Another way to optimize wine quality was examined by Grossmann et al. (1996, 1997), Eglinton et al. (2000) Cheraiti et al. (2005), Howell (2006), Favale et al. (2007) and Hayasaka et al. (2007). They carried out experiments with mixed yeast cultures of *Saccharomyces* yeasts. There is still not enough knowledge about the effects of the application of mixed cultures on wine quality and therefore, they are not widely used in commercial wineries.

Therefore the experiments of mixed cultures of *Saccharomyces* and non-*Saccharomyces* yeasts were carried out and the results (Section 4.3.1, 4.3.2) are discussed in this Section.

The sequential inoculation of non-*Saccharomyces* yeast exhibiting protease activity prior to *Saccharomyces* yeast was carried out. Fermentations were performed using grape juice treated with dimethyl dicarbonate (DMDC) and lysozyme in order to inhibit the natural microflora of must (Costa et al., 2008; Davidson, 2002; Divol et al., 2005; Margalit, 2004; Renouf et al., 2008; Vine et al., 2002; Zoecklein et al., 1999). The result of yeast growth on HDM-medium, the examination of the yeasts under microscope confirmed the growth of inoculated yeasts. No growth of lactic acid

bacteria was observed. This could indicate that the application of DMDC and lysozyme was effective in this experimental study.

Besides the expression of proteolytic activity, *H. uvarum* (H 097), *H. uvarum* (H 045) and *M. pulcherrima* (M 004) were selected to initiate the fermentation because they are species frequently found in the microflora of the must and at the early stage of fermentation (Sponholz & Dittrich, 1974; Jemec et al., 2001; Gafner, 2003 a; Sturm et al., 2003; Paraggio, 2004; Domizio et al., 2007; Nisiotou & Nychas, 2007). It was expected that proteases may hydrolyze proteins in must and liberate nitrogen-containing compounds; i.e. amino acids and small peptides. Subsequently, these nitrogen-containing compounds can serve as a source of nitrogen for yeast growth. Therefore after ca. 25 g/l sugar in must was consumed, *S. cerevisiae* species were introduced to the fermentation.

The growth kinetic was improved, when the species of *Hanseniaspora uvarum* (H 045) was used to begin the fermentation and when a later inoculation of *S. cervisiae* followed, i.e. H 045-S-EC, H 045/S-CM. The production of carbon dioxide was also higher and the concentration of reducing sugar at the end of fermentation was lower when this inoculation protocol was used. In addition, the greater consumption of sugar and the greater production of alcohol were in agreement with the growth kinetic. In contrast, the combined inoculation of *H. uvarum* and *S. cerevisiae* yielded slow fermentation kinetics in other works (Ciani et al., 2006; Dittrich & Grossmann, 2005). The authors pointed out that it was not comprehensible why this type of inoculation resulted in sluggish or stuck fermentations but that the problems were not caused by killer toxin.

Research carried out by Malcarino et al. (2005) suggests that conversion of a high concentration of sugars to ethanol was correlated to a high consumption of assimilable nitrogen in grape juice fermentation. The threshold concentration of assimilable nitrogen of approximately 140 mg N/l is considered to complete fermentation (Bisson, 1991; Zoecklein et al., 1999; Henschke & Jiranek, 1993). Concerning the concentration of FAN (68 mg/l) and the sugar concentration (210 g/l) in must, the nitrogen sources were probably insufficient for a sufficient yeast growth and the completion of fermentation (Section 4.3.1). This was indicated by a fermentation that lasted longer than 30 days in all variants and could be classified as sluggish. It is notable that higher concentrations of amino acids in the wine inoculated with *H. uvarum* may be derived from the protease activity of this yeast but it could also be derived from yeast autolysis. Autolysis of *Saccharomyces cerevisiae* involves the release of different products including amino acids (Cebollero et al., 2005; Alexandre

& Guilloux-Benatier, 2006; Cebollero & Gonzalez 2006) but there is no study about autolysis of non-*Saccharomyces* species.

Fermentation could proceed, although the concentration of the assimilable nitrogen in must was under the appropriate quantity. The number of viable cells of *H. uvarum* did not decrease dramatically until after the sugar exhaustion whereas *S. cerevisiae* dominated with the progress of fermentation. Therefore, the mixed culture fermentations had higher yeast population than single strain fermentation. A higher biomass production is expected to result in a higher uptake of yeast assimilable nitrogen. As *H. uvarum* has shown proteolytic activity in the previous study and thus yeast assimilable nitrogen may rise during fermentation and the higher nitrogen uptake can be masked.

A complete fermentation of the sugars was achieved by single inouculation of *S. cerevisiae* (S-EC) and sequential fermentation of *H. uvarum*/*S. cerevisiae* (H045/S-EC). Wines of the single inoculation of *S. cerevisiae*, S-EC and S-CM, had the concentrations of reducing sugars of 2.1 and 35.1 g/l, respectively and the concentrations of assimilable nitrogen of 90 and 94 mg/l, respectively. These differences indicated the influence of yeast strains on the demand of nitrogen for their growth during fermentation. The stuck fermentation of *S. cerevisiae* S-CM could be caused by a high nitrogen demand of this strain during fermentation and the nitrogen sources of the must could not be sufficient. Compared with wines from the inoculation protocol of H 045/S-EC and H045/S-CM, lower concentrations of the reducing sugars 0.5 and 6.9 g/l, respectively were obtained. It is likely that the use of the strain *H. uvarum* (H 045) is partly responsible for these lower concentrations of reducing sugars. These effects may be explained by two reasons. The first possibility is *H. uvarum* (H 045) may release nitrogen compounds and that the *S. cevesiae* strains could utilise them for their growth, subsequently this benefits the progress of the fermentation. The second possible explanation is the potential to secrete extracellular proteases of non-*Saccharomyces* yeasts as reported in the literature (Lagace & Bisson, 1990; Ogrydziak, 1993; Charoenchai et al., 1997; Dizy & Bisson, 2000) as well as in this work. The proteases of *H. uvarum* (H 045) possibly degraded protein substrates in the fermented juice during fermentation and split off nitrogenous compounds. In other words, *H. uvarum* (H 045) may support *S. cevesiae* strains by providing an additional nitrogen source, i.e. amino acids and small peptides. However, a broad range of factors, e.g. interaction between yeasts, requirement of nutrients, and influence of metabolites of one species on the other species, should also be considered as suggested by Fleet (2001).

Numerous factors affect the development of yeast growth and the composition of species in the total population in must during fermentation. These factors include grape variety, natural microflora, clarification of grape juice, composition of the juice, and inoculation with selected yeasts (Gafner, 2003 a; Nissen et al., 2004). The previous experiments of the investigation of extracellular proteases of non-*Saccharomyces* yeasts suggest that there is a good possibility that some strains of the wine yeasts are proteolytic. In this study the breakdown and utilization of grape juice proteins as a source of nitrogen for yeast growth of those yeasts in enological conditions were further considered. Like proteolytic strains, many non-*Saccharomyces* yeasts showing an undetectable activity of extracellular protease have the potential to produce greater or lesser concentrations of volatile compounds such as higher alcohols, acetic acid, acetaldehyde and esters. These compounds can influence the chemical composition and the sensory properties of the wine. The use of these non-*Saccharomyces* yeasts combined with *Saccharomyces* yeasts in winemaking should affect many important variables like the rate and completeness of fermentation, the concentration of many aroma and flavour constituents in the wine and removal of nitrogen compounds from the juice.

The study of Section 3.11.2 thus was aimed to demonstrate that despite the presence of natural yeasts in the must before inoculation with not only *Saccharomyces* but also with non-*Saccharomyces* and *Saccharomyces* yeasts can have effects on the non volatile and volatile metabolites in wine. The principal yeast species associated with wine fermentation selected from the previous studies were inoculated to the juices. Comparisons between inoculated and uninoculated fermentations with non-*Saccharomyces* yeasts were made. The study demonstrated that the inoculated strains of non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* strains could suppress and outgrow indigenous yeast species and dominate the fermentation. The similar effect of suppression of the natural yeast flora by the inoculated yeast strains was described (Santamaría et al., 2005; Domizio et al., 2007).

Fermentations initiated with non-*Saccharomyces* yeasts showed that the lag phase was increased and that the fermentation kinetic at the beginning of fermentation was slower than in fermentations initiated with *Saccharomyces* yeasts. In contrast, after such fermentations were sequentially inoculated with strains of *S. cerevisiae*, yeasts exhibited faster growth rate than in the single strain inoculation of *S. cerevisiae*. These non-*Saccharomyces* consistently grew during fermentation until the end of the stationary phase, although their population slowly decreased. This could suggest that

the inoculation of grape juice with mixed culture of *Saccharomyces* and non-*Saccharomyces* yeasts could influence the fermentation kinetics of the natural grape juices. As the result of that, those yeasts could contribute to the overall compostion of the resulting wines.

Considering the clarification of grape juice, the growth rate of yeasts was faster in fermentations of clarified must than unclarified must (Section 4.3.2). Moreover, the influence of inoculation protocols on the fermentation kinetics of the unclarifed must was not obviously different from the fermentation kinetics of the clarified must. Yeasts of the inoculation protocol *Torulaspora delbrueckii* / S*. cerevisiae* (T/S) exhibited slowed growth rates in fermentation of the unclarified must compared to the fermentation of clarified must. This result was not correlated with the general concept that the must with higher turbidity can support yeast growth better than the must with lower turbidity. It is possible that the clarification could eliminate some microflora and, therefore, decrease or increase the contribution of certain yeast species to fermentation. The clarification could also alter the chemical and physical composition of the juices by the removal of some grape components, e.g. grape solids and vitamins (Christmann, 2004 & Jung et al., 2005). This could also be a factor which influences yeast growth of mixed cultures of *Saccharomyces* and non-*Saccharomyces* species during fermentation. A recent study (Nissen et al., 2003) revealed that a cell-cell contact mechanism between mixed cultures of *K. thermotolerans, T. delbrueckii, and S. cerevisiae* was responsible for the individual growth behaviour. This mechanism may exist among mixed inoculants applied in this study and may affect the fermentation. Further studies are required to understand this effect in the fermentation of grape juice. Of particular interest was that non-*Saccharomyces* yeasts seemed to make a greater contribution to the fermentation by supporting *Saccharomyces* growth. More precise studies of the effect of such an inoculation protocol on musts with different turbidity levels are needed.

Lower concentrations of reducing sugars existed in wines where growth of non-*Saccharomyces* proceeded prior to the inoculation of *S. cerevisiae*. Furthermore, fermentation of grape juice with non-*Saccharomyces* and *Saccharomyces* yeasts resulted in a lower concentration of fructose than fermentation of grape juice without non-*Saccharomyces* yeasts. A preferential uptake of fructose by non-*Saccharomyces* species (Jolly, 2007) could be responsible for this effect. In contrast to wines obtained from fermentation of *S. cerevisiae* species which are glucophilic yeasts, often have a higher concentration of fructose (Guillaume et al., 2007).

Wines initially inoculated with non-Saccharomyces yeasts containing *T. delbrueckii* seems to have lower concentrations of the medium-chain fatty acids. It is possible that these fatty acids inhibited yeast growth (Magarlit, 2004). This suggests that mixed inoculations of non-*Saccharomyces* and non-*Saccharomyces* cultures could contribute to completeness of fermentation. The concentration of sulphur dioxide binding compounds varied greatly as they can be influenced by many factors, e.g. yeast strains, nitrogen metabolism, presence of compounds binding sulphur dioxide in wine (Dittrich & Grossmann, 2005; Sponholz, 1993). A sequential inoculation of *T. delbrueckii*/*S. cerevisiae* cultures produced less acetaldehyde than other inoculation protocols. A study conducted by Bely et al. (2008) demonstrated the same behaviour of a mixed culture fermentation of these 2 species.

The composition of odouriferous compounds of a wine fermented by the inoculation with a mixture between *Saccharomyces* and non-*Saccharomyces* yeasts was different to the composition of a wine fermented with *S. cerevisiae* inoculation alone. Different yeast strains affecting the odourifereous compounds of wines have been reported in many studies (Gil et al., 1996; Romano et al., 2003 a, 2003 b; Estevéz et al., 2004; Moreira et al., 2005; Torrens et al., 2008). An identical inoculation treatment of grape juices with a turbidity of 205 and 586 NTU grape juices also yielded different profiles of odouriferous compounds in the wine. The concentration levels of the odouriferous compounds of all wines obtained from variable treatments as shown in **Table 4-19** and **4-20** were within the ranges reported in earlier studies (Francis & Newton, 2005; Garde-Cerdán & Ancín-Aypilicueta, 2007; Favale et al., 2007).

The influence of inoculation protocols and juice clarification on the concentrations of esters showed a similar tendency as on the concentrations of higher alcohols. The ester profiles of wines resulting from the clarified must fermented with sequential cultures differed from the profile of the clarified must initially fermented with *S. cerevisiae*. These profiles could not be distinguished in wines from unclarifed must. Growth of *T. delbrueckii* prior to the inoculation of *S. cerevisiae* produced high amount of several esters, including acetic acid ethyl ester and acetic acid 3- methylbutyl ester which can noticeably impact the odouriferous profile. This inoculation protocol clearly showed higher concentrations of esters than other inoculation protocols. A similar observation was noted in the study conducted by Herraiz et al. (1990).

In comparison to the other yeast species used in this study *Torulaspora delbrueckii* produced relatively large amounts of higher alcohols, which can influence the flavour of the wines. This behaviour of *T. delbrueckii* was also demonstrated in other works

(Sponholz & Dittrich, 1974; Jolly et al., 2003). This effect was not pronounced in the fermentation of unclarified must and when the must fermented initially with *S. cerevisiae*. At low concentrations of less than 300 mg/l (Bell & Henschke, 2005), higher alcohols contribute to a wine's aroma complexity while higher levels can be responsible for disagreeable attributes. The concentrations of higher alcohols in all wines were lower than that amount.

The concentration of hexanol in wines of all treatments were under the odour threshold (8000 μg/l) as suggested by Francis & Newton (2005). The results showed no influence of grape juice clarification on the concentrations of hexanol, whereas clear must (less than 200 NTU) has been recommended for winemaking to avoid an increased concentration of this molecule in wine (Jung et al., 2006). Hexanol has been reported as a compound being originated from grapes, therefore its concentration was supposed to be increased in wine made from unclarified must which had plenty of particles from grapes. The concentrations of hexanol in wines from 205 NTU and 586 NTU musts were similar when the same inoculation protocols were compared. On the other hand, inoculation with non-*Saccharomyces* yeasts tended to reduce the concentration of hexanol in wines. It is likely that non-*Saccharomyces* yeast can influence the transformation of hexanol.

The esters of these fatty acids tended to increase in relation to higher concentrations of these acids found in wines. As these medium-chain fatty acids are formed by yeasts (Lambrechts & Pretorius, 2000), an impact of inoculation protocols on their concentrations was observed in this study. Lafourcade et al. (1984) reported that these fatty acids were fermentation inhibitors. The concentrations of fatty acids in the presented thesis were higher than the perception threshold (Francis & Newton, 2005), however those concentrations are commonly found in white wine.

Concentrations of fatty acids tended to decrease in wines which were initially inoculated with non-*Saccharomyces* yeasts as indicated in Table 4-19. The concentrations were considerably low in wines which were initially inoculated with non-*Saccharomyces* yeasts consisting of *T. delbrueckii* (T-MB). It should be noted that the concentrations of hexanoic, octanoic, and decanoic acid ethyl esters correlated well with the concentrations of the corresponding fatty acids in wines. The medium-chain fatty acids were the substrates for ethyl ester formation as has been reported by the research group of Saerens (2008).

Of the two monoterpenes detected in the wines presented in this study, i.e. linalool and α-terpineol, only the concentration of the linalool was situated over the threshold level (Clarke & Bakker, 2004). Yeast species, inoculation protocols, and clarification of grape juice did not affect the concentrations of monoterpenes in the wines. These factors which were described to influence the concentration of monoterpenes in other research works as already mentioned, did not play a role in this study.

The high sugar concentration (263 g/l) and the low concentrations of FAN (71 and 84 mg N/l) in the grape juices placed significant stress on the yeasts during alcoholic fermentation. Henschke and Jiranek (1993) stated that free α-amino nitrogen concentrations (FAN) of musts varied widely between 28-336 mg N/l. In present study, musts of 205 and 586 NTU contained concentrations of FAN of 71.1 and 84.0 mg N/l, respectively. These concentrations are in the level which is reported to be insufficient to sustain yeast growth necessary to complete fermentation (Bell & Henschke, 2005). As pointed out by Bisson and Butzke (2000) under enological conditions, mean values of 140-160 mg/l of amino nitrogen are considered to be sufficient for complete fermentation of reasonably ripened grapes, while 150 mg/l is proposed by Fleet (2001). In an experiment carried out in a synthetic grape juice with glucose (200 g/l), *S. cerevisiae* required a minimum of 267 mg N/l to complete fermentation (Mendes-Ferreira et al., 2004). Additionally, strains, growth conditions, and yeast starter preparation have an impact on the ability of the yeast to assimilate nitrogen (Manginot et al., 1998, Jiranek et al., 1990, & Coleman et al., 2007).

In the present study a relationship between nitrogen assimilated by yeasts and FAN remaining in the wine after fermentation could not be found. The concentration of FAN in wine was relatively high when compared to the original concentration found in juice and the FAN consumed by yeasts was not directly proportional to that which was available in the juice. It is interesting to note that the concentrations of FAN in wines obtained from fermentation initiated with non-*Saccharomyces* yeasts were higher than in wines made by fermentation initiated with *S. cerevisiae*. This may indicate that the proteolytic activity of non-*Saccharomyces* yeasts may provide nitrogen-containing compounds to fermented juice. Because of this, an impact of the inoculation treatments on the fermentation kinetics could be observed. The concentrations of nitrogen-containing compounds are presumably linked to the degree of production of other metabolites (Howell, 2006), e.g. wine produced from grape must with a low concentration of amino acids had a high content of total sulphur compounds (Moreira

et al., 2002). Likewise, a high concentration of total sulphur containing compounds was found in wine containing a low concentration of FAN in this study.

Concentrations of FAN in wines fermented by inoculants exhibiting protease activity, i.e. *H. uvarum* 097 and *M. pulcherrima* 004, were higher than in wines fermented without inoculation of these strains. It seems convincing that non-*Saccharomyces* yeasts produce extracellular proteases during the fermentation of grape juice. There is a possibility that these proteases can liberate nitrogen containing compounds from grape protein into the juice and that these liberated compounds can benefit the growth of non-*Saccharomyces* yeasts as well as the growth of *Saccharomyces* yeasts. Apart from the reaction of the extracellular protease, release of nitrogen-containing compounds of yeasts could affect the concentrations of assimilable nitrogen during fermentation. Release of amino acids into fermented grape juice at the end of fermentation has been reported (Monteiro & Bisson, 1991; Ough et al., 1990, 1991). The distribution of the amino acids at the end of fermentation is not well correlated with the starting composition of the juice, most likely reflecting the optimal yeast cellular pool levels for these compounds (Bisson, 1991). These compounds may be released into the wine during autolysis (Todd, 1995, Martínez- Rodríguez, 2001 a, 2001 b). In contrast, Bisson (1991) observed this release when more than 90% of the cells present in the wine are still viable. The author suggested that the release may have some metabolic or physiological role for the yeast, resulting perhaps in enhanced survival.

The availability of different assimilable nitrogen containing compounds for different yeast strains is important due to depletion of the preferred nitrogen source after the logarithmic growth phase of the cultures (Cramer et al., 2002). Furthermore, amino acids cannot penetrate into the yeast cell because higher concentrations of ethanol inhibit the uptake of amino acids by modification of transport-proteins (Henschke, 1997). The release of nitrogen-containing compounds could thus present an alternative assimilable nitrogen source for a given yeast (Marder et al., 1977; Yamada et al., 2005). These derivatives released during fermentation, either by yeasts or proteolytic activity, could also negatively or positively affect the final sensory quality of the wine (Martínez- Rodríguez et al., 2002). For instance, these released products containing nitrogen can influence the production of higher alcohols (Soufleros et al., 2003) or result in a risk of biogenic amine formation by lactic acid bacteria (Alcaide-Hidalgo et al., 2008). In particular this may also influence secondary fermentation in

sparkling wine making as well as malolactic fermentation (Alcaide-Hidalgo et al., 2008). Further investigation is required to explain this impact.

The concentration of amino acids and the concentration of FAN are usually used to indicate the amount of assimilable nitrogen in musts (Henschke & Jiranek, 1993). Investigation of amino acids and FAN in wines of the present study gave a clear correlation of both values. Comparing those values, the values based on amino acids without proline showed values that were a little higher than the values of FAN. It is certain that methods of measurement have an importance on the assessment of the concentration of amino nitrogen (Marcé et al., 1989, Magné & Larher, 1992; Filipe-Ribeiro & Mendes-Faia, 2007).

