Justus-Liebig-Universität Gießen, Faculty 09, Agricultural Sciences, Nutritional Sciences and Environmental Management
Hochschule Geisenheim University, Department of Microbiology and Biochemistry

Doctoral thesis

The potential of yeast proteins to substitute for traditional fining agents – technological and sensory aspects

submitted

by Bernd Christoph Lochbühler, born in Ulm/Donau, Germany

to Justus-Liebig-Universität Gießen, Faculty 09, Agricultural Sciences,
Nutritional Sciences and Environmental Management
in partial fulfillment of the requirements of the degree of Dr. agr.

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This work is dedicated to my family
who supported me in any way
during the course of all my studies

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"Yeast proteins as potential substitute of animal proteins used in fining"

List of abbreviations

ATP	Adenosine Triphosphate
BCIP	5-Bromo-4-Chloro-3-Indolylphosphate
BSA	Bovine Serum Albumin
BSE	Bovine Spongiform Encephalopathy
DAAD	Deutscher Akademischer Austauschdienst (German Academic Exchange Service)
DIN	Deutsche Industrienorm (German Industry Norm)
DNA	Deoxyribonucleic Acid
DTT	DL-dithiothreitol
EC	European Community
EP2	Specific type of molasses
EU	European Union
FTIR	Fourier Transform Infrared Spectroscopy
G	Gelatine (only used in figures)
HPLC	High Perfrormance Liquid Chromatography
HRP	Horseradish Peroxidase (only used in figures)
IDY	Inactive Dry Yeast Preparation
L	Lot (only used in graphs)
M	Molecular Mass Marker (only used in figures)
Meq	milli equivalent
NMR	Nuclear Magnetic Resonance
NTU	Nephelometric Turbidity Unit
O.D.	Optical Density (used as synonym for absorbance)
OIV	International Organization of Vine and Wine
PAGE	Polyacrylamide Gel Electrophoresis
PAS	Staining based on oxidation of sugars by Periodic Acid and subsequent Schiff reaction
PBS	Phosphate buffered saline solution
PMP	Polymethyl Pentene
PRP	Proline Rich Protein
PVDF	Polyvinylidene Flouride
PVP	Polyvinyl Pyrrolidone
PVPP	Polyvinyl Polypyrrolidone
RNA	Ribonucleic Acid
RPM	Rotations per Minute
SB-TI	Soybean Trypsin Inhibitor (only used in figures)
SDS	Sodium Dodecyl Sulphate

List of abbreviations continued

Si	Silica sol (only used in figuresand tables)
SD	Standard deviation (only used in annex)
T	Tannin (only used in figures and tables)
TBS	Tris Buffered Saline Solution
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultra Violet
YEPD	Yeast Extract Peptone Dextrose Medium
YP	Yeast Product
YPE	Yeast Protein Extract

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1 Introduction

1.1 Preliminary remarks

A study on yeast derivatives used in wine making was the initial project of this thesis.

There was a vast amount of products for oenological treatments based on inactive yeast on the market in 2008 and many were introduced since. Such products are / were for example claiming to provide nutrients to yeast or lactic acid bacteria, to have antioxidant effects on wine or to improve its organoleptic properties. Consequently, it was decided to focus the studies of this thesis on some fields of the vast variety of yeast derived oenological products.

Yeast autolysates were the first products that were examined at the beginning of the studies in 2008 and a contact to Professor Charpentier of "Université de Bourgogne", in Dijon, France, was established. Professor Charpentier has a big expertise and knowledge on yeast autolysis in wine conditions and the compounds released to wine during that processes. She offered the author the possibility to work some months in her laboratory in 2008 to learn more about the research on yeast autolysis, autolysates etc. Professor Charpentier and her group had started by 2006 a project on protein extracts released by yeast during autolysis.

It was furthermore examined if these extracts could replace traditional products based on animal proteins used for fining of grape must and wine.

A declaration of the use of some fining agents of animal origin, such as out of as milk or egg in wines, towards the consumer was discussed by the legislature of the European Union since 2007 as these proteins can cause allergies in sensitive people. Consequently, alternative sources for proteins for fining, that were not known to cause allergies, were looked for and yeast could be a promising source.

Professor Charpentier kindly offered to the author to contribute to that research what was agreed with pleasure.

The aim of studies presented herein was to obtain protein extracts from yeasts in autolysis that were less subject to protein hydrolysis than in the former researches done by other authors (cf. 1.2.).

The declaration of fining of must and wines with products out of egg and milk towards the consumer became finally obligatory in 2012 (regulation (EC) 579/2012). That legal requirement underlined that the research on alternative protein sources for fining agents was a subject meeting current demands of wine professionals.

Furthermore yeast protein extracts are allowed for fining of must and wine in the European Union since 2013 offering an alternative to the wine sector (regulation (EC) No 144/2013).

1.2 Objectives and stages of the studies

The development of protein extracts out of yeasts undergoing autolysis was one of the objectives of the studies of this thesis (cf. section 1.1.).

The other important task was to test if the protein extracts would meet the technological and sensory requirements of a fining agent for grape musts and wines.

Furthermore it would be interesting and necessary to analyze the protein composition of the yeast extracts to control the production process and to gain insight in the composition of promising fining agents out of yeast proteins.

The work of the studies of this thesis was therefore divided in three parts:

1. Mutagenesis and selection of yeast strains
    - Objective was to obtain yeasts that autolyse during stress conditions and release high concentrations of proteins
    - These proteins should have protein masses above 15 kDa
    - Proteins of low degree of hydrolysis were reported to precipitate more completely in wine (Yokotsuka and Singleton, 1987 and 1995) and to have a higher influence on phenolic compounds in wine (Tschiersch et al., 2010), which can be also desired
    - Work on extraction process to obtain YPE (yeast protein extracts)
2. Fining tests of must and wines with yeast protein extracts.
3. Partial characterization of yeast protein extracts and protein fining agents

Fining of wine always influences its sensorial characteristics (limpidity) and the reaction between proteins of the fining agents with tannins of the wine can also change colour, taste and astringency of wines (Maury et al., 2001).

Physico-chemical and sensorial analyses were used to evaluate the influence of fining with yeast protein extracts on parameters related to wine flavour.

1.3 Review of the literature

1.3.1 Yeast autolysis

1.3.1.1 General process

Autolysis of yeast has been an important subject in biotechnology as well as in food and beverage technology during the last decades. The event has been extensively described in several reviews (Babayan and Bezrukov 1985; Charpentier and Feuillat 1993; Fornairon-Bonnefond et al. 2002). Charpentier and Feuillat (1993) and Fornairon-Bonnefond et al (2002) have dealt with autolysis of yeast in wine and sparkling wine as well as Alexandre and Guilloux-Benatier (2006) in the case of sparkling wine.

The process of autolysis has been well described by Babayan and Bezrukov (1985), a work also cited by the other authors mentioned above. Autolysis is a passive process according to these authors happening after cell death. In a first step, the membranes of the yeast cell have lost their specific permeability and hydrolases are released. These hydrolases degrade intracellular macromolecules after enzyme activation or break down of enzyme inhibitors. Liberation of the hydrolyzed macromolecules out of the cell can occur if cell wall porosity has been increased by partial degradation of its polymers (cf. below). A further transformation of the compounds released by the cell can happen in the surrounding medium by chemical reactions that are partially also catalyzed by enzymes released together with the yeasts' macromolecules.

Autophagy may also play a role in the process of autolysis in case of yeasts used in food biotechnology as pointed out in the overview of Cebollero and Gonzalez (2007). Autophagy reactions are gene controlled processes taking place in still living cells. Autophagy means self-consumption resulting in transport of compounds and also of organelles of the cytoplasm in autophagosomes, vesicles having a bilayer membrane, to the vacuole. The outer membrane of the autophagosome fuses with the membrane of the vacuole and the inner membrane and the content of the autophagosome are degraded by enzymes. Formation of autophagosomes has been described in yeast cells during the second fermentation of sparkling wine (cf. also below).