The absorption of arginine and the excretion of proline were observable. Arginine is taken up by the yeasts when the supply of the other assimilable nitrogen sources is exhausted, whereas a net increase in proline is often observed due to the formation of arginine metabolism (Bisson, 1991; Ough et al., 1991). The large absorption of arginine by Harmony may be caused by a high population density and diversity of the yeasts. This could also suggest the importance of the inoculation strategy which can affect nitrogen metabolism of yeasts. The uptake of glutamine was almost complete due to it is preferential amino acid source for yeasts (Valero et al., 2003).

5.4 Influence of yeasts on polypeptides and proteins in winemaking

A large number of enological researches studied the nitrogen-containing compounds in *Saccharomyces* yeasts so that plenty of information is available. Understanding of the role of nitrogen containing compounds in *Saccharomyces* yeasts has increased due to the studies of many research groups. (Ough et al., 1991; Monteiro & Bisson, 1992; Cramer et al., 2002; Valero et al., 2003; Varela et al., 2004 ; Hernández-Orte et al., 2005, 2006 ; Garde-Cerdán & Ancín-Aypilicueta, 2007). Mostly, studies focused on ammonium and amino acids. Although many studies investigated peptides and proteins in wine, only those liberated during autolysis of *Saccharomyces* species were considered (Carnevillier et al., 2000; Alexandre et al., 2001; Martínez- Rodríguez et al. 2001 a, 2002; Guilloux-Benatier & Chassagne, 2003). The effect of the fermentation conditions of non-*Saccharomyces* yeasts on their release of nitrogen-containing compounds such as peptides and proteins, enzymes included, during fermentation is largely unknown. Therefore in the experiments of Section 4.4, peptides and proteins

were studied. The investigation of peptides and proteins in must and wine, and their impact on wine making were focused.

Grape juice and wine have various nitrogen compounds of high molecular weights, e.g. biogenic amines, nucleotides, pyrazine, amino acids, peptides, and proteins (Hsu & Heatherbell, 1987, Guilloux-Benatier & Chassagne, 2003; Gafner, 2003 b; Clarke & Bakker, 2004). These compounds have been examined for their impact on grape juice, wine composition, and sensory attributes of wine in a wide extent (Koch & Bretthauer, 1959; Murphey et al., 1989; Acedo et al., 1994; Waters et al., 1996; Moreno-Arribas et al., 1998; Desportes et al., 2001; Dambrouck et al., 2003; Pocock et al., 2003). In contrast nitrogen-containing molecules of yeasts with high molecular weights found in wines and relevant for winemaking are some of the least investigated compounds. Despite the low amount of these molecules, they may have a potential impact on wine composition. The release of peptides and proteins from yeasts and the composition of peptides and protein found in must and wines are therefore discussed here.

Yeasts were able to grow and multiply themselves in the synthetic medium very well (Section 4.4.1). *Saccharomyces* yeasts grew better than non-*Saccharomyces* yeasts. However, factors such as aeration and preparation of inoculation can essentially influence yeast growth (Russell, 2006). In the present study, *S. cerevisiae* (S-Rb) showed a remarkably higher proliferation rate than *S. cerevisiae* (S-EC). This suggests that the strains influence growth which can possibly influence the production of metabolites as well as proteins.

The results clearly showed the potential of yeasts both *Saccharomyces* and non-*Saccharomyces* species to release extracellular proteins. The synthesis and release of these proteins to the medium appeared during yeast growth. Species had a large impact on the level of protein production which could be noticed by the varied concentrations of proteins. Although these proteins may change conformation and function, they remained in the synthetic medium. These proteins could be released during yeast growth and remain in wine when those yeasts are inoculated to grape juice. They can possibly influence microorganisms in the ecosystem of wine fermentation in various ways, e.g. action against spoilage yeasts (Enrique et al., 2007). If those proteins are produced and present in musts and wines and what is their impact during fermentation and in final wines remains to be elucidated.

Ultrafiltration is an effective and fast way to remove materials that may interfere with the analysis of the molecules of interest, especially those present at a low concentration (Bollag et al., 1996; Pall Corporation, 2008). Therefore, the separations

of polypeptides and proteins in grape juices and wines were performed by using ultrafiltration technique.

Cultivation of non-*Saccharomyces* yeasts in pasteurized grape juices was undertaken to examine the influence of these yeasts on peptides and proteins in wine. The concentrations of soluble peptides (≥3-10 kDa) and proteins (≥10 kDa) were varied in fermented must. Variation among species and strains was observed, particularly there were remarkable differences in concentrations of peptides. Regarding the structure of proteins, they can react with numerous compounds and change the conformation depending on many factors, e.g. temperature and pH (Belitz et al., 2004). Grape juice and wine are complex matrices. There are a lot of compounds in grape juice and wine which can bind to proteins, e.g. phenolic compounds, and acids (Charpentier et al., 2004; Cosme et al., 2008). A change of conformation, solubility and hydrolysis of peptides and proteins can also occur (Bollag et al., 1996). This possibly explains lower concentrations of peptides and proteins detected in fermented must in comparison to in the synthetic medium.

Estimation of peptides and proteins in wines displayed great variability. Greater levels of peptides 3-10 kDa were found in wines from spontaneous fermentations. The vintage seems to be an important factor for the concentrations of peptides and proteins in wine because it influences the nitrogen-containing compounds and the diversity and population of yeasts in grape juice (Bisson, 1991; Henschke, 1993). It is well established that non-*Saccharomyces* yeasts dominate in the early stage of spontaneous fermentation, thus these yeasts may affect the concentrations of peptides and proteins in wine. These peptides and proteins produced by yeasts could influence wine composition in various modes of action, e.g. they may influence metabolism of microorganisms and have an impact on organoleptic characteristics. For example, they influence the metabolism of microorganisms by constituting nutrient sources and they can react with other wine compounds, and consequently influence sensory properties. It has been irregularly recognised that spontaneous fermentation results in wines which are rounder and more complex than wines from pure culture fementation of *S. cerevisiae*. Peptides and proteins which are produced by natural yeasts may be partly responsible for this characteristic. Nevertheless, other microorganisms, i.e. bacteria and molds may also provide peptides and proteins to grape juice and wine as well (Kwon, 2004). The process of winemaking such as wine clarification can also influence the amount of proteins in wines (Boyes et al., 1997). A comprehensive study of the role of these molecules related to winemaking should be encouraged to work.

In the last few decades the application of modern analytical techniques has permitted significant advances in wine protein research (Waters et al., 1991). Nevertheless, the common method of electrophoresis on polyacrylamide gel has usually been employed because it is able to separate protein with high solution (Bollag et al., 1996). Therefore, the variability of proteins in grape juices and wines were studied by using a simple method of alcohol precipitation in combination with electrophoresis in the present work. Analyses revealed that various sizes of proteins were present in grape juices. Due to the use of same procedure to fractionate proteins, the varying intensity of protein bands visible on gels suggests that vintage and grape variety are factors influencing in the concentration of proteins and protein composition. Grape cultivars affected the protein pattern as observed on SDS-PAGE gels of crude proteins of Riesling and Weissburgunder. The major component of Weissburgunder must was the 28 molecular weight protein and it was a single band stained on the lane. This visual band could not be interpreted as a single protein in the juice. The crude proteins precipitated by ethanol may contain other proteins too low to be detected with the Coomassie staining. The results confirmed the variability of proteins found in grape juice. Due to a large number of more than 80 different proteins in grape juice (Marchal et al., 2006), a broad range of protein molecular weights have been reported, e.g. 11-69.5 kDa (Murphey et al., 1989), 11.1-64.4 (Nakopoulou et al., 2006), 13-67 kDa (Ribéreau-Gayon et al., 2006 b), 16-200 kDa (Ruiz-Larrea et al., 1998), and 10-250 kDa (Wigand & Decker, 2007).

Enological products containing proteins used for various purposes (Gerland, 2001; Christmann, 2004; Lochbuehler, 2007) may remain in the resulting wines, even if they are applied in a tiny dose. Traces of these products have been already revealed in bottled wines as published by Marchal et al. (2000, 2002) and Stein-Hammer (2004). These proteins have various side effects apart from the desired one. Some of them have negative side effects such as being an allergen (Rolland et al., 2006; Wigand & Decker, 2007). According to Ishibashi et al. (1988) and Belitz et al. (2004), peptides and proteins are widely known to express specific taste and aroma. For examples, proteins like thaumatin have a threshold value of sweetness of 50 nm which is nearly 100,000 times lower than the value of sucrose and lysozyme has a threshold value of sweetness of 10 μM (Masuda & Kitabatake, 2006). There is a possibility that added proteins as well as grape and wine proteins may influence taste and flavour of wine.

Deliberate use of lysozyme in must could be detected in wine was report by Marchal et al. (2000, 2002) and Tirelli and Noni (2007). In samples of fermented must to which lysozyme has been added before fermentation, a protein with a molecular weight 14.6 kDa was found on the gels (**Figure 4-17**). This band had the same size as the protein of the original lysozyme preparation. The finding suggests the presence of lysozyme during fermentation and in wine which could be tracked by electrophoresis analysis. The probable detection of lysozyme reflects the influence of the addition of proteins during wine making on the protein composition of fermented must and wine. By microdensity technique of the band intensity present on gel, it could be possible to detect and quantify lysozyme.

Crude proteins of fermented must sampled during fermentation, wines obtained from different inoculations and wines from spontaneous fermentation had different protein patterns. These protein patterns suggest that the microbial manipulation of wine fermentation influences the protein composition of wine. Furthermore, variability in concentrations of proteins increased depending on the inoculation treatment and the development of fermentation. This was clearly shown in fermentation of Weissburgunder grape juice treated with different inoculation treatments. The visualization of protein bands with molecular weights in a range of 28 to ≥ 99 kDa after the inoculation indicates the production of these proteins by yeasts. It is likely that soluble proteins extremely increased as the intensity of the bands present on the gel rose with the progress of fermentation. This suggests that the composition of proteins during fermentation was related to the development of yeast growth. Therefore, the inoculated yeasts as well as other indigenous microorganisms can possibly involve in alterations of protein profiles of wines. During fermentation the viable population of non-*Saccharomyces* species decrease while *Saccharomyces* species become dominant after the first few days (Henick-Kling et al., 1994; Santamaría et al., 2005). The dead cells of non-*Saccharomyces* yeasts could undergo autolytic cycle which can result in the release of intracellular macromolecules and various degradation products into wine. This may partly explain the differences of protein patterns when non-*Saccharomyces* yeasts are involved in fermentation.

According to Waters et al. (1991, 1992) and Sarmento et al. (2000), proteins with molecular weights of 32kDa or 24 kDa were the most susceptible to heat-induced haze formation, whereas other protein fractions are not known. Further studies are required for the better understanding of peptides and proteins that are relevant in enology. SDS-PAGE is considerably effective to investigate the composition and pattern of proteins in must and wine made by different treatments, e.g. microbial

inoculation and manipulation in winery. The use of SDS-PAGE in combination with additional techniques, e.g. protein separation, protein purification, and staining methods, is a promising method for the basic study of protein pattern in grape juice, wine as well as monitoring of proteins during wine fermentation. Other electrophoresis techniques, e.g. two-dimensional gel electrophoresis, zymography, and isoelectric focusing can also be applied to characterize proteins in grape juice and wine to gain more fundamental knowledge.

6 CONCLUSION AND PERSPECTIVES

The investigation of extracellular proteolytic activity of yeasts confirms that such an activity was undetectable in *Saccharomyces cerevisiae* and that non-*Saccharomyces* yeasts can secrete proteases in variable amount depending on genera, species and strains. There was some evidence that the proteolytic activity may provide an alternative source of nitrogen for microorganisms during fermentation.

Genera, species, and strains had a great impact on the uptake of sugars and amino acids as well as on the production of ethanol and on the formation of other metabolites. The investigated non-*Saccharomyces* yeasts exhibited a limited capacity for completing fermentation in monoculture. Mixed culture inoculation of some strains of non-*Saccharomyces* yeasts and *Saccharomyces* yeasts modified the growth, the fermentation activity of the participating yeasts and the composition of volatile and non-volatile compounds in the resultant wines. The amount of absorption and excretion of amino acids differed depending on yeast strains. Furthermore, the inoculation strategy affected the behaviour of yeasts to excrete and take up amino acids. The excretion of a large amount of proline was noted under certain conditions.

The detected peptides and proteins indicated the release of these nitrogen-containing molecules by yeasts to the matrix. Proteins in wine were derived from grape juice, yeasts and added protein products. The quantity of proteins in white wines depended on fermentation treatments and vintages. The proportion of these proteins remaining in wines was influenced by many factors, i.e. grape variety, yeast diversity and their development during fermentation, vintage, the amount and type of protein additives, and the reaction of the proteins with components binding protein in the matrix.

The applicability of these results to other yeasts and juices from various varieties needs to be determined. This work nevertheless confirmed that the inoculation of non-*Saccharomyces* yeasts in natural grape juice can alter the composition of wine. The results also suggest that the use of non-*Saccharomyces* yeasts in inoculated fermentation in a controlled manner should be prudent because it could have an impact on patterns of fermentation kinetics of the participating yeasts and on their production of metabolites during fermentation. This can eventually influence the composition and sensorial characteristics of the wines. Improvement and detriment of wine quality will be better controlled by gaining more understanding of the behaviour of the mixed culture of *Saccharomyces* and non-*Saccharomyces* yeasts in wine fermentation.

Further studies are required to characterize the extracellular proteins produced by non-*Saccharomyces* yeasts and to study about their presence and stability during fermentation. Simultaneously, influence of these proteins and mannoproteins on volatile and non-volatile metabolites of wine should also be considered. Further experiments should also be undertaken about the role of autolysis of non-*Saccharomyces* yeasts on the wine composition.

7 Summary (in English)

Fifty yeast strains were investigated for extracellular protease activity. They were previously isolated from grapes and wines and belonged to genera *Metschnikowia*, *Hanseniaspora*, *Dekkera*, *Zygosaccharomyces*, *Saccharomycodes*, *Hansenula*, *Pichia*, *Debaromyces*, *Rhodotorula*, *Mycoderma*, *Kluyveromyces*, *Torulaspora*, and *Saccharomyces*. *Saccharomyces* yeasts showed no detectable protease activity, while most of the non-*Saccharomyces* yeasts exhibited proteolytic activity. The proteolytic activity varied within different strains of the same species. Members of the genus *Rhodotorula* exhibited a relatively high proteolytic activity. Nine of the fifty yeast strains were cultivated in grape juice and were analysed for the production of extracellular proteases. The proteolytic activity of *Metschnikowia pulcherrima* (M 004) was as high as that of *Rhodotolura* sp. (R3). Very low activity was found for *Hanseniспora uvarum* (H 045), whereas no activity was detected in the other yeasts. In a synthetic grape juice supplemented with grape proteins, the yeasts exhibiting protease activity did not show considerably higher growth than the yeasts exhibiting undetectable protease activity.

The characterization of eleven non-*Saccharomyces* yeasts during fermentation of pasteurized Riesling juice revealed differences in the concentrations of non-volatile and volatile metabolites. There was a great variation in the ability of yeasts to consume sugars and to influence the composition of odouriferous compounds of the fermented must. The analysis of reducing sugars in the fermented musts at the end of fermentation permitted the classification of yeasts into 3 groups. The species *Zygosaccharomyces bailii*, *Saccharomycodes ludwigii*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* were in the group of yeasts having a high fermentation rate. The species *Hanseniaspora uvarum* and *Metschnikowia pulcherrima* showed low fermentation activity, whereas *Rhodotorula glutinis* was in the third group of yeasts that were unable to ferment. The concentrations of other volatile and non volatile metabolites varied within genera, species and strains. *K. thermotolerans* was the only yeast to produce an extremely high amount of lactic acid. Yeasts of the genera *Zygosaccharomyces* and *Saccharomycodes* produced a relatively greater amount of higher alcohols than yeasts of the other genera. Acetic acid ethyl ester was the principal odouriferous compound produced by all yeast strains except *Rhodotorula glutinis* (N-21). The species *Saccharomycodes ludwigii* (N-15, N-13) produced extremely high amounts of this compound. *Zygosaccharomyces bailii* (N-29) produced higher quantities of fatty acids than the other yeast strains.

To find out whether yeasts exhibiting protease activity could support growth of *Saccharomyces* yeasts, the sequential inoculation of Non-*Saccharomyces* and *Saccharomyces* was performed in grape juice fermentation. 500 mg/l of DMDC and 250 mg/l of lysozyme were added to grape juice in order to suppress the natural microflora. The target concentration of residual sugars (less than 2 g/l) was achieved in the wine obtained from the sequential inoculation of *Hanseniaspora uvarum* (H 045) prior to *S. cerevisiae* (S-EC). The concentrations of assimilable nitrogen slightly increased in wines with sequential inoculation of *H. uvarum* (H 045, H 097) prior to *S. cerevisiae* (S-EC, S-CM).

The effect of the inoculation protocols of non-*Saccharomyces* and *Saccharomyces* yeasts on the formation of metabolites during the fermentation of clarified and unclarified Rielsing juice was investigated. The three inoculation strategies studied were: pure strain inoculation, where only *S. cerevisiae* was added to the grape juice; co-inoculation, where the yeast strains were simultaneously added to grape juice; and sequential inoculation, where the non-*Saccharomyces* species were added 4 days prior to *S. cerevisiae*. The fermentation kinetics were influenced by the inoculation protocols and by the turbidity level of musts. The concentrations of reducing sugars were lower and the concentrations of ethanol were slightly higher in wines obtained from the fermentation of clarified must where sequential inoculation of non-*Saccharomyces* yeast prior to *S. cerevisiae* was used. This effect was not observed in wines obtained from fermentation of unclarified must. The analyses of nitrogen and aroma compounds in the resultant wines have revealed that a wide variability in the production and transformation of these compounds during fermentation existed, depending on inoculation protocols and turbidity level of musts. The fermentation with mixed yeast cultures of clarified must resulted in a substantial increase of the concentration of higher alcohols and acetic acid ethyl ester compared to fermentation with *S. cerevisiae* alone. In contrast, wine fermented with sequential inoculation of *M. pulcherrima* (M 004) prior to *S. cerevisiae* had a lower level of higher alcohols compared to wines produced by other yeast inoculation protocols. The inoculation protocols affected the metabolism of amino acids. The mixed yeast ‘Harmony’ (Hmy) absorbed a substantially higher amount of arginine and γ-aminobutyric acid compared to other inoculation protocols. Alanine, arginine and γ-aminobutyric acid were taken up in a large quantity, whereas the excretion of proline was observed in all inoculation protocols.

Seven yeasts were cultivated in defined medium and cell free supernatants were used to determine the concentrations of soluble proteins. The content of soluble proteins in the medium after cultivation ranged from 3600 to 12100 μg/l. When the cultivation of yeasts in grape juice was performed under microaerobic conditions, the peptides and proteins in the resultant fermented juices ranged from 14 to 1500 μg/l and 1490 to 2301 μg/l, respectively. Four Riesling wines of the vintage 2005 and two Riesling wines of the vintage 2006 were also chosen to examine the content of peptides and proteins. The concentrations of soluble peptides and proteins of those wines ranged from 86 to 1516 μg/l and 40 to 699 μg/l, respectively.

The variability of proteins in grape juices and wines was studied by using a common technique of ethanol precipitation in combination with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Results from SDS-PAGE revealed that various sizes of proteins with a molecular weight of lesser than 14.4 kDa to greater than 99 kDa were present in grape juices and wines.

Deliberate use of lysozyme in must resulted in traces of this enzyme in the wines and musts during fermentation.

Crude proteins separated from musts, sampled during fermentation, and wines made by different strategies of inoculation were analysed by SDS-PAGE gels. The protein bands on the gels suggest that the composition and concentration of proteins in musts during fermentation are related to the progress of yeast growth, apart from other influencing factors.

Zusammenfassung

Es wurden fünfzig Hefestämme auf ihre extrazellulare Proteaseaktivität untersucht. Sie wurden vorher von Trauben und aus Weinen isoliert und gehörten zu den Gattungen *Metschnikowia*, *Hanseniaspora*, *Dekkera*, *Zygosaccharomyces*, *Saccharomycodes*, *Hansenula*, *Pichia*, *Debaromyces*, *Rhodotorula*, *Mycoderma*, *Kluyveromyces*, *Torulaspora*, und *Saccharomyces.*

Saccharomyces-Hefen zeigten keine nachweisbare Proteaseaktivität, während die meisten der Nicht-*Saccharomyces*-Hefen eine proteolytische Aktivität freisetzten. Die proteolytische Aktivität variierte zwischen verschiedenen Stämmen der gleichen Art. Hefen der Gattung *Rhodotorula* setzten eine relativ hohe proteolytische Aktivität frei. Neun der fünfzig Hefen wurden in Traubenmost kultiviert und auf die Produktion extrazellularer Proteasen untersucht. Die proteolytische Aktivität von *Metschnikowia pulcherrima* (M 004) war so hoch wie die von *Rhodotorula* sp. (R3). Eine sehr geringe Aktivität wurde bei *Hanseniaspora uvarum* (H 045) festgestellt, während keine Aktivität in den anderen Hefen detektiert wurde. In einem synthetischen Traubenmost, dem Traubenproteine zugesetzt worden waren, zeigten die Hefen mit proteolytischer Aktivität kein auffällig höheres Wachstum als die Hefen mit nicht nachweisbarer Proteaseaktivität.