1.3.1.2 Proteolysis

Proteolytic activity plays a key role in the release of compounds out of yeast cells during autolysis and will be outlined more in detail, as the release of proteins during yeast autolysis was an important part of the research work of the thesis.

Proteolysis in yeast, especially in *Saccharomyces cerevisiae*, has been reviewed by different authors (e.g. Achstetter and Wolf 1985, Hilt and Wolf 1992; van den Hazel et al. 1996 and Sorokin et al., 2009). The vacuole of the yeast is an important site of proteases and was supposed to be the place of unspecific broad proteolytic activity playing a role in nitrogen metabolism and stress response (Achstetter and Wolf 1985; van den Hazel et al. 1996). Proteolysis in the vacuole seems to be the basal degradation of long lived proteins and to perform bulk hydrolysis in case of starvation (van den Hazel

et al. 1996). Achstetter and Wolf (1985) described six different enzymes present at the vacuole, among which three were dependent on metal ions, but the authors pointed out that additional protease activity was found without knowing the specific enzymes. Proteinase ysc A and ysc B seem to perform the majority of proteolysis or at least 40 % (van den Hazel et al. 1996) in the vacuole and both enzymes were not reported to be metal dependent (Achstetter and Wolf, 1985; Hilt and Wolf, 1992). Proteinase A with an acidic pH-optimum is located in the vacuole and seems to play an important role in autolysis in wine-like conditions (cf. review of Charpentier and Feuillat 1993). Transport of proteins to be degraded to the vacuole can take place by autophagocytosis in autophagosomes having a double layer of membrane (reviewed by van den Hazel et al. 1996). Autophagocytosis is increased when cells have to face starvation (as reviewed by van den Hazel et al. 1996). Endocytosis is a transport mediated by vesicles, which is also important in the pathway of vacuolar proteolysis (van den Hazel et al. 1996). Three enzymes were described by (Achstetter, Wolf 1985) with location in the periplasmic space, between plasmalemma and cell wall. Two of these proteinases were metal dependent. Little was known about their function in the cell. At least 17 additional soluble proteinases were described by the two authors with unknown location. The soluble proteinases could probably be partially active outside the cell in the course of autolysis. This was proven for proteinase A in wine-like conditions (as reviewed by Charpentier and Feuillat 1993) A variety of membrane bound proteinases with unknown location were also described (Achstetter and Wolf 1985).

It could be concluded that *Saccharomyces cerevisiae* had a broad array of proteinases with different locations, activities, optima conditions and specificities. Achstetter and Wolf described the presence of at least 40 enzymes with proteolytic activity in *Saccharomyces cerevisiae* already in 1985. Protein degradation increased when yeast was exposed to stress such as nutrient limitations, heat and extreme pH values and vacuolar proteinase activity was increased when cells were exposed to mutagenic radiation (as reviewed by Achstetter and Wolf 1985; Hilt and Wolf 1992; van den Hazel et al. 1996).

Levels of proteolytic activity depend also on the growth phase of the cells and level is higher when cultures were in stationary phase (van den Hazel et al. 1996). Proteolysis plays also a crucial role in sporulation of yeast and degradation of proteins damaged by heat, radiation or oxidative stress (Achstetter and Wolf 1985; Hilt and Wolf 1992). A part of the vacuolar proteases is in glycosylated state in form of precursor or as mature enzyme, e.g. proteinase A, B and Carboxypeptidase Y having molecular masses of 42, about 33 and 61 kDa respectively in the mature form (van den Hazel et al. 1996).

Besides unspecific proteolysis in the vacuole yeast seem to have also a system to degrade specifically proteins in the cytoplasm or the nucleus (as reviewed by Hilt and Wolf 1992; Sorokin et al. 2009).

This proteolysis happens in the proteasome a complex multi protein structure always composed of a core unit of about 700 kDa and assocoiated with often two regulatory particles, which are also multi protein structures (Sorokin et al. 2009). Ubiquitin a small signal protein of 76 amino acids binds to target proteins and serves as a signal to proteolysis in the proteasome, but ubiquitin is removed before

proteolysis of the target molecule takes place. Proteolytic processing such as enzyme activation can also take place in the proteasome (as summarized by Sorokin et al. 2009). The expression of ubiquitin and of enzymes catalyzing binding of ubiquitin to target proteins is induced when yeast are confronted to starvation or heat. The proteasome has multiple (chymotrypsin-like, trypsin-like and peptidyl-glutamyl-peptide hydrolyzing) proteolytic activities. Caspase-like activity and threonine protease catalytic activity were also detected in the core unit of the proteasome (Sorokin et al. 2009). Proteolysis in the proteasome is possible in an ATP (adenosine triphosphate)-dependent (energy consuming) form but also in ATP-independent manner and in ubiquitin independent manner. Degradation of proteins in the proteasome seems to be important in case of short lived proteins and proteins that have become detrimental for the cell (van den Hazel et al. 1996).

1.3.1.3 Morphological changes in yeast cells during autolysis

Yeast cells in autolysis can also show morphological changes. The yeast cell wall as a whole persists during yeast autolysis (as reviewed by Babayan and Bezrukov 1985). Yeast cells performing the second fermentation of Champagne were observed by electron microscopy. The cells showed plasmolysis after three month and the inner layer of the cell wall composed mainly of ß-glucans disappeared, but cell walls persisted during fifteen years (Troton et al. 1989). *Saccharomyces cerevisiae* yeast showed cells that have shrunk after 5 hours of induced autolysis at 46°C in a light microscope, lost ovoid aspect and big vacuoles disappeared (Takeo et al. 1989). The cell wall formed granules after 5 hours of autolysis as observed under electron microscope (Takeo et al. 1989). Charpentier et al. (1986) observed by electron microscopy the cell wall of *Saccharomyces* species, which became wrinkled during induced autolysis in a wine-like medium. The work of Martinez-Rodriguez et al. (2001b) confirmed an ultra-structural change of the cell wall of *Saccharomyces cerevisiae,* which became rougher and got wrinkles during autolysis of yeast cells in wine-like medium or in sparkling wine. The volume and the extent of cytoplasm of the cell was reduced and cells showed a lot of small vesicles, which could be autophagosomes especially after 12 months of maturation of the yeast in a sparkling wine as observed under light microscope (Martinez-Rodriguez et al. 2001b).

It can be concluded that the cell wall of *Saccharomyces* yeasts is not completely broken down during autolysis, but it is modified. The cell wall of *Saccharomyces cerevisiae* has been extensively reviewed by Klis et al. (2002). It has to be kept in mind that the growth phase of a yeast culture influences cell wall composition. The cell wall is denser and thicker when cells are in stationary growth phase and stress factors like starvation, extreme temperatures or extreme pH seem also to induce strengthening of the cell wall structure e.g. the concentration in chitin increases. The inner part of the cell wall lying next to the cytoplasm membrane, consisting of ß-glucans, seemed to be mainly hydrolyzed during autolysis, at least, when autolysis took place in wine-like conditions at low temperature for months or years or at elevated temperatures for days. The glycosylated, mainly mannosylated proteins lying on the glucan layers seem to be less degraded (as reviewed by Charpentier and Feuillat 1993), but

proteolysis seemed also to occur in the cell wall when yeasts were autolysed in a wine-like medium at moderate (30°C) temperature (Charpentier et al. 1986).

1.3.1.4 Detection of yeast cells being in autolysis

Yeast colonies containing cells which are in autolysis can be detected by their release of alkaline phosphatase (Cabib and Duran 1975). Alkaline phosphatases were described as intracellular enzymes that were not released outside the cell and could be located bound on membranes or be in soluble form (Attias et al. 1970; Bauer and Sigarlakie 1975; Mitchell et al. 1981; Tonino and Steyn-Parvé 1963).
Different researchers have used 5-bromo-4-chloro-3-indolylphosphate (BCIP) as substrate of alkaline phosphatase (Giovani and Rosi 2007; Gonzalez et al. 2003; Molero et al. 1993). BCIP can be integrated in complex solid yeast media and will detect yeast colonies having cells in autolysis by staining these colonies blue to turquoise.