Die Charakterisierung von elf Nicht-*Saccharomyces*-Hefen während der Vergärung eines pasteurisierten Riesling-Mostes führte zu Unterschieden in der Konzentration von nicht flüchtigen und flüchtigen Metaboliten. Die Fähigkeit der Hefen den Zucker zu vergären und deren Einfluss auf die Zusammensetzung an geruchsaktiven Stoffen im fermentierten Most variierte stark. Die Analyse der reduzierten Zucker im fermentierten Most am Ende der Gärung erlaubte die Klassifikation der Hefen in drei Gruppen. Die Arten *Zygosaccharomyces bailii, Saccharomycodes ludwigii*, *Torulaspora delbrueckii* and *Kluyveromyces thermotolerans* waren der Gruppe von Hefen mit hoher Fermentationsrate zuzuordnen.

Die Arten *Hanseniaspora uvarum* und *Metschnikowia pulcherrima* zeigten eine geringe Gäraktivität und *Rhodotorula glutinis* befand sich in der dritten Hefegruppe, die nicht gärfähig waren. Die Konzentration anderer flüchtiger und nicht flüchtiger Metaboliten variierte innerhalb der Gattungen, Arten und Stämme. *K. thermotolerans* war die einzige Hefe, die eine extrem hohe Menge an Milchsäure produzierte. Hefen der Gattung *Zygosaccharomyces* und *Saccharomycodes* bildeten einen größeren Gehalt an höheren Alkoholen als die Hefen der anderen Gattungen. Essigsäureethylester war eine der geruchsaktiven Substanzen, die von allen Hefen außer *Rhodotorula glutinis* (N-21) produziert wurde. Die Spezies *Saccharomycodes*

ludwigii (N-15, N-13) bildete extrem hohe Mengen dieser Substanz. *Zygosaccharomyces bailii* (N-29) synthetisierte höhere Mengen an Fettsäuren als andere Hefestämme.

Um zu prüfen, ob Hefen mit höherer proteolytischer Aktivität das Wachstum von *Saccharomyces*-Hefen unterstützen können, wurde eine zeitlich versetzte Beimpfung mit Nicht-*Saccharomyces* und *Saccharomyces* Hefen in Traubenmost durchgeführt. Es wurden 500 mg/l DMDC und 250 mg/l Lysozym dem Traubenmost zugegeben, um die natürliche Mikroflora zu unterdrücken.
Die gewünschte Konzentration an Restzucker (weniger als 2 g/l) wurde in dem Wein erzielt, der mit sequentieller Beimpfung mit *Hanseniaspora uvarum* (H 045) vor *S. cerevisiae* (S-EC) hergestellt wurde.
Die Konzentrationen an hefeverfügbarem Stickstoff stiegen in den Weinen mit sequentieller Inokulation von *H. uvarum* (H 045, H 097) vor *S. cerevisiae* (S-EC, S-CM) etwas an.
Die Auswirkung der unterschiedlichen Beimpfung mit Nicht-*Saccharomyces*- und *Saccharomyces*-Hefen auf die Bildung von Metaboliten wurde in geklärtem und nicht geklärtem Riesling-Most geprüft. Die drei folgenden Inokulationsstrategien wurden untersucht: Inokulation des Traubenmostes mit Reinkultur von *S. cerevisiae*; Koinokulation (simultane Beimpfung des Traubenmostes mit Reinkulturen verschiedener Hefearten) und sequentielle Inokulation von Nicht-*Saccharomyces*-Reinkulturen vier Tage vor Zugabe der *S. cerevisiae* Kultur. Die Fermentationskinetiken wurden durch die verschiedenen Inokulationsstrategien und durch den Klärgrad der Moste beeinflusst. Die Konzentrationen an Restzucker waren niedriger und die Mengen an Alkohol waren etwas höher in den Weinen, bei deren Vergärung geklärter Most und sequentielle Inokulation von Nicht-*Saccharomyces*-Hefen vor *S. cerevisiae* eingesetzt worden waren. Dieser Effekt wurde nicht in Weinen aus der Vergärung mit ungeklärtem Traubenmost festgestellt. Die Analyse von Stickstoff- und Aromakomponenten in den vergorenen Weinen verdeutlicht, dass eine große Variabilität in der Produktion und Veränderung dieser Komponenten während der Gärung in Abhängigkeit der verschiedenen Inokulationsstrategien vorlag. Eine Vergärung ungeklärten Mostes mit Hefemischkulturen führte im Vergleich zu einer Gärung mit ausschließlich *S. cerevisiae* zu einem deutlichen Anstieg in den Konzentrationen an höheren Alkoholen und Essigsäureethylester. Im Gegensatz dazu hatte der Wein mit sequentieller Vergärung von *M. pulcherrima* (M 004) vor *S. cerevisiae* eine geringere Konzentration an höheren Alkoholen im Vergleich zu

Weinen, die gemäß den anderen Inokulationsprotokollen hergestellt wurden. Die unterschiedlichen Inokulationsstrategien beeinflussten auch den Metabolismus der Aminosäuren.
Die Hefemischkultur ‚Harmony' (Hmy) verwertete eine deutlich höhere Menge Arginin und γ-Aminobuttersäure im Vergleich zu den Vergärungen gemäß den anderen Inokulationsprotokollen. Alanin, Arginin und γ-Aminobuttersäure wurden in hohen Mengen aufgenommen, während die Exkretion von Prolin bei Einsatz jedes Inokulationsprotokolls festgestellt wurde.

Sieben Hefen wurden in definierten Medien kultiviert und der zellfreie Überstand wurde dazu benutzt, die Konzentration der löslichen Proteine zu bestimmen. Der Gehalt an löslichen Proteinen im Medium nach der Kultivierung lag zwischen 3600 und 12100 µg/l. Bei einer Hefekultivierung in Traubenmost unter mikroaeroben Bedingungen bewegten sich die Gehalte an Peptiden und Proteinen in den resultierenden vergorenen Mosten jeweils zwischen 14 bis 1500 µg/l und 1490 bis 2301 µg/l. Vier Riesling Weine des Jahrgangs 2005 und zwei Riesling Weine des Jahrgangs 2006 wurden ebenfalls ausgewählt, um den Gehalt an Peptiden und Proteinen zu untersuchen. Die Konzentrationen an löslichen Peptiden und Proteinen in diesen Weinen befanden sich jeweils zwischen 86 bis 1516 µg/l und 40 bis 699 µg/l. Die Variabilität der Proteine in Traubenmosten und Weinen wurde mittels einer üblichen Technik untersucht, nämlich der Fällung mit Ethanol in Verbindung mit Sodiumdodecylsulfat-Polyacrylamid-Gelelektrophorese (SDS-PAGE). Die Ergebnisse der SDS-PAGE zeigten, dass verschiedene Proteingrößen mit einem Molekulargewicht von weniger als 14,4 kDa bis mehr als 99 kDa im Traubenmost und Wein vorhanden waren.
Das bewusste Einsetzen von Lysozym im Most hatte zur Folge, dass Spuren dieses Enzyms in den Weinen und Mosten während der Gärung zu finden waren.
Die während der Vergärung von Traubenmosten und aus vergorenen Weinen, die gemäß verschiedener Inokulationsstrategien hergestellt wurden, abgetrennten Rohproteine wurden mit SDS-PAGE Gelen analysiert. Die Proteinbanden auf den Gelen lassen vermuten, dass die Zusammensetzung und Konzentration von Proteinen in Mosten während der Gärung neben anderen Einflussfaktoren mit dem Wachstumsfortschritt der Hefen zusammenhängt.

8 REFERENCES

Acedo, M. I., Pueyo, E., & Polo, M. C. (1994). Preliminary studies on peptides in wine by HPLC. *American Journal of Enology and Viticulture, 45*, 167-172.

Aehle, W. (2004). *Enzymes in industry: production and applications.* Weinheim: Wiley-VCH.

Alcaide-Hidalgo, J. M., Moreno-Arribas, M. V., Polo, M. C., & Pueyo, E. (2008). Partial characterization of peptides from red wines: changes during malolactic fermentation and aging with lees. *Food Chemistry, 107,* 622-630.

Alexandre, H., Heintz, D., Chassagne, D., Guilloux-Benatier, M., Charpentier, C., & Feuillat, M. (2001). Protease A activity and nitrogen fractions released during alcoholic fermentation and autolysis in enological conditions. *Journal of Industrial Microbiology and Biotechnology, 26,* 235-240.

Ancín, C., Torrea, D., Fraile, P., & Garde, T. (2004). Amino acids and volatile compounds in the fermentation of inoculated musts: biogenic amines in the wines. In F. Shahidi & D. K. Weerasinghe (Eds.), *Nutraceutical beverages: chemistry, nutrition, and health effects* (pp. 302-313). Washington, DC: American Chemical Society.

Ansorge, A. (2007). Influence of vinification processes on contents of biogenic amines in wine. 8th International Symposium Innovations in Enology, (pp. 133-140). Stuttgart.

Bakalinsky, A. T. & Boulton, R. (1985). The study of an immobilized acid protease for the treatment of wine proteins. *American Journal of Enology and Viticulture, 36*, 23-29.

Barata, A., Caldeira, J., Botellheiro, R. Pagliara, D., Malfeito-Ferreira, M., & Loureiro, V. (2008). Survival patterns of *Dekkera bruxellensis* in wines and inhibitory effect of sulphur dioxide. *International Journal of Food Microbiology, 121,* 201-207.

Barnett, J. A., Payne, R. W., & Yarrow, D. (2000). *Yeasts: characteristics and indentification.* Cambridge: Cambridge University Press.

Barrett, A. J., Rawlings, N. D., & Woesser, J. F. (2004). *Handbook of Proteolytic Enzymes.* Amsterdam: Academic Press.

Baumgartner, D., Bill, R., & Roth, I. (2001). Traubenmostanalyse mit Hilfe der FTIR-Spektroskopie. *Schweizerische Zeischrift fuer Obst-und Weinbau, 2*, 46-48.

Béhalová, B., & Beran, K. (1979). Activation of proteolytic enzymes during autolysis of disintegrated baker's yeast. *Folia Microbiology, 24,* 455-461.

Belitz., H.D., Grosch, W., & Schieberle, P. (2004). *Food chemistry*. Berlin: Springer.

Bell, S., & Henschke, P. (2005). Implications of nitrogen nutrition for grapes, fermentation and wine. In R. Blair, M. Francis, & I. Pretorius, (Eds), *Advances in wine science* (pp. 45-91). The Australian Wine Research Institute.

Beltran, G., Novo, M., Rozes, N., Mas, A., & Guillamon, J. M. (2004). Nitrogen catabolite repression in *Saccharomyces cerevisiae* during wine fermentations. *FEMS Yeast Research, 4,* 625-632.

Beltran, G., Rozes, N., Mas, A., & Guillamon, J. M. (2007). Effect of low-temperature fermentation on yeast nitrogen metabolism. *World Journal of Microbiology and Biotechnology, 23, 809-815*.

Beltran, G., Novo, M., Guillamón, J. M., Mas, A., & Rozès, N. (2008). Effect of fermentation temperature and culture media on the yeast lipid composition and wine volatile compounds. *International Journal of Food Microbiology, 121,* 169-177.

Bely, M., Stoeckle, P., Masneuf-Pomarède, I., & Dubourdieu, D. (2008). Impact of mixed *Torulaspora delbrueckii-Saccharomyces cerevisiae* culture on high-sugar fermentation. *International Journal of Food Microbiology, 122,* 312-320.

Bergdolt, C. (2007). Mischkulturen aus verschiedenen Hefearten im Vergleich zur Reinzuchthefe- und Spontangaerung bei der Wesswinbereitung. Diplomarbeit, Fachhochschule Wiesbaden, Fachbereich Geisenheim, Germany.

Beynon, R. J., & Bond, J. S. (1990). *Proteolytic enzymes: a practical approach.* Oxford: Oxford University Press.

Bilinski, C. A., & Stewart, G. G. (1990). Yeast proteases and brewing. In H. Verachtert, & R. D. Mot (Eds.), *Yeast Biotechnology and Biocatalysis* (pp. 147-162). New York: Marcel Dekker, Inc.

Bilinski, C.A., Russell, I, & Stewart, G. G. (1987). Applicability of yeast extracellular proteinases in brewing: physiological and biochemical aspects. *Applied and Environmental Microbiology, 53,* 495-499.

Bilinski, C. A., Emery, C. J., Zygora, P. S., Russell, I., & Stewart, G. G. (1988). Applicability of yeast extracellular proteinases in beer stabilization. *Critical Reviews in Biotechnology, 8,* 111-120.

Bio-Rad Laboratories. (2007). Instructions for the Bio-Rad protein assay. Munich: Bio-Rad Laboratories.

Bisson, L. F. (1991). Influence of nitrogen on yeast and fermentation of grapes. *International symposium on Nitrogen in Grapes and Wine*, (pp. 78-89). Davis: American society for Enology and viticulture

Bisson, L.F., & Butzke, C.E. (2000). Diagnosis and rectification of stuck and sluggish fermentations. *American Journal of Enology and Viticulture, 51*, 168-177.

Boehringer Mannheim. (1984). Methoden der enzymatischen Lebensmittelanalytik mit Einzelreagenzien. Mannheim, Germany.

Bollag, D. M., Rozychi, M. D. & Edelstein, S. (1996). *Protein methods.* New York: John Wiley & Sons.

Bolumar, T., Sanz, Y., Aristoy, M., & Toldrá, F. (2005). Protease B from *Debaryomyces hansenii*: purification and biochemical properties. *International Journal of Food Microbiology, 98,* 167-177.

Boulton, C., & Quain, D. (2006). *Brewing yeast and fermentation*. Oxford: Blackwell.

Boulton, R. B., Singleton, V. L., Bisson, L., & Kunkee, R. (1999). *Principles and practices of winemaking.* Maryland: Aspen Publishers.

Boze, H., Moulin, G., & Galzy, P. (1992). Production of food and fodder yeasts. *Critical Reviews in Biotechnology, 12,* 65-86.

Bradford, M.M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. *Analytical Biochemistry, 72,* 248-254.

Brueckner, H., & Koza, A. (2003). Solution phase synthesis of the 14-residue peptaibol antibiotic trichovirin I. *Amino Acids, 24,* 311-323.

Brueckner, H., & Paetzold, R. (2006). Sind D-aminosaeuren gute molekulare Marker in Lebensmitteln? Pro und Kontra. *Lebensmittelchemie, 60,* 141-142.

Brueckner, H., & Westhauser, T. (2003). Chromatographic determination of L- and D-amino acids in plants. *Amino Acids, 24,* 43-55.

Brueckner, H., Justus, J., & Kirschbaum, J. (2001). Saccharide induced racemization of amino acids in the course of the Maillard reaction. *Amino Acids, 21,* 429-433.

Brueckner, H., Kirschbaum, J., & Paetzold, R. (2007). Molekulare Uhren, Spiegelmolekuele und der Zahn der Zeit. *Spiegel der Forschung, 1,* 60-67.

Brunner, U. (2006). Massgeschneiderte Gaerung mit Hefe-Mischungen. *Schweizerische Zeischrift fuer Obst-und Weinbau, 23*, 6-9.

Buzzini, P. & Vaughan-Martini, A. (2006). Yeast biodiversity and biotechnology. In C. Rosa & G. Péter (Eds.), *Biodiversity and ecophysiology of yeasts: the yeast handbook* (pp. 533-559). Berlin: Springer.

Cabaroglu, T., Selli, S, Canbas, A., Lepoutre, J., & Guenata, Z. (2003). Wine flavour enhancement through the use of exogenous fungal glycosidases. *Enzyme and Microbial Technology, 33,* 581-587.

Carnevillier, V., Charpentier, C., & Feuillat, M. (2000). Production de Peptides par *Saccharomyces cerevisiae* au Cours de la Fermentatio et de L'Autolyse sur Moût Chardonnay. *6e Symposium International d'œnologie,* (pp. 287-289). Paris.

Carrau, F. M., Medina, K., Boido, E., Farina, L., Gaggero, C., Dellacassa, E., Versini, G. & Henschke, P. A. (2005). De novo systhesis of monoterpenes by *Saccharomyces cerevisiae* wine yeasts. *FEMS Microbiology Letters, 243*, 107-115.

Caruso, M., Fiore, C., Contursi, M., Salzano, G., Paparella, A., & Romano, P. (2002). Formation of biogenic amines as criteria for the selection of wine yeasts. *World Journal of Microbiology and Biotechnology, 18,* 159-163.

Cebollero, E., & Gonzalez, R. (2006). Induction of autophagy by second-fermentation yeasts during elaboration of sparkling wines. *Applied and Environmental Microbiology, 72,* 4121-4127.

Cebollero, E., Carrascosa, A. V., & Gonzalez, R. (2005). Evidence for yeast autophagy during simulation of sparkling wine aging: a reappraisal of the mechanism of yeast autolysis in wine. *Biotechnology Progress, 21,* 614-616.

Charoenchai, C., Fleet, G. H., Henschke, P. A., & Todd, B. E. N. (1997). Screening of non-*Saccharomyces* wine yeasts for the presence of extracellular hydrolytic enzymes. *Australian Journal of Grape and Wine Research, 3,* 2-8.

Charpentier, C., Escot, S., Gonzalez, E., Dulau, L., & Feuillat, M. (2004). The influence of yeast glycosylated proteins on tannins aggregation in model solution. *International des Sciences de la Vigne et du Vin, 38*, 209-218.

Cheraiti, N., Guezenec, S., & Salmon J. M. (2005). Redox interactions between *Sacharomyces cerevisiae* and *Sacharomyces uvarum* in mixed culture under enological conditions. *Applied and Environmental Microbiology, 71,* 255-260.

Christmann, M. (2004). *Moderne Mostvorklaerung: Theoretische Grundlagen der Mostvorklaerung aus der Sicht der Weissweinbereitung.* Neustadt: Meininger Verlag.

Ciani, M., & Comitini, F. (2006). Influence of temperature and oxygen concentration on the fermentation behaviour of *Candida stellata* in mixed fermentation with *Saccharomyces cerevisiae*. *World Journal of Microbiology and Biotechnology, 22,* 619-623.

Ciani, M. & Maccarelli, F. (1998). Oenological properties of non-*Saccharomyces* yeasts associated with wine-making. *World Journal of Microbiology and Biotechnology, 14,* 199-203.

Ciani, M., Beco, L., & Comitini, F. (2006). Fermentation behaviour and metabolic interactions of multistarter wine yeast fermentations. *International Journal of Food Microbiology, 70,* 239-245.

Clarke, R. J., & Bakker, J. (2004). Wine flavor chemistry. Oxford: Blackwell Publishing.

Clemente-Jimenez, J. M., Mingorance-Cazorla, M., Martinez-Rodriguez, S., Heras-Vazquez, F. J. L., & Rodriguez-Vico, F. (2004). Molecular characterization and oenological properties of wine yeasts isolated during spontaneous fermentation of six varieties of grape must. *Food Microbiology, 21,* 149-155.

Clemente-Jimenez, J. M., Mingorance-Cazorla, L., Martinez-Rodriguez, S., Las Heras-Vazquez, F. J., & Rodriguez-Vico, F. (2005). Influence of sequential yeast mixtures on wine fermentation. *International Journal of Food Microbiology, 98,* 301-308.

Colagrande, O., Silva, A., & Fumi, M.D. (1994). Recent applications of biotechnology in wine production. *Biotechnology Progress, 10,* 2–18.

Coleman, M., Fish, R., and Block, D. (2007). Temerature-dependent kinetic model for nitrogen-limited wine fermentations. *Applied and Environmental Microbiology, 73,* 5875-5884.

Combina, M., Elía, A., Mercado, L., Catania, C., Ganga, A., & Martinez, C. (2005). Dynamics of indigenous yeast populations during spontaneous fermentation of wines from Mendoza, Argentina. *International Journal of Food Microbiology, 99,* 237-243.

Conterno, L., & Delfini, C. (1994). Peptidase activity and the ability of wine yeasts to utilise grape must proteins as sole nitrogen source. *Journal of Wine Research, 5,* 113-126.

Conterno, L., & Delfini, C. (1996). Peptidasic activity and ability of wine yeasts to utilise grape proteins as sole nitrogen source. In A. Lonvaud-Funel (Ed.), *5e Symposium International d' Oenologie* (pp. 240-245). London: Lavoisier Tec & Doc.

Cosme, F., Ricardo-da-Silva, J. M., & Laureano, O. (2008). Interactions between protein fining agents and proanthocyanidins in white wine. *Food Chemistry, 106*, 536-544.

Costa, A., Barata, A., Malfeito-Ferreira, M., & Loureiro, V. (2008) Evaluation of the inhibitory effect of dimethyl dicarbonate (DMDC) against wine microorganisms. *Food Microbiology, 25,* 422-427.