1.3.1.5 Compounds released during autolysis

The autolysis of yeast cells leads to the release of intracellular compounds, mainly macromolecules and their degradation products into the surrounding medium (cf. review of Babayan and Bezrukov 1985). Proteins, peptides and amino acids were released during autolysis and the intracellular break down and liberation seemed to be the highest at temperatures between 40 and 50°C and at a slightly acidic pH (Hernawan and Fleet 1995; Kollar et al. 1993; Vosti and Joslyn 1954).
Glutamic acid, phenylalanine, leucine, alanine and arginine were the prevalent free aminoacids found in the autolysates (Hernawan and Fleet 1995). Alanine was also among the prevalent amino acids released during autolysis in wine-like conditions at moderate temperature (30°C) and also asparagine was liberated in higher amounts (Martinez-Rodriguez et al. 2001b; Perrot et al. 2002).
Most of the nitrogen compounds released during autolysis at moderate temperature (30°C) in a wine-like medium were peptides (Martinez-Rodriguez and Polo 2000; Martinez-Rodriguez et al. 2001a; Perrot et al. 2002). Perrot et al. (2002) found that amino acids and peptides were mainly released in nearly equal concentrations. Proteins were also liberated but in a more than hundred fold lower concentration. The peptides and proteins were mainly composed of glycine, glutamine and glutamic acid, alanine, lysine, asparagine and aspartic acid, proline, threonine and serine. Guilloux-Benatier and Chassagne (2003) confirmed that proteins made up only below 10% of the nitrogen compounds released during autolysis in wine-like media. They found mainly alanine, leucine, γ-aminobutyric acid and valine as amino acids liberated in free form and the basically the same amino acid composition in peptides and proteins as observed by (Perrot et al. 2002).
Degradation products of cellular DNA (deoxyribonucleic acid) and RNA (ribonucleic acid) have been found in autolysates of yeasts of the genus *Saccharomyces* (Hernawan and Fleet 1995; Kollar et al. 1993; Vosti and Joslyn 1954; Zhao and Fleet 2003, 2005). Products out of RNA predominated as the cells contained between 0.2 g/100 g and 1.5 g/100 g of DNA, but roughly ten times more RNA (Zhao and Fleet 2003, 2005). The conditions of autolysis determined which concentrations of the nucleotides

of 2', 3' or 5' type were in the autolysate. 2' nucleotides were not naturally present in the cell but formed by chemical modification. Nucleotides were the prevalent form of RNA degradation products. 5'IMP (inosine monophosphate) formed out of the precursor 5'AMP (adenosine monophosphate) and 5'GMP (guanosine monophosphate) were reported to be potent flavour enhancers as cited by (Zhao and Fleet 2003, 2005).

Polysaccharides and reducing sugars were also found in yeast autolysates of *Saccharomyces* yeasts (Hernawan and Fleet 1995; Kollar et al. 1993). Polysaccharides and glycosylated proteins may mainly derive from the partial degradation of the yeast cell wall cf. above.

Lipids were analyzed in yeast autolysates by Hernawan and (Fleet 1995). They found no phospholipids in the autolysates. Comuzzo et al. (2006) found 8.5 g/100 g of lipids in a yeast extract used in wine. Release of lipids was also investigated during autolysis in wine-like conditions. 2 to 9 % of the lipid material was set free during autolysis and triacyl glycerides and sterol esters prevailed (Pueyo et al. 2000).

1.3.1.6 Influence of yeast autolysis in wine on its chemical composition and sensory character

Several reviews on yeast autolysis during wine and sparkling wine production have been published in the last 20 years (Alexandre and Guilloux-Benatier 2006; Charpentier and Feuillat 1993; Fornairon-Bonnefond et al. 2002).

In the following, only some effects of compounds released during yeast autolysis on wine's character will be outlined according to the reviews mentioned and some other studies are also cited.

Peptides out of the proteolysis of a heat shock protein (Hsp P 12) were isolated from yeast autolysate and they increased the perception of sweetness in dry red wine (Marchal et al., 2011).

Amino acids released during autolysis can be precursors of aroma compounds found in some types of wine like threonine, which can be transformed to 3-hydroxy-4,5-dimethyl-2(5H)-furanone (as reviewed by Charpentier and Feuillat, 1993) also named sotolon. Sotolon seems to be especially a contributor to aroma in wines aged with flor yeast like "Sherry fino" and "vin jaune"(Martin et al. 1992). Mannoproteins that can be released from the yeast cell wall during autolysis (cf. above) can modulate the perception of bitterness caused probably by polyphenols in conditions similar to wine (Vidal et al. 2004). Furthermore mannoproteins can modify the volatility of aroma compounds found in wine, like norisoprenoids, alcohols and esters in wine-like conditions (Chalier et al. 2007; Lubbers et al. 1994b). Nucleotides, that are released from autolysing yeast cells, are well known as flavour enhancing compounds in the food industry and showed such an effect in champagne but only at concentrations higher than naturally present (Charpentier et al. 2005). The flavour enhancing effect of nucleotides can be reinforced by glutamic acid also set free during yeast autolysis (cf. above) and such a combined effect could not be ruled out in sparkling wines (Charpentier et al. 2005).

Lipids released during autolysis (cf. above) can set free fatty acids, which can be degraded to hexanoic, octanoic and decanoic acid. These fatty acids have aroma thresholds in the range of mg/l (as reviewed by Francis and Newton 2005) and are described as rather unpleasant (sweat, cheese).

On the other hand esters can be formed out of these fatty acids with alcohols naturally present in wine and these esters having fruity aroma notes can positively modulate wine's aroma profile (Francis and Newton 2005). Higher alcohols and aldehydes were also released from yeast cells during autolysis (Chung as cited by Alexandre and Guilloux-Benatier 2006).

Furthermore the insoluble yeast lees that are in contact with wine during autolysis can adsorb various compounds and thus influence wine's sensory profile. An example is the adsorption of polyphenolic compounds such as anthocyanins, phenolic acids but also proanthocyanidins (Mazauric and Salmon 2005, 2006; Vasserot et al. 1997). Their potential sensory influence of phenolic compounds will be outlined later in the section 1.3.4. Another important role of yeast lees is the protection of wine against oxidation by binding oxygen (Fornairon et al. 1999; Fornairon-Bonnefond and Salmon 2003) and by release of reductive compounds containing cysteine (Tirelli et al. 2010).

Yeast cell walls can adsorb typical wine aroma compounds such as methyl-2-propanol or ethyl esters of fatty acids. Yeast lees are also able to bind aroma compounds causing off flavours such as some thiols (Lavigne 1996; Vasserot et al. 2003) or volatile phenols (Pradelles et al. 2008).

1.3.2 Creation of yeast mutants showing inducible autolysis and their use during wine making

Gonzalez et al. (2003) and Giovani and Rosi (2007) created mutants by UV (ultra violet) radiation out of *Saccharomyces* yeast strains, but Giovani and Rosi used spores in order to get a diploid strain in homozygous state. They screened the mutants for release of alkaline phosphatase, an autolysis marker (on BCIP medium cf. above), during a heat shock of 37°C. Mutants which were positive on BCIP-test showed abnormal cell morphology, like plasmolysis and cytoplasmic granules bigger than in the mother strain, after five days of heat shock (Gonzalez et al. 2003). Plasmolysis, deformation of cell walls and burst cells were also observed by Giovani and Rosi (2007) after heat shock of 37°C. Some of the mutants showed a higher release of proteins at temperatures equal to or higher than 25°C (until 37°C) and a higher liberation of amino acids at 25°C (Gonzalez et al. 2003). Nunez et al. (2006) worked with mutants of Gonzalez et al. (2003) and found a mutant liberating more polysaccharides with mannose as the main sugar compound during second fermentation of sparkling wine for nine month. The sparkling wine elaborated with this mutant had better foaming characteristics while not showing differences in the other sensory descriptors. Giovani and Rosi (2007) reported that some mutants released more polysaccharides during fermentation of a medium simulating grape juice than the mother strain at 28°C while preserving the same viability as the mother strain (Giovani and Rosi 2007).