Cramer, A. C., Vlassides, S., & Block, D. E. (2002) Kinetic model for nitrogen-limited wine fermentations. *Biotechnology and Bioengineering, 77,* 49-60.

Dambrouck, T., Marchal-Delahaut, L., Parmentier, M., Maujean, A., & Jeandet, P. (2003). Immuno detection of proteins from grapes and yeast in a white wine. *Journal of Agricultural and Food Chemistry, 51,* 2727-2732.

Davidson, P. M. (2002). Chemical preservatives and natural antimicrobial compounds. In M. P. Doyle, L. R. Beuchat, & T. J. Montville (Eds.), *Fundamentals and frontiers* (pp. 593-627). New York: ASM Press.

Delfini, C., & Vormica, J. V. (2001). *Wine microbiology: science and technology.* New York: Marcel Dekker.

Demain, A. L., Phaff, H. J., & Kurtzman, C. P. (1998). The industrial and agricultural significance of yeasts. In C. P. Kurtzman & J. W. Fell (Eds.), *The yeast: a taxonomic study* (pp. 13-20). Amsterdam: Elsevier.

Desportes, C., Charpentier, M., Duteurtre, B., Maujean, A., & Duchiron, F. (2001). Isolation, identification, and organoleptic characterization of low-molecular-weight peptides from white wine. *American Journal of Enology and Viticulture, 52*, 376-380.

Dickinson, J. R. (2004). Nitrogen metabolism. In J. R. Dickinson & M. Schweizer (Eds.), *The metabolism and molecular physiology of Saccharomyces cerevisiae* (pp. 104-116) Boca Raton: CRC Press.

Dittrich, H. H., & Grossmann, M. (2005). Mikrobiologie des Weines. Stuttgart: Ulmer.

Divol, B., Strehaiano, P., & Lonvaud-Funel., A. (2005). Effectiveness of dimethyldicarbonate to stop alcoholic fermentation in wine. *Food Microbiology, 22,* 169-178.

Dizy, M., & Bisson, L, F. (2000) Proteolytic activity of yeast strains during grape juice fermentation. *American Journal of Enology and Viticulture, 51*, 155-167.

Domizio, P., Lencioni, L., Ciani, M., Di Blasi, S., Peontremosesi, C., & Sabatelli, M. P. (2007). Spontaneous and inoculated yeast populations dynamics and their effect on organoleptic characters of Vinsanto wine under different process conditions. *International Journal of Food Microbiology, 115,* 281-289.

Erbsloeh Geisenheim. (1997). Technical datasheet of Ferm-N and ammonium analysis.

Favale, S. Pietromarchi, P., & Ciolfi, G. (2007). Metabolic activity and interactions between two strains, *Saccharomyces cerevisiae* r.f. bayanus (SBC2) and Saccharomyces cerevisiae r.f. uvarum (S6U), in pure and mixed culture fermentations. Vitis, 46, 39-43.

Fell, J.W., & Statzell-Tallman, A. (1998). 94. *Cryptococus* Vuillemin. In C. P. Kurtzman & J. W. Fell (Eds.), The yeast: a taxonomic study (pp. 742-767). Amsterdam: Elsevier.

Fernández, M., Ubeda, J. F., & Briones, A. I. (2000). Typing of non-*Saccharomyces* yeasts with enzymatic activities of interest in wine-making. *International Journal of Food Microbiology, 59,* 29-36.

Fernández-González, M., Di Stefano, R. & Briones, A. (2003). Hydrolysis and transformation of terpene glycosides from muscat must by different yeast species. *Food Microbiology, 20*, 35-41.

Ferreira, J., Dutoit, M., & Dutoit, W. J. (2006). The effects of copper and high sugar concentrations on growth, fermentation efficiency and volatile acidity production of different commercial wine yeast strains. *Australian Journal of Grape and Wine Research, 12,* 50-56.

Ferreira, R. B., Picarra-Pereira, M. A., Monteiro, S., Loureiro, V. B., & Teixeira, A. R. (2002). The wine proteins. *Trends in Food Science & Technology, 12,* 230-239.

Feuillat, M. (2005). Use of yeasts in Burgundy and in other regions: fermentation and aging on lees. *Les XVII^{e} scientitiques Lallemand* (pp. 27-32). La Rioja : Lallemand.

Filipe-Ribeiro., L. & Mendes-Faia, A. (2007). Validation and comparison of analytical methods used to evaluate the nitrogen status of grape juice. *Food Chemistry, 100,* 1272-1277.

Fleet, G. (1992) Spoilage yeasts. *Critical Reviews in Biotechnology, 1,* 1-44.

Fleet, G.H. (1993). The microorganisms of wine making: isolation enumeration and identification. In G. H. Fleet (Ed.), *Wine Microbiology and Biotechnology* (pp. 1-26). Chur: Harwood Academic Publishers.

Fleet, G.H. (1999). Microorganisms in food ecosystems. *International Journal of Food Microbiology, 50,* 101-117.

Fleet, G. H. (2001). Wine. In M. P. Doyle, L. R. Beuchat, & T. J. Montville (Eds.), *Food Microbiology: fundamentals and frontiers* (pp. 747-772). Washington: ASM Press.

Fleet, G. H. (2003). Yeast interactions and wine flavor. *International Journal of Food Microbiology, 86,* 11-22.

Fleet, G. H. (2006). The commercial and community significance of yeasts in food and beverage production. In A. Querol & G. H. Fleet (Eds.), *Yeasts in Food and Beverages* (pp. 1-12). Heidelberg: Springer.

Fleet, G. H. (2007). Yeasts in foods and beverages: impact on product quality and safety. *Current Opinion in Biotechnology, 18,* 1-6.

Fleet, G. H. & Heard, G. (1993). Growth during fermentation. In G. H. Fleet (Ed.), *Wine Microbilology and Biotechnology* (pp. 27-54). Chur: Harwood Academic Publishers.

Forgarty, W. M., & Kelly, C. T (1990). *Microbial enzymes and biotechnology*. New York: Elsevier Science.

Fornairon-Bonnefond, C., Camarasa, C., Mouthounet, M. & Salmon, J.M. (2002). New trends on yeast autolysis and wine ageing on lees: a bibliographic review. *International des Sciences de la Vigne et du Vin, 36*, 49-69.

Francis, P. S. (2006). The determination of urea in wine: a review. *Australian Journal of Grape and Wine Research, 12,* 97-106.

Francis, I. L., & Newton, J. L. (2005). Determining wine aroma from compositional data. *Australian Journal of grape and Wine Research, 11,* 114-126.

Fugelsang, K. C., & Edwards, C. G. (2007). Wine microbiology: practical applications and procedures. New york: Springer.

Fukui, M., & Yokotsuka, K. (2003). Content and origin of protein in white and red wines: changes during fermentation and maturation. *American Journal of Enology and Viticulture, 54,* 178-188.

Fukui, N., Kogin, A., Furukubo, S., Kondo, H., Yomo, H., & Kakimi, V. (1996). Evaluation of yeast activity and its application to our industrial brewing. Technical Quarterly*, 33,* 166-169.

Gafner, J. (2002). Gaerstockungen: erkennen, verhindern, kurieren. *Schweizerische Zeischrift fuer Obst-und Weinbau, 22*, 594-595.

Gafner, J. (2003 a). Guter Wein ist kein Zufall. *Schweizerische Zeischrift fuer Obst-und Weinbau, 8*, 13-15.

Gafner, J. (2003 b). Mikroorganismen beim biologischen Saeureabbau und Weinausbau. *Schweizerische Zeischrift fuer Obst-und Weinbau, 11*, 6-11.

Ganeva, V., Galutzov, B., & Teissie, J. (2002). Electroinduced release of invertase from saccharomyces cerevisiae. *Biotechnology Letters, 24,* 1853-1856.

Ganga, M.A., & Martinez, C. (2004). Effect of wine yeast monoculture practice on the biodiversity of non-*Saccharomyces* yeasts. *Journal of Applied Microbiology, 96,* 76-83.

Garcia, A., Carcel, C., Dulau, L., Samson, A., Aguera, E., Agosin, E., & Guenata, Z. (2002). Influence of a mixed culture with *Debaryomyces vanriji* and *Saccharomyces cerevisiae* on the volatiles of a Muscat wine. *Journal of Food Science, 67,* 1138-1143.

Garde-Cerdán, T. & Ancín-Aypilicueta, C. (2007). Effect of the addition of different qualities of amino acids to nitrogen-deficient must on the formation of esters, alcohols, and acids during wine alcoholic fermentation. *LWT-Food Science and Technology, 41,* 501-510.

Gawel, R. Sluyter, S.V., & Waters, E. (2007). The effects of ethanol and glycerol on the body and other sensory characteristics of Riesling wines. *Australian Journal of Grape and Wine Research, 13,* 38-45.

Genovés, S., Gil, J.V., Vallés, S., Casas, J.A., & Manzanares, P. (2005). Assessment of the aromatic potential of Palomino Fino grape must using glycosidases. *American Journal of Enology and Viticulture, 56*, 188-191.

Gerland, C. (2001) Use of lysozyme in enology: main applications and secondary effects. *Intervitis Interfructa: 6. Internationales Symposium Innovationen in der Kellerwirtschaft Intervitis Interfructa*, (pp. 251-260). Stuttgart.

Gil, J. V., Mateo, J. J., Jiménez, M., Pastor, A., & Huerta, T. (1996). Aroma compounds in wine as influenced by apiculate yeasts. *Journal of Food Science, 61,* 1247-1250.

Godden, P., & Gishen, M. (2005). Trends in the composition of Australian wine. *Australian and New Zealand Wine Industry Journal, 20,* 21-46.

Grbin, P. R., & Henschke, P. A.(2000). Mousy off-flavour production in grape juice and wine by *Dekkera* and *Brettanomyces* yeasts. *Australian Journal of Grape and Wine Research, 6,* 255–262.

Grbin, P. R., Costello, P. J., Herderich, M., Markides, A. J., Henschke, P. A. & Lee, T. H. (1995). Developments in the sensory, chemical and microbiological basis of mousy taint in wine. In C. S. Stockley, A. N. Sas & R. S. Johnstone & T. H. Lee (Eds.), *Proceedings of the ninth Australian wine industry technical conference* (pp. 57-61). Underdale: Winetitles.

Grossmann, M. (2006). Terroir-Charakter durch lokale Hefeflora?. *Der Deutsche Weinbau, 4,* 6.

Grossmann, M. (2007). Spontangaerung als Ausdruck von Tradition? *Der Deutsche Weinbau, 7,* 84-89.

Grossmann, M. & Begerow GmbH & Co., Langenlonsheim, Germany (1990). Protocol for preparation of HDM-media, company internal recipe.

Grossmann, M., Munol, H., & Rapp, A. (1997). Influence of oligo-strain yeast cultures on fermentation profiles in comparison to one-strain cultures. *American Journal of Enology and Viticulture, 4*8, 258.

Grossmann, M., Linsenmeyer, H., Muno, H. & Rapp, A. (1996). Use of oligo-strain yeast cultures to increase complexity of wine aroma. *Viticultural and Enological Science, 51,* 175-179.

Guilloux-Benatier, M., & Chassagne, D. (2003). Comparison of components released by fermented or active dried yeasts after aging on lees in a model wine. *Journal of Agricultural and Food Chemistry, 51,* 746-751.

Hansen, E. H., Nissen, P. Sommer, P., Nielsen, J. C., & Arneborg, N. (2001). The effect of oxygen on the survival of non-*Saccharomyces* yeasts during mixed culture fermentations of grape juice with *Saccharomyces cerevisiae*. *Journal of Applied Microbiology, 91,* 541-547.

Hayasaka, Y., Birse, M., Eglinton, J., & Herderich, M. (2007). The effect of *Saccharomyces cerevisiae* and *Saccharomyces bayanus* yeast on colour properties and pigment profiles of a Cabernet Sauvignon red wine. *Australian Journal of Grape and Wine Research, 13,* 176-185.

Heard, G. M. (1999). Novel yeasts in winemaking-looking to the future. *Food Australia, 51,* 347-352.

Heard, G. M., & Fleet, G. H. (1986). Occurrence and growth of yeast species during the fermentation of some Australian wines. *Food Technology in Australia, 38,* 22-25.

Heidkamp, F. (2005). Isolierung und Selektionierung von Nicht-*Saccharomyceten* aus einer grossen Gewächs-Lage. Diplomarbeit, Fachhochschule Wiesbaden, Fachbereich Geisenheim, Germany.

Henick-Kling, T & Oarjm Y, H. (1994). Considerations for the use of yeast and bacterial starter cultures: SO_2 and timing of inoculation. *American Journal of Enology and Viticulture, 45,* 464-469.

Henick-Kling, T., Edinger, W., Daniel, P., & Monk, P. (1998). Selective effects of sulphur dioxide and yeast starter culture addition on indigenous yeast populations and sensory characteristics of wine. *Journal of Applied Microbiology, 84,* 865-867.

Henschke, P. A. (1997). Wine yeasts. In F. K. Zimmermann & K. D. Entian (Eds.), *Yeast sugar metabolism* (pp. 527-560). Lancaster: Technomic Publishing.

Henschke, P. A, & Jiranek, V. (1991). Hydrogen sulfide formation during fermentation: effect of nitrogen composition in model grape must. *International symposium on nitrogen in grapes and wine* (pp. 172–184), Seattle, Davis: American Society for Enology and Viticulture.

Henschke, P.A., & Jiranek V. (2002). Yeast-metabolism of nitrogen compounds. In G. H. Fleet (Ed.), *Wine Microbilology and Biotechnology* (pp. 77-164). Chur: Harwood Academic Publishers.

Herbert, P., Cabrita, M. J., Ratola, N., Laureano, O., & Alves, A. (2005). Free amino acids and biogenic amines in wines and musts from the Alentejo region: evolution of amines during alcoholic fermentation and relationship with variety, sub-region and vintage. *Journal of Food Engineering, 66,* 315-322.

Hernández -Orte, P., Bely, M., Cacho, J., & Ferreira, V. (2006 a). Impact of ammonium additions on volatile acidity, ethanol, and aromatic compound production by different *Saccharomyces cerevisiae* strains during fermentation in controlled synthetic media. *Australian Journal of Grape and Wine Research, 12,* 150-160.

Hernández-Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2005). Effect of the addition of ammonium and amino acids to musts of Airen variety on aromatic composition and sensory properties of the obtained wine. *Food Chemistry, 89,* 163-174.

Hernández -Orte, P., Ibarz, M. J., Cacho, J., & Ferreira, V. (2006 b). Addition of amino acids to grape juice of the Merlot variety: effect on amino acid uptake and aroma generation during alcoholic fermentation. *Food Chemistry, 98,* 300-310.

Hernández-Orte, P., Cersosimo, M., Loscos, N., Cacho, J., Garcia-Moruno, E. G., & Ferreira, V. (2008). The development of varietal aroma from non-floral grapes by yeasts of different genera. *Food Chemistry, 107,* 1064-1077.

Herraiz, T., Reglero, G., Herraiz, M., Martin-Alvarez, P. J., & Cabezudo, M. D. (1990). Influence of the yeast and type of culture on the volatile composition of wines fermented without sulphur dioxide. *American Journal of Enology and Viticulture, 41,* 313-318.

Howell, K., Cozzolino, D., Bartowsky, E., Fleet, G. H., & Henschke, P. A. (2006). Metabolic profiling as a tool for revealing *Saccharomyces* interaction during wine fermentation. *FEMS Yeast Research, 6,* 91-101.

Hsu, J. & Heatherbell, D. A. (1987). Isolation and characterization of soluble proteins in grapes, grape juice, and wine. *American Journal of Enology and Viticulture, 38*, 6-10.

Iranzo, J. F. U., Perez, A. I. B., & Canas, P. M. I. (1998). Study of the oenological characteristics and enzymatic activities of wine yeasts. *Food Microbiology, 15,* 399-406.

Irmler, S., Raboud, S., Beisert, B., Rauhut, D., & Berthoud, H. (2008). Cloning and characterization of two *Lactobacillus casei* genes encoding a cystathionine lyase. *Applied and Environmental Microbiology, 74,* 99-106.

Ishibashi, N., Arita, Y., Kanehisa, H., Kouge, K., Okai, H., & Fukui, S. (1987). Bitterness of leucine-containing peptides. *Agricultural and Biological Chemistry, 51,* 2389-2394.

Ishibashi, N., Kubo, T., Chino, M., Fukui, H., Shinoda, I., Kiduchi, E., and Okai, H. (1988) Taste of proline-containing peptides. *Agricultural and Biochemistry,* 52, 95-98.

Jakob, L. (1995). *Lexikon der Oenologie* (3rd ed). Neustadt: Meininger.

Jay, J. M., Loessner, M., & Golden, D. A. (2005). *Modern food microbiology* (7th ed.). New York: Springer.

Jemec, K. P., Cadez, N., Zagorc, T., Bubic, V., Zupec, A., & Raspor, P. (2001). Yeast population dynamics in five spontaneous fermentations of Malvasia must. *Food Microbiology, 18,* 247-259.

Jeune, C. L., Erny, C., Demuyter, C., & Lollier, M. (2006). Evolution of the population of *Saccharomyces cereviae* from grape to wine in a spontaneous fermentation. *Food Microbiology, 23,* 709-716.

Jiranek, V., Langridge, P., & Henschke, P. A. (1990). Nitrogen requirement of yeast during wine fermentation. In P. J. Williams, D. M. Davidson & T. H. Lee (Eds.), *Proceeding of seventh Australian wine industry technical conference* (pp. 166-171). Adelaide: Winetitles.

Jones, E. W. (1991 a). Tackling the protease problem in *Saccharomyces cerevisiae*. *Methods in Enzymology, 194,* 428-453.

Jones, E. W. (1991 b). Three proteolytic systems in the yeast *Saccharomyces cerevisiae*. *Journal of Biological Chemistry, 266,* 7963-7966.

Jolly, N. (2007). The use of fructophilic yeast for a co-inoculated fermentation of grape juice. *8th International Symposium Innovations in Enology* (pp. 87-95). Stuttgart.

Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2003 a). The effect of non-*Saccharomyces* yeasts on fermentation and wine quality. *South African Journal of Enology and Viticulture, 24*, 55-62.

Jolly, N. P., Augustyn, O. P. H., & Pretorius, I. S. (2003 b). The use of *Candida pulcherrim*a in combination with *Saccharomyces cerevisiae* for the production of Chenin blanc wine. *South African Journal of Enology and Viticulture, 24*, 63-69.

Jolly, N., Augustyn, O. P. H., & Pretorius, I. S. (2006). The role and use of non-*Saccharomyces* yeasts in wine production. *South African Journal of Enology and Viticulture, 27*, 15-39.

Jung, R., Seckler, J., Freund, Langen, M., Dollt, M., Rupp, A., & Niederberger, G. (2005). Wodurch wird die Vergaerung beeinflusst?. *Der Deutsche Weinbau, 19,* 28-33.

Kapsopoulou, K., Kapaklis, A., & Spyropoulos, H. (2005). Growth and fermentation characteristics of the wine yeast *Kluyveromyces thermotolerans* isolated in Greece. *World Journal of Microbiolology and Biotechnology, 21,* 1599-1602.

Kapsopoulou, K., Mourtzini, A. & Anthoulas, M. (2006). Biological acidification during grape must fermentation using mixed cultures of *Kluyveromyces thermotolerans* and

Saccharomyces cerevisiae. *World Journal of Microbiolology and Biotechnology, 23,* 735-739.

Klar, A. J. S., & Halvorson, H. O. (1975). Proteinase activities of *Saccharomyces cerevisiae* during sporulation. *Journal of Bacteriology, 124*, 863-869.

Koch, J., & Bretthauer, G. (1959). Zur Kenntnis der Eiweißstoffe des Weines II. Mitteilung Einfluss der Mosterhitzung auf die Eiweißstabilität der Weißweine. *Lebensmitteluntersuchung und –Forschung, 106,* 361-367.

Koch, J., & Sajak, E. (1959). A review and some studies on grape protein. *American Journal of Enology and Viticulture, 10*, 114-123.

Kurtzman, C. P. (1998). 51. *Torulaspora* Lindner. In C. P. Kurtzman & J. W. Fell (Eds.), *The yeast: a taxonomic study* (pp. 404-408). Amsterdam: Elsevier.

Kurtzman, C.P. (1998) 38. *Lodderomyces* van der Walt. In C. P. Kurtzman & J. W. Fell (Eds.), *The yeast: a taxonomic study* (pp. 254-255). Amsterdam: Elsevier.

Kurtzman, C. P. & Droby, S. (2001). *Metschnikowia fructicola*, a new ascosporic yeast with potential for biocontrol of postharvest fruit rots. *Systematic and Applied Microbiology, 24,* 395-399.

Kurtzman, C. P., & Fell, J. W. (2006). Yeast systematics and Phylogeny: implications of molecular identification methods for studies in ecology. In C. Rosa & G. Péter (Eds.), *Biodiversity and ecophysiology of yeasts: the yeast handbook* (pp. 11-30). New York: Springer.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, 227,* 680-685.

Lagace, L. S. & Bisson. L.F. (1990). Survey of yeast acid proteases for effectiveness of wine haze reduction. *American Journal of Enology and Viticulture, 41,* 147-155.

Lambrechts, M. G., & Pretorius, I. S. (2000). Yeast and its importance to wine aroma: a review. *South African Journal of Enology and Viticulture, 21*, 97-129.

Large, P. J. (1986). Degradation of organic nitrogen compounds by yeasts. *Yeast, 2,* 1-34.

Leitao, M.C., Teixeira, H.C.,Barreto Crespo, M.T., and San Romao, M.V. (2000) Biogenic amines occurrence in wine: amino acid decarboxylase and proteolytic activities expression by *Oenococcus oeni*. *Journal of Agricultural and Food Chemistry, 48,* 2780 -2784.

Lochbuehler, B. C. (2007). Entwicklung der Aromaprofile von Weissweinen bei sur-lies-Ausbau mit Einsatz von Hefenaehrstoffen und Hefezellafuschlussprodukten. Masterarbeit, Justus-Liebig-University Giessen.

Longo, E., Cansado, J., Agrelo, D., & Villa, T. G. (1991). Effect of climatic conditions on yeast diversity in grape musts from northwest Spain. *American Journal of Enology and Viticulture, 42*, 141-144.

Loureiro, V., & Malfeito-Ferreira, M. (2003). Spoilage yeasts in the wine industry. *International Journal of Food Microbiology, 86,* 23-50.

Magné, C. & Larher, F. (1992). High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry, 200,* 115-118.

Maicas, S., & Mateo, J. J. (2005). Hydrolysis of terpenyl glycosides in grape juice and other fruit juices*. Applied Microbiology and Biotechnology, 67,* 322-335.

Malacrino, P., Tosi, E., Caramia, G., Prisco, R., & Zapparoli, G. (2006). The vinification of partially dried grapes: a comparative fermentation study of *Saccharomyces cerevisiae* strains under high sugar stress. *Letters in Applied Microbiology, 40,* 466-472.

Malfeito-Ferreira, M. Tereco, M., & Loureiro, V. (1997). Fatty acid profiling: a feasible typing system to trace yeast contamination in wine bottling plants. *International Journal of Food Microbiology, 38,* 143-155.

Manginot, C., Roustan, J. L., & Sablayrolles, J. M. (1998). Nitrogen demand of different yeast strains during alcoholic fermentation: importance of the stationary phase. *Enzyme and Microbial Technology, 23,* 511-517.

Marcé, R.M., Calull, M., Guasch, J., & Borrull, F. (1989). Determination of free amino acids in wine by HPLC using precolumn derivatization with phenylisothiocyanate. *American Journal of Enology and Viticulture, 40,* 194-198.

Marchal, R., Chaboche, D., Douillard, R., & Jeandet, P. (2002). Influence of lysozyme treatments on champagne base wine foaming properties. *Journal of Agricultural and Food Chemistry, 50,* 1420-1428.

Marchal, R., Warchol, M., Cilindre, C., & Jeandet, P. (2006). Evidence for protein degradation by *Botrytis cinerea* and relationships with alteration of synthetic wine foaming properties. *Journal of Agricultural and Food Chemistry, 54,* 5157-5165.

Marchal, R., Chaboche, D., Delahaut, L., Gandon, J.P. & Jeandet, P. (2000). Detection and quatification of lysozyme in Champagne wines. *Journal of Agricultural and Food Chemistry, 48,* 3225-3231.

Marder, R., Becker, J. M., & Naider, F. (1977). Peptide transport in yeast: utilization of leucine- and lysine-containing peptides by *Saccharomyces cerevisiae*. *Journal of Bacteriology, 131,* 906-916.

Margalit, Y. (2004). *Concepts in wine technology.* San Francisco: The Wine Appreciation Guild.

Martínez-Rodríguez, A. J., & Polo, M. C. (2000). Characterization of the nitrogen compounds released during yeast autolysis in a model wine system. *Journal of Agricultural and Food Chemistry, 48,* 1081-1085.

Martínez-Rodríguez, A. J., Carrascosa, A. V., & Polo, M. C. (2001 b). Release of nitrogen compounds to the extracellular medium by three strains of *Saccharomyces cerevisiae* during induced autolysis in a model wine system. *International Journal of Food Microbiology, 96,* 235-252.

Martínez-Rodríguez, A. J., Polo, M. C., & Carrascosa, A. V. (2001). Structural and ultrastructural changes in yeast cells during autolysis in a model wine system and in sparkling wines. *International Journal of Food Microbiology, 71,* 45-51.

Martínez-Rodríguez, A. J., Carrascosa, A. V., Barcenilla, J. M., Pozo-Bayón, A., & Polo, M.C. (2001 a). Autolytic capacity and foam analysis as additional criteria for the selection of yeast strains for sparkling wine production. *Food Microbiology, 18,* 183-191.

Martínez-Rodríguez, A. J., Carrascosa,A. V., Martín-Álverez, P. J., Moreno-Arribas, V., & Polo, M. C. (2002). Influence of the yeast strain on the changes of the amino acids, peptides and proteins during sparkling wine production by the traditional method. *Journal of Industrial Microbiology and Biotechnology, 29,* 34-322.

Masuda, T., & Kitabatake, N. (2006). Developments in biotechnological production of sweet proteins. *Journal of Bioscience and Bioengineering, 102,* 75-389.

McDonald, J. K. (1985). An overview of protease specificity and catalytic mechanisms: aspects related to nomenclature and classification. *Histochemical Journal, 17,* 773-785.

Mendes-Ferreira, A., Mendes-Faia, A., & Leao, C. (2004). Growth and fermentation patterns of *Saccharomyces cerevisiae* under different ammonium concentrations and its implications in winemaking industry. *Journal of Applied Microbiology, 97,* 540-545.

Mendes-Ferreira, A., Olmo, M., García-Martínez, J., Jiménez-Martí, E., Leao, C., Mendes-Faia, A., & Pérez-Ortín, J. E. (2007a). *Saccharomyces cerevisiae* Signature genes for predicting nitrogen deficiency during alcoholic fermentation. *Applied and Environmental Microbiology, 73, 5*363-5369.

Mendes-Ferreira, A., Olmo, M., García-Martínez, J., Jiménez-Martí, E., Mendes-Faia, A., Pérez-Ortín, J. E., and Leao, C. (2007b). Transcriptional Response of *Saccharomyces cerevisiae* to different nitrogen concentrations during alcholic fermentation. *Applied and Environmental Microbiology, 73,* 3049-3060.

Milewski, S., Andruszkiewicz, R., & Borowski, E. (1988). Substrate specificity of peptide permeases in *Candida albicans*. *FEMS Microbiology Letters, 50,* 73-78.

Miller, M.W. & Phaff, H.J. (1998) 45. *Saccharomycodes* E.C. Hansen. In C. P. Kurtzman & J. W. Fell (Eds.), *The yeast: a taxonomic study* (pp. 372-373). Amsterdam: Elsevier. Amsterdam.

Monteiro, F. F. & Bisson, L. F. (1991). Amino acid utilization and urea formation during vinification fermentions. *American Journal of Enology and Viticulture, 42,* 199-208.

Monteiro, F. F. & Bisson, L. F. (1992). Nitrogen supplementation of grape juice II: effect on amino acid and urea release following fermentation. *American Journal of Enology and Viticulture, 43,* 11-17.

Monteiro, S., Picarra-Pereira, M. A., Loureiro, V. B., Teixeira, A. R. & Ferreira, R. B. (2007). The diversity of pathogenesis-related proteins decreases during grape maturation. *Phytochemistry, 68,* 416-425.

Moreira, N., Mendes, F., Pereira, O., Pinho, P. G., Hogg, T., & Vasconcelos, I. (2002). Volatile sulphur compounds in wines related to yeast metabolism and nitrogen composition of grape musts. *Analytica Chimica Acta, 458,* 157-167.

Moreno-Arribas, V., Bartolomé, B., & Cierva, J. (1998). Isolation and characterization of individual peptides from wine. *Journal of Agricultural and Food Chemistry, 46,* 3422-3425.

Moreno-Arribas, V., Pueyo, E., & Polo, M. C. (1996). Peptides in Musts and wines: changes during the manufacture of Cavas (Sparkling wines). *Journal of Agricultural and Food Chemistry, 44,* 3783-3788.

Moreno-Arribas, V., Pueyo, E., Nieto, F. J., Martín-Álvarez, P. J., & Polo, M. C. (2000). Influence of the polysaccharides and the nitrogen compounds on foaming properties of sparkling wines. *Food chemistry, 70,* 309-317.

Murphey, J. M., Powers, J. R., & Spayed, S. E. (1989). Estimation of soluble protein concentration of white wines using Coomassie brilliant blue G-250. *American Journal of Enology and Viticulture, 40*, 189-193.

Nakopoulou, Z. G., Vafopoulou-Mastrogiannaki, A., & Soufleros, E. H. (2006). Protein characterization of Roditis Greek grape variety and Sauvignon Blanc and changes in certain nitrogen compounds during alcoholic fermentation. *Journal International des Sciences de la Vigne et du Vin, 40*, 165-175.

Nelson, G., & Young, T. W. (1986). Yeast extracellular proteolytic enzymes for chill-proofing beer. *Journal of the Institute of Brewing, 92,* 599-603.

Nguyen, T. H., Fleet, G. H., & Rogers, P. L., (1998). Composition of the cell walls of several yeast species. *Applied Microbiology and Biotechnology, 50*, 206-212.

Nisiotou, A., & Nychas, G. E. (2007). Yeast populations residing on healthy or *Botrytis*-infected grapes from a vineyard in Attica, Greece. *Applied and Environmental Microbiology, 73,* 2765-2768.

Nissen, P., Nielsen, D., & Arneborg, N. (2003). Viable *Saccharomyces cerevisiae* cells at high concentrations cause early growth arrest of non-*Saccharomyces* yeasts in mixed cultures by a cell-cell contact-mediated mechanism. *Yeast, 20,* 331-341.

Nissen, P., Nielsen, D., & Arneborg, N. (2004). The relative glucose uptake abilities of non-*Saccharomyces* yeasts play a role in their coexistence with *Saccharomyces cerevisiae* in mixed cultures. *Applied Microbiology and Biotechnology, 64,* 543-550.

Oganesjanz, L, Telegin, J., & Kardasch, N. (2007) Lysatpräparate zur Qualitätssteigerung. *Der Deutsche Weinbau, 2,* 31.

Ogrydziak, D. M. (1993). Yeast extracellular proteases. *Critical Reviews in Biotechnology. 13*, 1-55.

Osborne, J. P., & Edwards, C. (2006). Inhibition of malolactic fermentation by *Saccharomyces* during alcoholic fermentation under low- and high-nitrogen conditions: a study in synthetic media. *Australian Journal of Grape and Wine Research, 12,* 69-78.

Ough, C. S., Huang, Z., An, D., & Stevens, D. (1991). Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation: effects on urea excretion and reabsorption. *American Journal of Enology and Viticulture, 42,* 26-39.

Ough, C. S., Stevens, D., Sendovski, Z., Huang, Z., & An, D. (1990). Factors contributing to urea formation in commercially fermented wines. *American Journal of Enology and Viticulture, 41,* 68-73.

Paetzold, R., & Brueckner, H. (2007). D-Amino Acids - New Frontiers in Amino Acid and Peptide Research. In R. Konno, H. Brueckner, A. D'Aniello, G. H. Fisher & H. Homma (Eds.), *Gas Chromatographic Determination of D-Amino Acids in Food and Beverages* (pp. 351-366). New York: NovaScience Publishers.

Paetzold, R., Nieto-Rodriguez, A., & Brueckner, H. (2003). Chiral gas chromatographic analysis of amino acids in fortified wines. *Chromatographia Supplement, 57,* 207-212.

Paraggio, M. (2004). Biodiversity of a natural population of *Saccharomyces cerevisiae* and *Hanseniaspora uvarum* from Aglianica del Vulture. *Food Technology and Biotechnology, 42,* 165-168.

Patterson, C. A. & Ingledew, W. M. (1999). Utilization of peptides by a lager brewing yeast. *Journal of the American Society of Brewing Chemists, 57,* 1-8.

Patz, C. D., David, A., Thente, K., Kuerbel, P., & Dietrich, H. (1999). Wine analysis with FTIR spectrometry. *Viticultural and Enological Sciences, 54,* 80-87.

Payne, J. M & Smith, M. W. (1994). Peptide Transport by Micro-organisms. *Advances in Microbial Physiology, 36,* 1-80.

Pocock, K. F., Hayasaka, Y., McCarthy, M. G., & Waters, E. (2000). Thaumatin-like proteins and chitinases, the haze-forming proteins of wine, accumulate during ripening of grape (*Vitis vinifera*) berries and drought stress does not affect the final levels per berry at maturity. *Journal of Agricultural and Food Chemistry, 48,* 1637-1643.

Pocock, K. F., Hoj, P. B., Adams, K. S., Kwiatkowski, M. J., & Waters, E.J. (2003). Combined heat and proteolytic enzyme treatment of white wines reduces haze forming protein content without detrimental effect. *Australian Journal of Grape and Wine Research, 9,* 56-63.

Poll Life Sciences. (2006). Technical Guide: protein sample preparation and analysis application manual. Poll Corporation, Ann Arbor, USA.

Prior, B. (1997). Einfluss der Stickstoffversorgung auf die loeslichen Amiosaeuren in den Organen von *Vitis vinifera* L. (c.v) Riesling) und auf die Qualitaet des Mostes und des Weines. Dissertation, Justus-Liebig-University Giessen, Geisenheimer Berichte Bd.32.

Radler, F. (1992). Metabolism of organic acids. In G. H. Fleet (Ed.), *Wine Microbilology and Biotechnology* (pp. 77-164). Chur: Harwood Academic Publishers.

Rainier, S., & Pretorius, I. S. (2000). Selection and improvement of wine yeasts. *Annals of Microbiology, 50,* 15-31.

Rapp, A. (1998). Volatile flavour of wine: Correlation between instrumental analysis and sensory perception. *Narung, 42,* 351-363.

Rauhut, D. (1993). Yeast-production of sulphur compounds. In G. H. Fleet (Ed.), *Wine Microbilology and Biotechnology* (pp. 183-224). Chur: Harwood Academic Publishers.

Rauhut, D. (1996). Qualitaetsmindernde schwefelhaltige Stoffe im Wein: Vorkommen, Bildung, Beseitigung. Dissertation, Justus-Liebig-University Giessen, Geisenheimer Berichte Bd.24.

Rauhut, D. (2003). Impact of volatile sulphur compounds on wine quality. In J. C. Davidian, D. Grill, L. J. De Kok, I. Stulen, M. J. Hawkesford, E. Schnug & H. Rennenber (Eds). *Sulphur transport and assimilation in plants* (pp. 121-131). Leiden: Backhuys Publishers.

Rauhut, D. (2006). Wine aromas and analysis. Workshop RMUT 2006. Forschungsanstalt Geisenheim, Geisenheim, Germany.

Rauhut, D., Kuerbel, H., MacNamara, K., & Grossmann, M. (1998). Headspace GC-SCD monitoring of low volatile sulphur compounds during fermentation and in wine. *Analusis, 26,* 142-145.

Rauhut, D., Beisert, B., Berres, M., Gawron-Scibek, M., & Kuerbel, H. (2005). Pulse flame photometric detection: an innovative technique to analyse volatile sulphur compounds in wine and other beverages. In T. Hofmann, M. Rothe, M. & P. Schieberle, *State of the art in flavour chemistry and biology* (pp. 363-368). Garching: Deutsche Forschungsanstalt fuer Lebensmittelchemie (pp. 363-368).

Rauhut, D., Kuerbel, H., Prior, B., Loehnertz, O., & Grossmann, M. (1997). Synthetic media to monitor the different ability of yeast strains to produce undesirable volatile sulphur compounds. *American Journal of Enology and Viticulture, 4*8, 212.

Rementeria, A., Rodriguez, J. A., Cadaval, A., Amenabar, R., Muguruza, J. R., Hernando, F. L., & Sevilla, M.J. (2003). Yeast associated with spontaneous fermentation of white wines from the “Txakoli de Bizkaia” region (Basque country, North Spain). *International Journal of Food Microbiology, 86,* 201-207.

Remize, F., Sablayrolles, J. M., & Dequin, S. (2000). Re-assessment of the influence of yeast strain and environmental factors on glycerol production in wine. *Journal of Applied Microbiology, 88,* 371-378.

Renouf, V., Strehaiano, P., & Lonvaud-Funel., A. (2008). Effectiveness of dimethyldicarbonate to prevent *Brettanomyces bruxellensis* growth in wine. *Food Control, 19,* 208-216.

Renouf, V., Perello, M. C., Strehaiano, P., & Lonvaud-Funel, A. (2006). Global survey of the microbial ecosystem during alcoholic fermentation in winemaking. *Journal International des Sciences de la Vigne et du Vin, 40*, 101-116.

Rensburg, P., & Pretorius, I. S. (2000). Enzymes in winemaking: harnessing natural catalysts for efficient biotransformations : a review. *South African Journal of Enology and Viticulture, 21*, 52-73.

Ribéreau-Gayon, P., Dubourdieu, D., Donèche, B., & Lonvaud, A. (2006 a). *Handbook of Enology Volume 1: the microbiology of wine and vinifications.* (2nd ed.). Chichester: John Wiley & Sons.

Ribéreau-Gayon, P., Glories, Z., Maujean, A. & Dubourdieu, D. (2006 b). *Handbook of Enology Volume 2:* the chemistry of wine stabilization and treatments. (2nd ed.). Chichester: John Wiley & Sons.

Roeder, C., Wallbrunn, C., & Froehlich, J. (2007). Detektion und Untersuchung der Verbreitung der Wine-relevanten Schaedlingshefe *Dekkera* (*Brettanomyces*) *bruxellensis* in Rheinhessen. *Deutsche Lebensmittel-Rundschau, 103,* 353-359.

Rojas, V., Gil, J. V., Pinaga, F., & Manzanares, P. (2001). Studies on acetate ester production by non-*Saccharomyces* wine yeasts. *International Journal of Food Microbiology*, *70,* 238-289.

Rolland, J. M., Apostolou, E., Deckert, K., Leon, M. P., Doughlass, J. A., Glaspole, I. N., Bailey, M., Stockley, C. S., & O'Hehir, R. E. (2006). Potential food allergens in wine: Double-blind, placebo-controlled trial and basophil activation analysis, *Nutrition, 22, 9,* 882-888.

Romano, P., Suzzi, G., Domizio, P., & Fatichenti, F. (1997). Secondary products formation as a tool for discriminating non-*Saccharomyces* wine strains. *Antonie van Leeuwenhoek, 71,* 239-242.

Romano, P., Fiore, C., Praggio, M., Caruso, M., & Capece, A. (2003). Function of yeast species and strains in wine flavour. *International Journal of Food Microbiology, 86,* 169-180.

Romano, P., Caruso, M., Capece, A., Lipani, G., Paraggio, M., & Fiore, C. (2003). Metabolic diversity of *Saccharomyces cerevisiae* strains from spontaneously

fermented grape musts. *World Journal of Microbiology and Biotechnology, 19,* 311-315.

Rosi, I., & Costamagna, L. (1987). Screening for extracellular acid protease(s) production by wine yeasts. *J.Inst.Brew, 93,* 322-324.

Rothman, J. H. & Stevens, T. H. (1986). Protein sorting in yeast: mutants defective in vacuole biogenesis mislocalize vacuolar proteins into the late secretory pathway. *Cell, 47,* 1041-1051.

Roussis, I. G., Lambropoulos, I., & Tzimas, P. (2007). Protection of volatiles in a wine with low sulphur dioxide by caffeic acid or glutathione. *American Journal of Enology and Viticulture, 58*, 274-282.

Rueck, C. (2005). Auswirkungen verschiedener Pflanzenschutzstrategien im ökologischen Weinbau auf Hefeflora, Gärverlauf und Aromaprofil der Weine. Diplomarbeit, Fachhochschule Wiesbaden, Fachbereich Geisenheim, Germany.

Russell, I. (2006). Yeast. In F. G. Priest & G. G. Stewart (Eds.), *Handbook of Brewing.* (pp. 527-560). Boca Raton, CRC Press.

Saerens, S. M. G., Delvaux, F., Verstrepen, K. J., Dijck, V. P., Thevelein, J. M., & Delvaux, F. R. (2008). Parameters affecting ethyl ester production by *Saccharomyces cerevisiae* during fermentation. *Applied and Environmental Microbiology, 72,* 454-461.

Sala, C., Mestres, M., Martí , M. P., Busto, O., & Guasch, J. (2002). Headspace solid-phase microextraction analysis of 3-alkyl-2-methoxypyrazines in wines. *Journal of Chromatography A,* 953, 1-6.

Santamaría, P., Garijo, P., López, R., Tenorio, C., & Gutiérrez, A. R. (2005). Analysis of yeast population during spontaneous alcoholic fermentation: effect of the age of the cellar and the practice of inoculation. *International Journal of Food Microbiology, 103,* 49-56.

Santos, A., & Marquina, D. (2004). Killer toxin of *Pichia membranifaciens* and its possible use as a biocontrol agent against grey mould disease of grapevine. *Microbiology, 150,* 2527-2534.

Sarmento, M. R., Oliveira, J. C., & Boulton, R. B. (2000). Selection of low swelling materials for protein adsorption from white wines. *International Journal of Food Science and Technology, 35,* 41-47.

Scanes, K. T., Hohmann, S., & Prior, B. A. (1998). Glycerol production by the yeast *Saccharomyces cerevisiae* relevance to wine: a review. *South African Journal of Enology and Viticulture, 19*, 17-24.

Schuller, D. Corte-Real., M., & Leao, C. (2000). A differential medium for the enumeration of the spoilage yeast *Zygosaccharomyces bailli* in wine. *Journal of Food Protection, 63, 1*570-1575.

Shallow, D. A., Barrett-Bee, K. J., & Payne, J. W. (1991). Evaluation of the dipeptide and oligopeptide permeases of *Candida albicans* as uptake routes for synthetic anticandidal agents. *FEMS Microbiology Letters, 70,* 9-14.

Siler, C. E., & Morris, J. R. (1996). High alcohol fermentation of grape juice concentrate. In T. Henick-Kling, T. E. Wolf & E. M. Harkeness (Eds.), *Proceeding fourth International Symposium on Cool Climate Viticulture and Enology* (pp. 97-99). American Society for Enology and Viticulture.

Silva, P., Cardoso, H., & Gerós, H. (2004). Studies on the wine spoilage capacity of *Brettanomyces/Dekkera* spp. *American Journal of Enology and Viticulture, 55,* 65-72.

Sipiczki, M. (2006). *Metschnikowia* strains isolated from Botrytized grapes antogonize fungal and bacterial growth by iron depletion. *Applied and Environmental Microbiology. 72,* 6716-6724.

Soden, A., Francis, I. L., Oakey, H., & Henschke, P. A. (2000). Effect of co-fermentation with *Candida stellata* and *Saccharomyces cerevisiae* on the aroma and composition of Chardonay wine. *Australian Journal of Grape and Wine Research, 6,:* 21-30.

Soden, A., Francis, I. L., Gockowiak, H., Lee, T. H., & Henschke, P. A. (1999). The use of non-*Saccharomyces* yeasts in winemaking. In R. J. Blair, A. N. Sas, P. F. Hayes & P. B. Høj (Eds.), *Proceedings of the Tenth Australian Wine Industry Technical Conference* (pp.166-176). Urrbrae, Winetitles.

Sommer, P., Bunte, A., Stolpe, E., & Kaldahl, P. (2007). Flavour enhancement by use of mixed yeast starter cultures of *Torulaspora delbrueckii*, *Kluyveromyces thermotolerans* and *Saccharomyces cerevisiae* in wine. 8th International Symposium Innovations in Enology, (pp. 282-283). Stuttgart.

Soufleros, E. H., Bouloumpasi, E., Tsarchopoulos, C., Biliaderis, C. G. (2003). Primary amino acid profiles of Greek white wines and their use in classification according to variety, origin and vintage. *Food Chemistry, 80,* 261-273.

Sponholz, W. R. (1993). Sulphur dioxide and wine microorganisms. In G. H. Fleet (Ed.), *Wine Microbilology and Biotechnology* (pp. 373-393). Chur: Harwood Academic Publishers.

Sponholz, W. R. & Dittrich, H. H. (1974). Die Bildung von SO_2-bindenden Gaerungs-Nebenprodukten, hoeheren Alkoholen und Estern bei einigen Reinzuchthefestaemmen und bei einigen fuer die Weinbereitung wichtigen“wilden“ Hefen. *Die Wein-Wissenschaft, 6,* 301-313.

Sponholz, W. R., & Rauhut, D. (1992). Red wine production with low β-glucosidase activity yeast strains. *American Journal of Enology and Viticulture, 43*, 393.

Stein-Hammer, C. (2004). Schoenungsmittel: allergens Potential. *Der Deutsche Weinbau, 5,* 28-29.

Stoelben, T. C. (2007). Auswirkungen verschiedener Pflanzenschutzstrategien im oekologischen- und biodynamischen Weinbau auf Hefeflora, Gaerverlauf und Aromaprofil der Weine. Diplomarbeit, Fachhochschule Wiesbaden, Fachbereich Geisenheim, Germany.

Strauss, M. L. A., Jolly, N. P., Lambrechts, M. G., & Rensburg, P. (2001). Screening for the production of extracellular hydrolytic enzymes by non-Saccharomyces wine yeasts. *Journal of Applied Microbiology, 91,* 182-190.

Sturley, S. L. & Young, T. W. (1988). Extracellular protease activity in a strain of *Saccharomyces cerevisiae*. *J.Inst.Brew, 94,* 23-27

Sturm, J., Grossmann, M., & Schnell, S. (2006). Influence of grape treatment on the wine yeast populations isolated from spontaneous fermentations. *Journal of Aplied Microbiology, 101,* 1241-1248.

Swiegers, J. H. & Pretorius, I. S. (2007). Modulation of volatile sulphur compounds by wine yeast. *Applied Microbiology and Biotechnology, 74,* 954–960.

Swiegers, J. H., Bartowsky, E. J., Henschke, P. A., & Pretorius, I. S. (2005). Yeast and bacterial modulation of wine aroma and flavour. In R. Blair, M. Francis, & I. Pretorius, (Eds), *Advances in wine science* (pp. 159-187). The Australian Wine Research Institute.

Taillandier, P., Portugal, F. R., Fuster, A., & Strehaiano, P. (2007). Effect of ammonium concentration on alcoholic fermentation kinetics by wine yeasts for high sugar content. *Food Microbiology, 24,* 95-100.

Thomas, K. C. & Ingledew, W. M. (1992). Production of 21% (v/v) ethanol by fermentation of very high gravity (VHG) wheat mashes. *Journal of Industrial Microbiology and Biotechnology, 10,* 61-68.

Thomas, K. C. & Ingledew, W. M. (1992). The relationship of low lysine and high arginine to efficient ethanolic fermentation of wheat mash. *Canadian Journal of Microbiology, 38,* 626-634.

Tirelli, A., & Noni, I. (2007). Evaluation of lysozyme stability in young red wine and model systems by a validated HPLC method. *Food Chemistry, 105,* 1564-1570.

Todd, B. E. N. (1995). Aspects of yeast autolysis in sparkling wine production. In C. S. Stockley, A. N. Sas & R. S. Johnstone & T. H. Lee (Eds.), *Proceeding of the ninth Australian wine industry technical conference* (pp. 33-38). Underdale: Winetitles.

Torrens, J., Urpí, P., Riu-Aumatell, M., Vichi, S., López-Tamames, E., & Buxaderas, S. (2008). Different commercial yeast strains affecting the volatile and sensory profile of cava base wine. *International Journal of Food Microbiology,* DOI:10.1016/j.ijfoodmicro.2008.02.023.

Valero, E., Mauricio J. C., Milán, M. C., & Ortega, J. M. (1999). Changes in the urea content of wine under different fermentation and aging conditions by two *Saccharomyces cerevisiae* races. *Biotechnology Letters, 21,* 555-559.

Valero, E., Millán, C., Ortega, J., & Mauricio, J. (2003). Concentration of amino acids in wine after the end of fermentation by *Saccharomyces cerevisiae* strains. *Journal of the Science of Food and Agriculture, 83,* 830-835.

Varela, C., Pizarro, F., & Agosin, E. (2004). Biomass content governs fermentation rate in nitrogen-deficient wine musts. *Journal of Applied and Environmental Microbiology, 70,* 3392-3400.

Vaughan-Martini, A., & Martini, A. (1998). 44. *Saccharomyces* Meyen ex Reess. In C. P. Kurtzman & J. W. Fell (Eds.), *The yeast: a taxonomic study* (pp. 358-371). Amsterdam: Elsevier.

Vincenzi, S., Mosconi, S., Zoccatelli, G., Pellegrina, C.D., Veneri, G., Chignola, R., Peruffo, A., Curioni, A., & Rizzi, C. (2005). Development of a new procedure for protein recovery and quantification in wine. *American Journal of Enology and Viticulture, 56*, 182-187.

Vine, R. P., Harkness, E. M., & Linton, S. J. (2002). *Wine making: from grape growing to marketplace*. (2nd ed.). New York: Kluwer Acdemic.

Von Wallbrunn, C. (2007). Identification and monitoring: risk assessment of genetically modified yeast during wine making. 8th International Symposium Innovations in Enology, (pp. 125-132). Stuttgart.

Waters, E. J. (1994). A *Saccharomyces* mannoprotein that protects wine from protein haze. *Carbohydrate Polymers, 23,* 185-191.

Waters, E. J., Shirley, N. J., & Williams, P. (1996). Nuisance proteins of wine are grape pathogenesis-related proteins. *Journal of Agricultural and Food Chemistry, 44,* 3-5.

Waters, E.J., Wallace, W., & Williams, P.J. (1991). Heat Haze Characteristics of fractionated wine proteins. *American Journal of Enology and Viticulture, 42*, 123-127.

Waters, E. J., Wallace, W., & Williams, P. J. (1992). Identification of heat –unstable wine proteins and their resistance to peptidases. *Journal of Agricultural and Food Chemistry, 40,* 1514-1519.

Waters, E. J., Peng, Z., Pocock, K. F., & Williams, P.J. (1995 a). Proteins in white wine, I: procyanidin occurrence in soluble proteins and insoluble protein hazes and its relationship to protein instability. *Australian Journal of Grape and Wine Research, 1,* 86-93.

Waters, E. J., Peng, Z., Pocock, K.F., & Williams, P. J. (1995 b). Proteins in white wine, II: their resistance to proteolysis is not due to either phenolic association or glycosylation. *Australian Journal of Grape and Wine Research, 1,* 94-99.

Waters, E. J., Hayasaka, Y., Tattersall, D. B., Adams, K. S., & Williams,P. (1998). Sequence analysis of grape (*Vitis vinifera*) berry chitinases that cause haze formation in wines. *Journal of Agricultural and Food Chemistry, 46,* 4950-4957.

Waters, E. J., Alexander, G., Muhlack, R., Pocock, K. F., Colby, C., & O'Neill, B. K. (2005). Preventing protein haze in bottled white wine. *Australian Journal of Grape and Wine Research, 11,* 215-225.

Wigand, P. & Decker, H. (2007). Proteine im Wein. *Deutsche Lebensmittel-Rundschau, 103,* 52-57.

Wylie, E. B., & Johnson, M. (1961). Effect of penicillin on the cell wall of *Escherichia coli. Biochimica et Biophysica Acta, 59,* 450-457.

Yamada, T., Furukawa, K., Hara, S., & Mizoguchi, H. (2005). Effect of amino acids on peptide transport in sake yeast. *Journal of Bioscience and Bioengineering, 99,* 383-389.

Yanniotis, S., Kotseridis, G., Orfanidou, A., & Petraki, A. (2007). Effect of ethanol, dry extract and glycerol on the viscosity of wine. *Journal of Food Engineering, 81,* 300-403.

Zhijun, L., Yongning, W., Gong, Z., Yunfeng, Z., & Changhu, X. (2007). A survey of biogenic amines in Chinese red wines. *Food Chemistry, 105,* 1530-1535.

Zoecklein, B. W., Fugelsang, K. C., Gump, B. H., & Nury, F. (1999). *Wine analysis and production*. New York: Kluwer Academic.

Electronic literatures

1. www.chem.qmul.ac.uk/iubmb/enzyme/
2. www.chr-hansen.com
3. www.pall.com/laboratory_1003.asp
4. www.begerow.com

APPENDIX

Results of the variants and their replications of the experimental studies are presented in the Appendix. R1 is used as abbreviation for the variant and R2 is used for the corresponding replication.

Table A-1 Proteolytic activity of yeasts grown in synthetic medium

Yeast species	Strain/ Code*	Proteolytic activity (unit)	
		R1	R2
Metschnikowia pulcherrima	N-1	0.54	0.38
Hanseniaspora uvarum	N-2	0.45	0.25
Dekkera bruxellensis	N-4	0.03	0.03
Dekkera bruxellensis	N-5	nd	nd
Dekkera bruxellensis	N-6	0.04	0.06
Dekkera bruxellensis	N-7	0.18	0.20
Dekkera bruxellensis	N-8	0.19	0.19
Zygosaccharomyces mellis	N-9	0.39	0.31
Zygosaccharomyces mellis	N-10	0.54	0.50
Zygosaccharomyces bailii	N-11	0.73	1.13
Zygosaccharomyces bailii	N-12	0.54	0.34
Saccharomycodes ludwigii	N-13	1.01	1.01
Saccharomycodes ludwigii	N-14	nd	nd
Saccharomycodes ludwigii	N-15	1.01	0.99
Hansenula saturnus	N-16	0.40	0.36
Hansenula sp.	N-17	nd	nd
Pichia farinosa	N-18	0.11	0.11
Debaromyces hansenii	N-19	n.d.	n.d.
Debaromyces nicotianae	N-20	0.15	0.17
Rhodotorula glutinis	N-21	nd	nd
Mycoderma bispora	N-22	0.39	0.59
Mycoderma bispora	N-23	nd	nd
Hansenula anomala	N-24	nd	nd

One unit of enzyme is defined as that amount of enzyme which releases the colour equivalent of 1 μg of tyrosine in 1min.

nd denotes not detectable.

Table A-1 (continued) Proteolytic activity of yeasts grown in synthetic medium

Yeast species	Strain/ Code*	Proteolytic activity (unit)	
		R1	R2
Metschnikowia pulcherrima	N-25	0.12	0.22
Kloeckera apiculata	N-26	nd	nd
Hanseniaspora uvarum	N-27	0.20	0.18
Zygosaccharomyces bailii	N-28	n.d.	n.d.
Zygosaccharomyces bailii	N-29	0.83	1.13
Brettanomyces sp.	N-30	nd	nd
Brettanomyces sp.	N-31	nd	nd
Zygosaccharomyces bailii	Z-CM	1.04	0.98
Kluyveromyces thermotolerans	K-MB	0.14	0.19
Torulaspora delbrueckii	T-MB	0.05	0.11
Hanseniaspora uvarum	H 045	1.22	1.22
Hanseniaspora uvarum	H 097	1.36	1.74
Hanseniaspora uvarum	H 182	1.36	1.66
Hanseniaspora uvarum	H 155	0.73	0.87
Hanseniaspora uvarum	H 030	1.11	0.79
Hanseniaspora uvarum	H 210	0.25	0.25
Hanseniaspora uvarum	H 146	1.07	1.09
Metschnikowia pulcherrima	M 004	1.08	1.03
Rhodotorula sp.	R-1	1.08	1.37
Rhodotorula sp.	R-2	0.49	0.24
Rhodotorula sp.	R-3	20.69	21.52
Rhodotorula sp.	R-4	2.38	2.57
Saccharomyces cerevisiae	S-CM	nd	nd
Saccharomyces cerevisiae	S-CEG	nd	nd
Saccharomyces cerevisiae	S-CY	nd	nd
Saccharomyces cerevisiae	S-EC	nd	nd
Saccharomyces cerevisiae	S-S6U	nd	nd

One unit of enzyme is defined as that amount of enzyme which releases the colour equivalent of 1 μg of tyrosine in 1min.

nd denotes not detectable.

Table A-2 Protease activity, total soluble solid (TSS) and viable yeast cells in grape juice after 3 days of growth

Yeasts	Protease activity (U)		TSS (^{O}Brix)		Yeast cells/ml	
	R1	R2	R1	R2	R1	R2
H. uvarum (H 045)	0.31	0.39	16.0	16.0	1.1×10^8	1.4×10^8
H. uvarum (H 097)	nd	nd	13.5	13.5	3.5×10^7	4.0×10^7
K. thermotolerans (K-MB)	nd	nd	6.0	6.0	3.8×10^8	3.0×10^8
M. pulcherrima (M 004)	10.0	11.48	8.0	8.0	4.1×10^8	4.8×10^8
Rhodotorula sp. (R-3)	11.60	9.00	17.5	17.5	2.1×10^8	3.0×10^8
Rhodotorula sp. (R-4)	nd	nd	17.0	18.0	1.5×10^8	1.1×10^8
S. ludwigii (N-13)	nd	nd	5.5	5.5	9.4×10^7	7.5×10^7
T. delbrueckii (T-MB)	nd	nd	6.5	6.0	3.3×10^8	3.2×10^8
Z. bailli (Z-CM)	nd	nd	7.0	7.0	1.2×10^8	1.7×10^8

One unit of enzyme is defined as that amount of enzyme which releases the colour equivalent of 1 μg of tyrosine in 1min. Systematic names of yeasts are noted in **Table 4-1**. nd denotes not detectable.

Table A-3 OD_{600} values of synthetic media containing different sources of nitrogen

	Beginning						14 days after inoculation					
	WON		IP		WN		WON		IP		WN	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
M 004	0.209	0.259	0.222	0.236	0.223	0.289	1.035	1.269	1.321	1.249	2.096	2.440
H 097	0.211	0.244	0.217	0.233	0.281	0.299	1.083	1.193	1.361	1.249	2.290	2.308
T-MB	0.280	0.351	0.293	0.331	0.300	0.321	1.491	1.833	2.897	2.705	5.100	4.783
K-MB	0.399	0.389	0.374	0.414	0.355	0.401	1.531	1.642	1.685	1.563	1.793	1.929

Nitrogen sources; without nitrogen (WON), with ammonium sulfate (WN) and with isolated protein(IP)

Systematic names of yeasts are noted in **Table 4-1**.

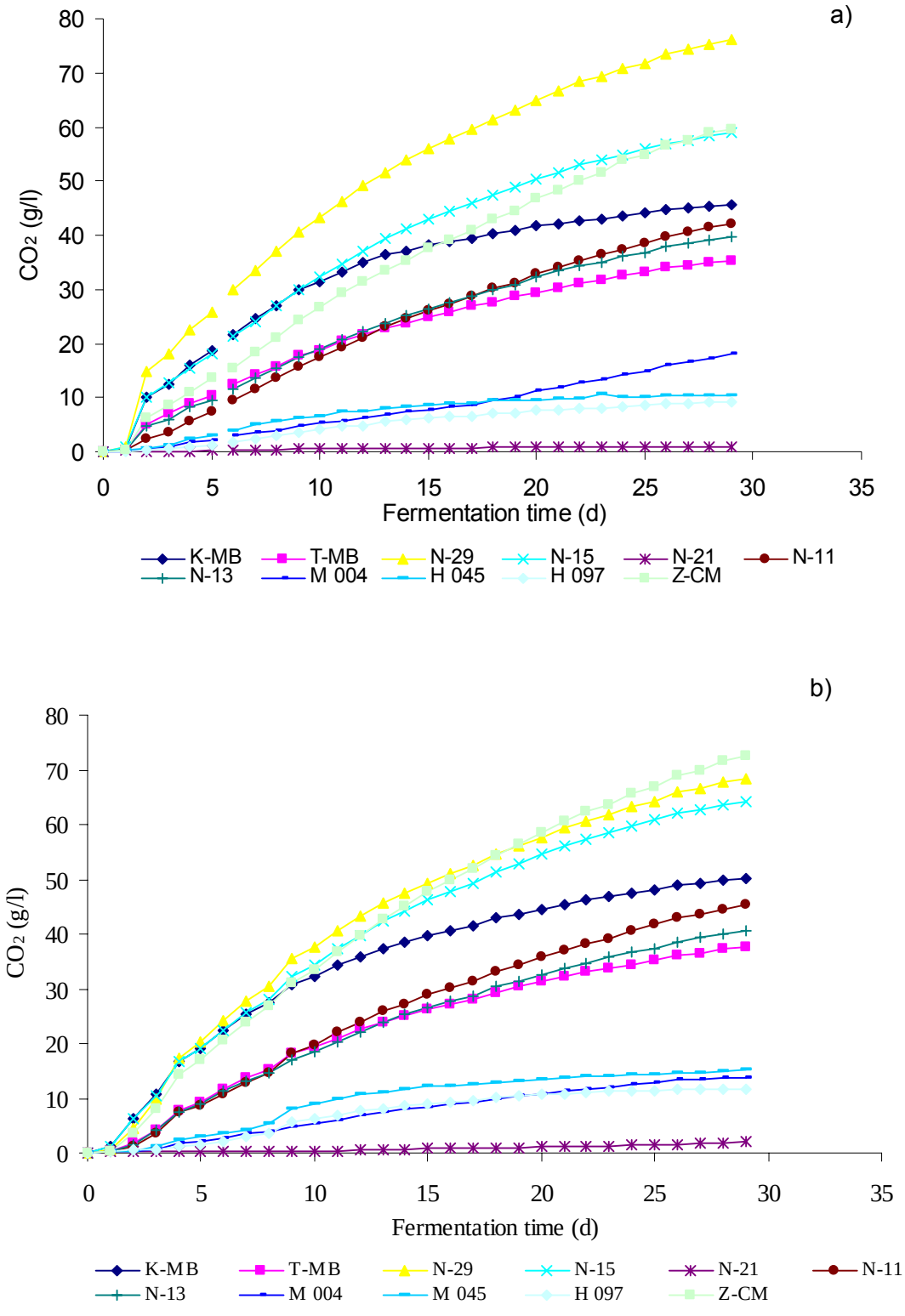


Figure A-1 Fermentation kinetics of non-*Saccharomyces* yeasts during fermentation of grape juice; systematic names of yeasts are noted in **Table 4-1**; a) and b) are data from R1 and R2 respectively.

Table A-4 Composition of end products obtained from grape juice fermentation with non-*Saccharomyces* yeasts

Products	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
Reducing Sugar(g/l)	82.9	93.4	52.1	81.2	182.8	91.8	100.1	139.1	145.5	139.8	42.3
Alcohol (%v/v)	43.4	23.6	65.3	53.4	1.6	35.2	32.7	11.6	13.2	10.5	60.5
Glycerol (g/l)	4.1	2.2	5.1	6.3	1.1	6.0	3.8	3.1	2.6	2.6	5.8
Volatile acid (g/l)	0.2	0.3	0.3	0.4	0.3	0.9	0.6	0.3	0.7	0.6	0.5
pH	3.2	3.2	3.1	3.1	3.2	3.4	3.2	3.2	3.2	3.2	3.4
Total Acidity (g/l)	6.4	4.4	5.5	5.7	5.3	6.3	5.1	4.6	5.1	4.9	5.3

Table A-5 Concentrations of sulphur-containing compounds in end products obtained from grape juice fermentation with non-*Sccharomyces* yeasts

Products	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
H_2S (µg/l)	2.3	2.1	nd	1.1	nd	nd	nd	nd	nd	nd	nd
MeSH (µg/l)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
EtSH (µg/l)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DMS (µg/l)	1.1	0.8	1.1	0.8	0.7	1.4	1.3	1.3	1.3	0.8	1.4
CS_2 (µg/l)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
MeSAc (µg/l)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DMDS (µg/l)	nd	nd	nd	nd	nd	nd	6.0	7.0	7.1	nd	7.0
EtSAc (µg/l)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DEDS (µg/l)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd
DMTS (mg/l)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd

nd denotes not detectable.

Data are from R2 with sampling during the fermentation.

H_2S - hydrogen sulfide, MeSH – methanethiol, EtSH – ethanethiol, CS_2 - carbon disulfide, DMS - dimethyl sulfide, MeSAc - thioacetic -S- methyl ester, EtSAc - thioacetic -S- ethyl ester, DMDS - dimethyl disulfide, DEDS - diethyl disulfide, DMTS - dimethyl trisulfide

Table A-6 Concentrations of assimilable nitrogen in fermented grape juice at the end of fermentation expressed as free α-amino nitrogen (FAN)

	Yeasts*										
FAN (mg/l)	K-MB	T-MB	N-29	N-15	N-21	N-11	N-13	M 004	H 045	H 097	Z-CM
R1	44	40	19	54	44	75	47	33	50	54	56
R2	49	39	24	50	40	77	39	35	42	51	55

*Systematic names of yeasts are noted in **Table 4-1**

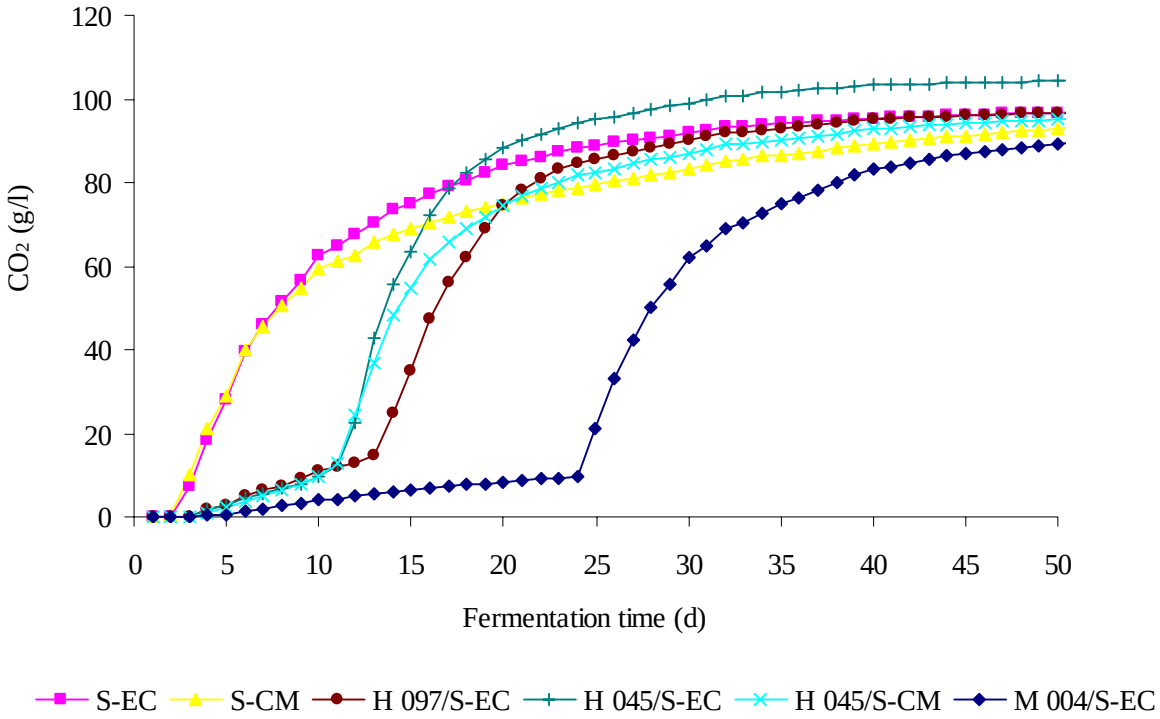


Figure A-2 Growth kinetics of grape juice fermentation with single and sequential inoculation; systematic names of yeasts and inoculation protocols are noted in **Table 3-2**.

Table A-7 Composition of wines from fermentation of single and sequential inoculation

	S-EC	S-CM	H097/S-EC	H045/S-EC	H045/S-CM	M004/S-EC
Reducing sugar (g/l)	1.5	11.9	1.4	1.0	4.4	2.1
Alcohol (%v/v)	13.5	12.8	13.2	13.2	13.1	13.2
Glycerol (g/l)	5.3	5.0	7.0	6.1	5.9	6.2
Volatile acid (g/l)	0.5	0.5	0.6	0.5	0.6	0.5
pH	3.7	3.6	3.7	3.7	3.7	3.6
Total acidity (g/l)	5.1	5.2	5.1	5.2	5.5	5.2

*Systematic names of yeasts and inoculation protocols are noted in **Table 3-2**

Table A-8 Assimilable nitrogen expressed as free α-amino nitrogen (FAN) in wines from fermentation of single and sequential inoculation

FAN (mg/l)	S-EC	S-CM	H097/S-EC	H045/S-EC	H045/S-CM	M004/S-EC
R1	32	33	37	41	37	33
R2	35	35	37	38	41	35

*Systematic names of yeasts and inoculation protocols are noted in **Table 3-2**

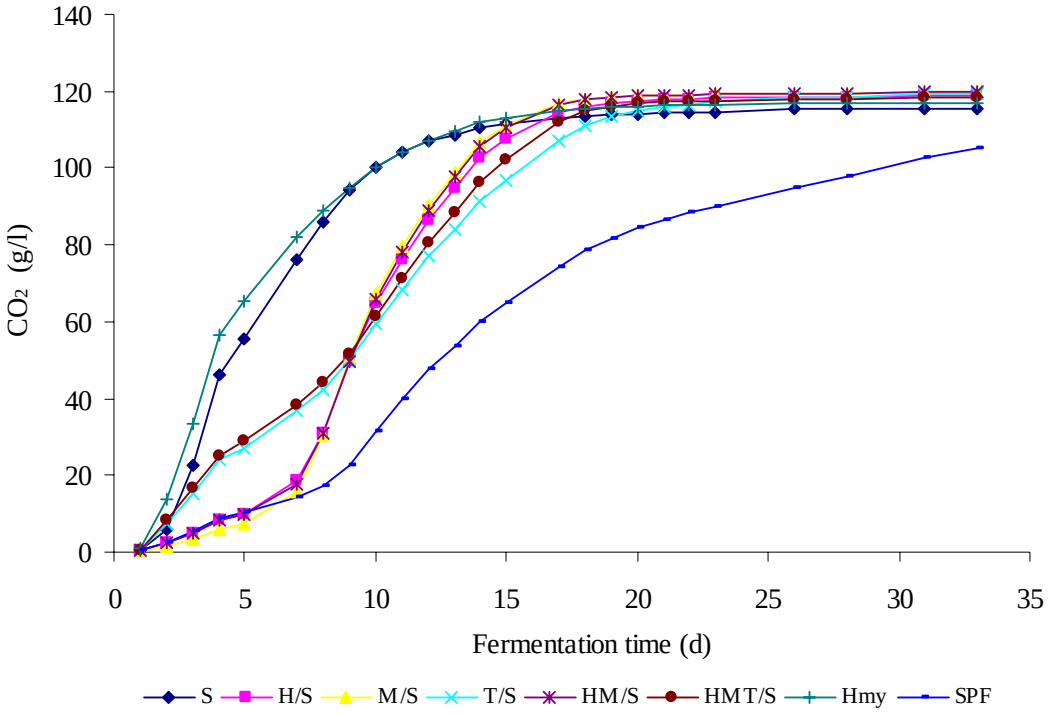


Figure A-3 Effect of inoculation treatment on fermentation kinetics during fermentation of clarified Riesling must (R 1) ; systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

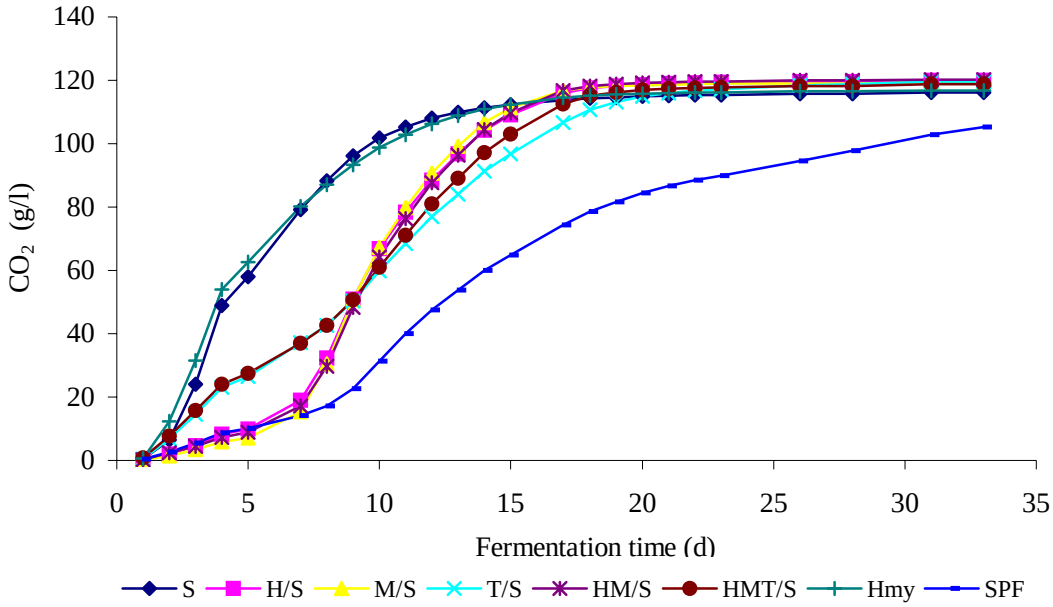


Figure A-4 Effect of inoculation treatment on fermentation kinetics during fermentation of clarified Riesling must (R2) ; systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

.

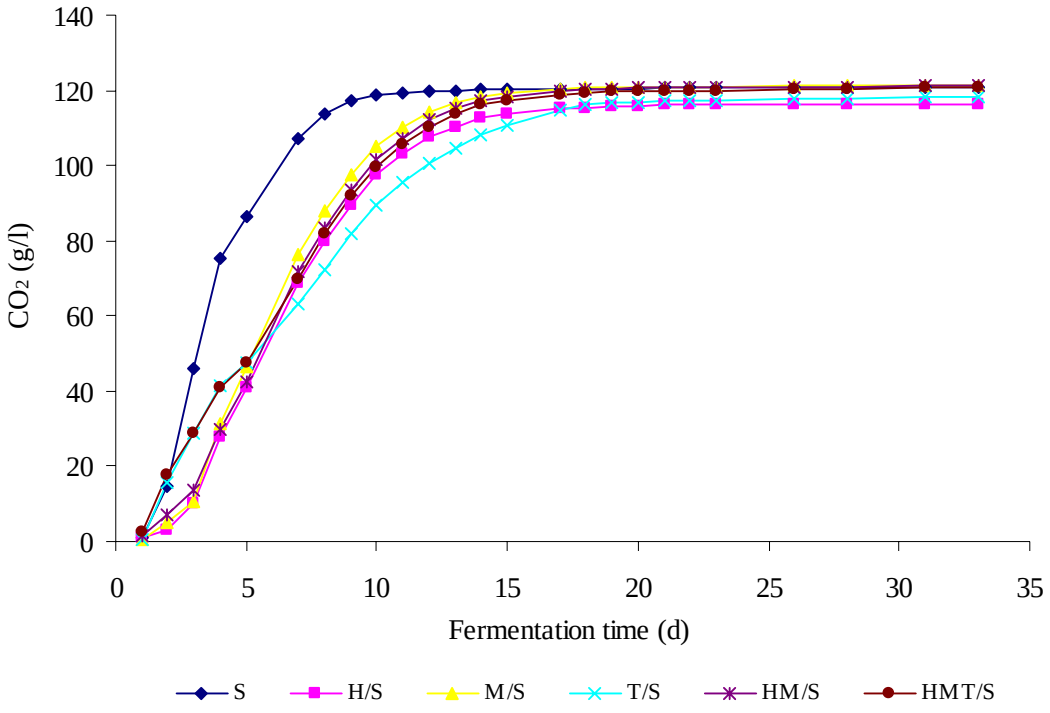


Figure A-5 Effect of inoculation treatment on fermentation kinetics during fermentation of unclarified Riesling must (R1) ; systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

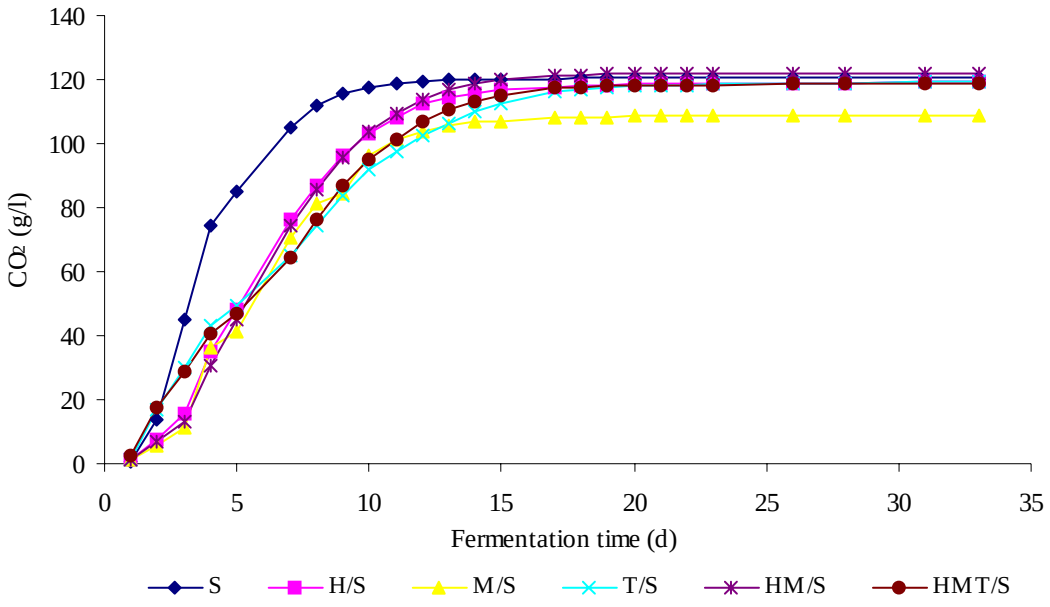


Figure A-6 Effect of inoculation treatment on fermentation kinetics during fermentation of unclarified Riesling must (R2) ; systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table A-9 Composition of the wines produced by fermentation of clarified Riesling grape juice fermented with different inoculation protocols

Composition		S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Reducing sugar(g/l)	R1	11.4	4.1	3.7	4.1	3.6	3.6	10.5
	R2	10.7	3.8	3.6	4.0	3.3	3.7	11.5
Alcohol (%v/v)	R1	15.6	16.0	15.9	16.0	16.0	15.9	15.8
	R2	15.6	16.0	16.0	16.0	16.0	15.9	15.8
Glycerol (g/l)	R1	12.8	13.4	13.6	12.9	13.4	13.4	12.1
	R2	12.7	13.3	13.5	13.0	13.5	13.4	12.1
Volatile acid (g/l)	R1	0.65	0.65	0.71	0.54	0.65	0.59	0.54
	R2	0.66	0.64	0.70	0.55	0.66	0.57	0.54
pH	R1	3.6	3.6	3.7	3.6	3.7	3.6	3.5
	R2	3.6	3.6	3.6	3.6	3.7	3.6	3.5
TA (g/l)	R1	7.3	7.3	7.3	7.1	7.3	7.2	7.2
	R2	7.3	7.3	7.3	7.2	7.3	7.2	7.2

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table A-10 Composition of the wines produced by fermentation of unclarified Riesling grape juice fermented with different inoculation protocols

Composition		S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
Reducing sugar(g/l)	R1	4.5	2.8	3.0	3.6	3.1	3.1
	R2	3.5	2.9	3.1	3.6	2.9	3.4
Alcohol (%v/v)	R1	16.0	16.0	16.0	15.9	16.0	15.9
	R2	16.0	16.0	16.0	16.0	16.0	15.9
Glycerol (g/l)	R1	13.8	15.0	14.8	14.4	14.5	14.9
	R2	14.1	14.3	14.4	14.3	14.5	15.1
Volatile acid (g/l)	R1	0.49	0.59	0.54	0.56	0.58	0.60
	R2	0.49	0.57	0.56	0.55	0.59	0.60
pH	R1	3.5	3.6	3.6	3.6	3.6	3.6
	R2	3.6	3.6	3.6	3.6	3.6	3.6
TA (g/l)	R1	7.1	7.0	7.0	6.9	7.1	7.0
	R2	7.1	7.0	7.1	6.9	7.0	7.1

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table A-11 Concentrations of metabolites binding sulphur dioxide in wines from clarified and unclarified Riesling grape juice fermented with different inoculation protocols

Composition		S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Clarified must								
Acetaldehyde (mg/l)	R1	37	32	33	27	34	23	47
	R2	39	32	29	25	29	27	38
Pyruvate (mg/l)	R1	21	18	19	28	18	27	19
	R2	22	18	18	25	17	28	20
Ketoglutarate (mg/l)	R1	35	40	41	48	37	46	45
	R2	33	41	44	52	40	45	47
Unclarified must								
Acetaldehyde (mg/l)	R1	37	27	28	21	27	27	-
	R2	34	29	28	24	28	33	-
Pyruvate (mg/l)	R1	15	14	12	29	16	27	-
	R2	17	13	11	29	15	25	-
Ketoglutarate (mg/l)	R1	57	66	61	51	58	49	-
	R2	57	66	61	51	58	49	-

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

R1 and R2 were no sampling during fermentation.

Table A-12 Concentration of amino acids in clarified must and wines fermented with different inoculation protocols

R	Amino acids (mg/l)	Must	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
1	Alanine	102	34	38	33	28	36	22	47
2			37	55	40	30	41	28	55
1	Arginine	486	111	113	94	190	114	121	35
2			119	188	125	217	140	128	41
1	Asparagine	9	Trace	Trace	Trace	Trace	Trace	Trace	Trace
2			Trace	Trace	Trace	Trace	Trace	Trace	Trace
1	Aspartic acid	26	32	32	29	28	33	26	27
2			34	37	36	29	35	32	29
1	Citrulline	11	7	5	8	8	9	6	7
2			8	11	8	8	8	7	0
1	Cystine	0	6	6	5	6	6	5	7
2			3	5	3	3	4	3	4
1	Glutamine	111	Trace	Trace	Trace	Trace	Trace	Trace	Trace
2			Trace	4	5	3	5	3	3
1	Glutamic acid	45	33	37	31	27	32	25	39
2			29	37	34	28	32	27	41
1	Glycine	7	16	17	16	21	18	18	17
2			16	22	18	21	18	23	16
1	Histidine	30	11	12	9	19	10	18	14
2			16	15	16	22	14	20	16
1	Isoleucine	8	6	6	5	4	8	4	8
2			8	9	7	5	7	7	8
1	Leucine	4	23	22	24	15	23	18	29
2			23	24	23	15	30	19	28

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table A-12 (continued) Concentration of amino acids in clarified must and wines fermented with different inoculation protocols

R	Amino acids (mg/l)	Must	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
1	Lysine	19	31	27	26	22	29	24	40
2			39	39	38	25	32	16	42
1	Methionine	Trace	5	5	4	4	3	3	6
2			5	6	5	3	5	4	7
1	Ornithine	7	9	8	8	8	9	7	4
2			15	18	16	11	13	5	9
1	Phenylalanine	13	15	14	9	9	12	18	19
2			16	22	16	12	15	13	19
1	Proline	276	380	366	415	345	399	364	418
2			401	388	404	346	350	348	410
1	Serine	57	13	9	11	10	13	10	15
2			16	16	16	12	13	13	17
1	Threonine	45	8	8	7	5	8	7	10
2			7	9	10	7	7	6	10
1	Tyrosine	12	13	16	5	11	14	6	19
2			17	17	16	14	15	12	18
1	Valine	19	20	20	10	16	20	17	22
2			13	18	12	8	8	9	15
1	GABA	171	99	106	65	106	98	92	15
2			128	164	142	125	126	119	11

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table A-13 Concentration of amino acids in clarified must and wines fermented with different inoculation protocols

R	Amino acids (mg/l)	Must	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
1	Alanine	102	31	41	37	36	47	43
2			30	43	39	38	42	42
1	Arginine	445	38	35	34	30	37	37
2			35	34	32	31	30	34
1	Asparagine	9	Trace	Trace	Trace	Trace	Trace	Trace
2			Trace	Trace	Trace	Trace	Trace	Trace
1	Aspartic acid	27	33	30	28	31	33	35
2			32	33	33	31	30	33
1	Citrulline	11	2	7	8	8	8	6
2			Trace	Trace	Trace	7	6	6
1	Cystine	0	6	6	7	8	8	8
2								
1	Glutamine	108	Trace	Trace	Trace	Trace	Trace	Trace
2			3	4	4	2	2	3
1	Glutamic acid	45	41	46	46	39	50	46
2			31	32	37	34	39	39
1	Glycine	8	14	22	18	24	23	25
2			12	18	15	24	20	24
1	Histidine	26	16	14	13	25	15	22
2			16	16	20	22	12	22
1	Isoleucine	7	10	7	8	7	8	9
2			10	9	10	8	8	9
1	Leucine	3	35	28	27	26	28	31
2			33	29	32	26	23	29

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table A-13 (continued) Concentration of amino acids in clarified must and wines fermented with different inoculation protocols

R	Amino acids (mg/l)	Must	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
1	Lysine	21	48	39	37	38	40	44
2			47	50	52	42	40	36
1	Methionine	Trace	8	9	10	11	12	13
2			8	7	8	6	6	8
1	Ornithine	7	1	2	3	4	5	6
2			1	4	4	9	9	8
1	Phenylalanine	10	23	18	18	18	20	21
2			20	23	21	18	17	21
1	Proline	251	431	431	422	431	401	431
2			405	411	403	408	379	373
1	Serine	58	16	16	15	16	17	17
2			15	17	17	17	17	18
1	Threonine	46	12	10	10	10	11	11
2			11	10	13	11	10	10
1	Tyrosine	10	15	15	13	15	16	16
2			17	15	15	16	20	17
1	Valine	21	27	25	24	24	25	27
2			14	15	13	14	13	16
1	GABA	163	20	110	83	119	121	65
2			12	85	15	127	123	121

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**

Table A-14 Concentrations of odouriferous compounds in wines from clarified Riesling grape juice fermented with different inoculation protocols

R	Compounds (μg/l)	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
1	2-methyl propanol	44800	43700	46800	43300	40200	36900	74900
2		42900	42800	44300	52100	49400	46900	81100
1	3-Methyl butanol	80500	83300	91900	105000	89200	83100	67400
2		90800	92300	91800	128200	119200	113000	75800
1	2-Methyl butanol	23900	20300	28600	29200	26200	18200	15900
2		25300	21900	21500	34700	30100	30400	18500
1	2-Phenylethanol	18600	18500	18700	22700	17300	19500	14500
2		17400	19800	18900	26700	22200	26600	15500
1	Hexanol	597	636	435	435	307	326	542
2		604	596	447	483	431	426	534
1	Ethyl acetate	198400	225500	154500	227300	146900	153000	123800
2		157600	171800	156200	293500	226400	250500	126600
1	Isoamyl acetate	692	775	764	2767	791	2611	527
2		801	738	691	3152	820	2998	578
1	Acetic acid 2-methylbutylester	20	25	37	136	44	91	16
2		44	20	28	143	64	126	14
1	Acetic acid hexylester	30	33	25	78	27	58	36
2		35	34	23	80	27	65	39
1	Phenylethyl acetate	4	4	4	4	4	4	4
2		4	4	4	3	4	4	4
1	Acetic acid 2-phenylethylester	57	73	63	199	63	191	34
2		53	68	61	191	62	209	43
1	Propionic acid ethylester	57	48	46	82	48	69	31
2		64	40	45	104	53	79	28
1	Ethyl isobutyrate	48	43	52	54	48	50	76
2		52	41	46	78	65	67	80

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nq denotes not quantifiable.

Table A-14 (continued) Concentration of odour compounds found in Riesling wine from different inoculation treatments in clarified grape juice

R	Compounds (μg/l)	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
1	Butanoic acid ethylester	247	260	249	261	255	226	274
2		261	246	235	301	249	214	268
1	Lactic acid ethylester	nq	nq	nq	nq	nq	nq	nq
2		nq	nq	nq	nq	nq	nq	nq
1	Caproic acid ethylester	939	827	873	514	803	460	939
2		973	844	808	546	799	464	919
1	Succinic acid diethylester	357	338	315	487	301	521	791
2		333	304	348	556	297	646	777
1	Caprylic acid ethylester	1156	989	985	487	847	431	1097
2		1116	1042	953	499	967	468	1254
1	Capric acid ethylester	497	391	355	260	273	146	390
2		466	413	361	169	341	162	475
1	Caproic acid	6600	5900	5700	4700	5400	3700	6300
2		6500	5800	6000	4200	5900	4000	6200
1	Caprylic acid	6500	5400	5300	4100	5000	2800	6000
2		6400	5400	5400	2800	4600	2700	5800
1	Capric acid	2500	2000	2300	1600	1600	800	2100
2		2700	2300	1900	800	1500	700	2100
1	trans-Linalooloxide	nq	nq	nq	nq	nq	nq	nq
2		nq	nq	nq	nq	nq	nq	nq
1	cis-Linalooloxide	nq	nq	nq	nq	nq	nq	nq
2		nq	nq	nq	nq	nq	nq	nq
1	Linalool	93	106	104	103	104	100	103
2		103	106	102	108	96	101	961
1	α-Terpineol	40	35	33	32	35	31	36
2		33	36	34	33	32	32	31
1	Geraniol	nd	nd	nq	nq	nq	nq	nq
2		nd	nd	nq	nq	nq	nq	nq

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nd and nq denotes not detectable and not quantifiable, respectively.

Table A-15 Concentrations of odouriferous compounds in wines from unclarified Riesling grape juice fermented with different inoculation protocols

R	Compounds (μg/l)	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
1	2-methyl propanol	60400	58300	60100	63100	61000	50900
2		56400	52200	49800	52900	58200	64600
1	3-Methyl butanol	139800	119600	119400	148600	115100	110200
2		124200	101000	96800	117500	123200	139700
1	2-Methyl butanol	36600	37000	35600	41200	34900	33300
2		30700	28800	28200	31700	38600	44700
1	2-Phenylethanol	37700	22000	27700	37000	24300	26000
2		31200	18800	21100	28000	24400	34800
1	Hexanol	605	590	513	557	515	358
2		624	457	370	442	443	350
1	Ethyl acetate	218500	243000	219900	227300	240500	160000
2		188300	218600	143700	219000	242900	236500
1	Isoamyl acetate	828	521	490	690	705	822
2		631	591	505	672	593	905
1	Acetic acid 2-methylbutylester	42	61	40	58	50	40
2		31	61	35	26	39	70
1	Acetic acid hexylester	15	6	1	7	13	8
2		5	9	7	6	8	8
1	Phenylethyl acetate	5	4	6	4	4	5
2		6	5	5	5	4	5
1	Acetic acid 2-phenylethylester	58	30	33	78	49	79
2		41	35	40	72	37	72
1	Propionic acid ethylester	71	49	44	75	42	57
2		79	40	32	70	41	72
1	Ethyl isobutyrate	70	55	65	79	53	74
2		69	48	51	76	45	106

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nq denotes not quantifiable.

Table A-15 (continued) Concentrations of odouriferous compounds in wines from unclarified Riesling grape juice fermented with different inoculation protocols

R	Compounds (μg/l)	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
1	Butanoic acid ethylester	257	339	359	243	382	235
2		246	383	339	238	351	292
1	Lactic acid ethylester	nq	nq	nq	nq	nq	nq
2		nq	nq	nq	nq	nq	nq
1	Caproic acid ethylester	963	721	870	414	944	422
2		830	921	965	380	915	524
1	Succinic acid diethylester	865	759	200	1201	659	1226
2		878	606	757	1088	662	1359
1	Caprylic acid ethylester	1171	720	1005	438	1071	447
2		1030	907	1068	376	944	535
1	Capric acid ethylester	356	193	276	201	337	156
2		296	237	309	134	268	142
1	Caproic acid	6600	5400	6000	4100	6200	3800
2		5700	5700	6400	3800	6300	4300
1	Caprylic acid	5600	4000	5100	2700	5100	2800
2		5000	5000	5800	2500	4700	2900
1	Capric acid	1800	1000	1500	800	1500	800
2		1600	1400	1700	700	1200	700
1	trans-Linalooloxide	nq	nq	nq	nq	nq	nq
2		nq	nq	nq	nq	nq	nq
1	cis-Linalooloxide	nq	nq	nq	nq	nq	nq
2		nq	nq	nq	nq	nq	nq
1	Linalool	104	102	114	108	112	105
2		109	110	111	107	103	111
1	α-Terpineol	33	33	39	35	36	33
2		34	35	36	36	31	34
1	Geraniol	nq	nq	nq	nq	nq	nq
2		nq	nq	nq	nq	nq	nq

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nq denotes not quantifiable.

Table A-16 Assimilable nitrogen expressed as free α-amino nitrogen (FAN) in wines from clarified and unclarified musts fermented with different inoculation protocols

FAN (mg/l)	S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Clarified must							
R1	65.9	69.8	70.7	69.2	74.7	64.1	50.2
R2	64.2	65.1	66.3	60.2	66.1	59.6	44.1
Unclarified must							
R1	51.6	63.9	55.7	69.2	67.4	74.7	
R2	47.0	59.6	56.9	60.9	65.5	73.7	

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
R 1 and R 2 were no sampling during fermentation.

Table A-17 Concentrations of sulphur-containing compounds in wines from clarified Riesling grape juice fermented with different inoculation protocols

.

Composition		S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB	Hmy
Total SO_2 (mg/l)	R1	17	18	12	6	12	4	22
	R2	18	10	11	5	11	4	20
H_2S (μg/l)	R1	21	17	18	13	22	17	26
	R2	22	15	19	11	20	16	25
MeSH (μg/l)	R1	5	5	5	5	5.	5	6
	R2	5	5	6	5	5	5	7
EtSH (μg/l)	R1	nd	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd	nd
DMS (μg/l)	R1	3	3	3	2	3	3	2
	R2	3	3	3	3	3	3	3

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nd denotes not detectable.

R 1 and R 2 were no sampling during fermentation.

H_2S - hydrogen sulfide, MeSH – methanethiol, EtSH – ethanethiol, CS_2 - carbon disulfide, DMS - dimethyl sulfide, MeSAc - thioacetic -S- methyl ester, EtSAc - thioacetic -S- ethyl ester, DMDS - dimethyl disulfide, DEDS - diethyl disulfide, DMTS - dimethyl trisulfide

Table A-17 (continued) Concentrations of sulphur-containing compounds in wines from clarified Riesling grape juice fermented with different inoculation protocols

CS_2 (μg/l)	R1	5	4	1	4	9	<1	2
	R2	5	12	1	4	4	<1	1
MeSAc (μg/l)	R1	nd	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd	nd
DMDS (μg/l)	R1	nd	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd	nd
EtSAc (μg/l)	R1	nd	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd	nd
DEDS (μg/l)	R1	nd	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd	nd
DMTS (mg/l)	R1	nd	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd	nd

sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nd denotes not detectable.

R 1 and R 2 were no sampling during fermentation.

H_2S - hydrogen sulfide, MeSH – methanethiol, EtSH – ethanethiol, CS_2 - carbon disulfide, DMS - dimethyl sulfide, MeSAc - thioacetic -S- methyl ester, EtSAc - thioacetic -S- ethyl ester, DMDS - dimethyl disulfide, DEDS - diethyl disulfide, DMTS - dimethyl trisulfide

Table A-18 Concentrations of sulphur-containing compounds in wines from unclarified Riesling grape juice fermented with different inoculation protocols

Composition		S-EC	H 097*	M 004*	T-MB*	H 097* M 004	H 097* M 004 T-MB
Total SO_2 (mg/l)	R1	14	5	7	6	7	4
	R2	11	9	8	3	7	8
H_2S (μg/l)	R1	66	42	56	21	46	39
	R2	43	40	47	28	47	45
MeSH (μg/l)	R1	10	9	10	7	9	8
	R2	12.6	8	9	5.6	8	7
EtSH (μg/l)	R1	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd
DMS (μg/l)	R1	3	3	3	3	3	3
	R2	3	3	3	2	3	3

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nd denotes not detectable.

R 1 and R 2 were no sampling during fermentation.

H_2S - hydrogen sulfide, MeSH – methanethiol, EtSH – ethanethiol, CS_2 - carbon disulfide, DMS - dimethyl sulfide, MeSAc - thioacetic -S- methyl ester, EtSAc - thioacetic -S- ethyl ester, DMDS - dimethyl disulfide, DEDS - diethyl disulfide, DMTS - dimethyl trisulfide

Table 18 (continued) Concentrations of sulphur-containing compounds in wines from unclarified Riesling grape juice fermented with different inoculation protocols

CS_2 (μg/l)	R1	5	8	84	79	7	7
	R2	18	10	9	18	25	25
MeSAc (μg/l)	R1	11	nd	nd	nd	nd	nd
	R2	11	nd	nd	nd	nd	nd
DMDS (μg/l)	R1	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd
EtSAc (μg/l)	R1	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd
DEDS (μg/l)	R1	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd
DMTS (mg/l)	R1	nd	nd	nd	nd	nd	nd
	R2	nd	nd	nd	nd	nd	nd

* sequential inoculation with *S. cerevisiae*

† Systematic name of yeasts and inoculation protocols are shown in **Table 3-3**
nd denotes not detectable.

R 1 and R 2 were no sampling during fermentation.

H_2S - hydrogen sulfide, MeSH – methanethiol, EtSH – ethanethiol, CS_2 - carbon disulfide, DMS - dimethyl sulfide, MeSAc - thioacetic -S- methyl ester, EtSAc - thioacetic -S- ethyl ester, DMDS - dimethyl disulfide, DEDS - diethyl disulfide, DMTS - dimethyl trisulfide

Table A-19 Viable yeast cells in the medium

Yeasts	Viable cells (Cells/ml)			
	Beginning		3-day fermentation	
	R 1	R2	R 1	R2
M. pulcherrima (M 004)	1.5×10^6	1.4×10^6	3.3×10^7	3.8×10^7
H. uvarum (H 097)	1.2×10^6	1.2×10^6	8.3×10^7	7.9×10^7
S. cerevisiae (S-EC)	1.1×10^6	1.2×10^6	5.0×10^7	4.7×10^7
T. delbrueckii (T-MB)	1.2×10^6	1.2×10^6	5.2×10^7	5.0×10^7
K. thermotolerans (K-MB)	1.1×10^6	1.2×10^6	3.3×10^7	3.3×10^7
S. cerevisiae (S-Rb)	7.7×10^6	8.1×10^6	5.2×10^7	5.2×10^7
Mixed yeasts (Hmy)	8.3×10^6	8.1×10^6	1.2×10^8	1.4×10^8

Table A-20 Concentrations of extracellular proteins released by species of non-*Saccharomyces* into synthetic defined medium

Yeasts	Protein (µg/l)	
	R1	R2
M. pulcherrima (M 004)	9300	8600
H. uvarum (H 097)	3600	3600
S. cerevisiae (S-EC)	11000	12100
T. delbrueckii (T-MB)	7400	7600
K. thermotolerans (K-MB)	3300	4000
S. cerevisiae (S-Rb)	10400	10400
Mixed yeasts (Hmy)	9300	9900

Table A-21 Estimation of the concentrations of polypeptides and proteins in grape juice fermented with different yeast strains

Molecules	Concentration (μg/l)						
	K-MB	T-MB	N-21	N-11	M 004	H 097	Z-CM
Polypeptides (3-10 kDa)							
R1	85	100	360	14	360	1500	180
R2	91	108	387	15	387	1070	194
Proteins (>10 kDa)							
R1	1990	1490	1480	2189	2080	2000	2140
R2	2139	1602	1591	2344	2236	1700	2301

Systematic names of yeasts are noted in **Table 4-1**.

Table A-22 Concentrations of polypeptide and protein biomolecules in wines with different fermentation condition.

Molecules	Concentration (μg/l)					
	TM14-RH	TM15-SPF	CT12-RH	CT12-SPF-1	CT14-RH	CT-14-SPF2
Polypeptides (3-10 kDa)						
R1	80	1410	301	500	270	500
R2	86	1516	280	538	290	538
Proteins (>10 kDa)						
R1	42	150	650	380	460	450
R2	40	161	699	409	495	484

Description of wine are noted in **Table 3-4**.

ACKNOWLEDGEMENTS

I wish to extend my cordially thanks to all who contributed to the successful accomplishment of this work. I wish to express my sincere thanks and gratitude to Prof. Dr. Doris Rauhut for her fruitful supervision as well as her tireless encouragement and support with kind understanding.

High appreciation and sincere gratitude are extended to Prof. Dr. Hans Brückner for letting me explore this interesting and stimulating research subject and for supervising this work with a perfect balance of the necessary guidance.

Sincere gratitude is due to Prof. Dr. Sylvia Schnell for her kind support and suggestions to achieve this study program.

I would like to express my thanks to Prof. Dr. Klaus Schaller who adopted me in the Geisenheim Research center with technical infrastructure where I conducted all the experimental work.

The valuable suggestions, unreserved guidance, and continuous support of Prof. Dr. Manfred Grossmann are gratefully acknowledged.

Sincere gratitude is due to Prof. Dr. Hans Juerg Gafner, Agroscope Changins-Waedenswil ACW, Waedenswil, Switzerland for his provision of yeast cultures.

Working with the colleagues at Section of Microbiology and Biochemistry of the Geisenheim Research Center has been a wonderful experience. I am grateful to all members for their continuous contribution and understanding.

Royal Thai Government and Rajamangala University of Technology Lanna (RMUTL) for the provision of necessary financial support and the allowance throughout the period of my doctoral program, AB-Enzmyes GmbH, Darmstadt, Germany where I learned the high-quality analytical techniques of protein purification, National Wine and Grape Industry Center (NWGIC) where I fulfill the internship, Prof. Dr. Hans Juerg Gafner for his provision of yeast cultures, Asst. Prof. Dr. Wanphen Jitjaroen who proposed me the opportunity to study in Germany, Dr. Christian von Wallbrunn and Mr. Bernd Lochbuehler for sharing his enthusiasm of analytical biochemistry and many helpful discussions are also acknowledged with thanks.

I heartily appreciate staff of Food Science and Technology Department, Lampang Agricultural Research and Training Center, RMUTL for their support, encouragement, and much understanding during my absence.

CURRICULUM VITAE

Name	Ni-orn Chomsri
Date of birth	28 June 1970
Place of birth	Nakornsawan, Thailand
Nationality	Thai

Academic Credentials

1989-1990	Certificate, Boonyawat Witthayalai School, Lampang, Thailand
1991-1992	Diploma (Agro-Products Processing), Rajamangala University of Technology Lanna, Lampang, Thailand
1993-1994	B.Sc. (Food Agro Industry), Maejo University , Chiang Mai, Thailand
1998-2000	M.Sc. (Biotechnology), Chiang Mai University, Chiang Mai, Thailand

Professional experience

1994-Present	Lecturer at Rajamangala University of Technology Lanna, Thailand

Erklärung

„Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der „Satzung der Justus-Liebig-Universität Giessen zur Sicherung guter wissenschaftlicher Praxis“ niedergelegt sind, eingehalten.“

Geisenheim, den 09.Mai 2008

Unterschrift: .....