1.3.3 Fining of must and wine

1.3.3.1 General considerations

Fining of must and especially wine is an old practice in wine making, which had traditionally the aim of clarification of wine and also of improvement of its sensory characters, like colour but also taste and astringency.

The process of fining consists of the addition of proteins, traditionally of animal origin, like egg white, casein of milk, isinglass or gelatine, to the wine or must. The proteins will be precipitated in the wine/must by tannins, which are naturally present in the wine or which are added in some cases, mainly in white and rosé wines. This precipitation is connected with a temporary increase in wine's turbidity and with flocculation, often formation of flakes visible by eye. The precipitate of proteins and tannins will settle down in a later stage and other particles causing turbidity in the wine/must will connect to the precipitating protein-tannin composite. The turbidity of the liquid will be thus decreased. The speed of settlement by gravity of the tannin-protein-particles is dependent on several factors, namely the diameter of the particles, the difference of density between the liquid and the particle and also the viscosity of the liquid (in ideal conditions the law of Stokes would be valid; for further explanations the reader is referred to Ribéreau-Gayon et al. 2004b pp. 383-390).

The factor viscosity of the must/wine can limit a successful fining, i.e. complete settlement of particles causing turbidity. Must or wine can present high levels of viscosity caused by pectin (composed of polygalacturonic acid, polysaccharides and also glycoproteins) extracted out of the grapes or by glucans present especially in musts/wines that have been produced out of grapes infected with grey rot (*Botrytis cinerea*). Polysaccharides and glycoproteins can also act as "protecting colloids" avoiding interactions between proteins and tannins and thus inhibiting the formation of composites that are able to settle down by gravity (cf. Ribéreau-Gayon et al. 2004b pp. 383-390 and de Freitas et al. 2003).

Tannins can be cause of excessive astringency and bitterness of wines and of colour deviations and their precipitation by the protein fining agents can thus in some cases improve wine's sensory quality. This will be outlined in the following passages.

1.3.3.2 Composition of polyphenols of must and wine and their quantification or estimation

The phenolic compounds in must and wines have been extensively reviewed in Ribéreau-Gayon et al. (2004b, pp.179-259), but the different categories of polyphenolic compounds will be mentioned and detailed in the following passage. The text of this passage is based on Ribéreau-Gayon et al. (2004b, pp.179-259), unless otherwise stated.

Phenolic acids and their derivatives are found in white must, white wines and red wines. They derive from benzoic acid or cinnamic acid and are found in concentrations of 10 to 30 mg/l in white must or wine and in ten times higher concentrations in red wines. Derivatives of the two phenolic acids are often glycosylated and derivatives of cinnamic acid are mostly esterified with tartaric acid.

A study of the concentrations of phenolic acids in German white wines of the varieties Riesling, Silvaner, Traminer and of Bacchus, Müller-Thurgau and Rieslaner, which are cross-breeds having Riesling as one parent, showed that all varieties had concentrations from 1 to 10 mg/l of the phenolic acids caffeic acid, ferulic acid and coumaric acid as well as their esters with tartaric acid (Pour Nikfardjam et al. 2007). Furthermore wines of all these varieties contained 10 to 20 mg/l tyrosol, which should be derived from yeast metabolism according to Ribéreau-Gayon et al. (2004b, p. 180).

Flavonoids are another important group of phenolic compounds in white must, wine and red wine and can be also found in the grape in glycosylated form. Red wine contains some 100 mg/l of flavonoids in form of aglycones and white wine around 100 times lower concentrations.

Anthocyanins are the red pigments in grapes and are only found in the skin of the grape berries except in case of teinturier varieties in which anthocyanins are also found in the pulp. They are present in glycosylated forms that are in most grape varieties also acylated with p-coumaric, caffeic or acetic acid. Anthocyanins have a positive charge in their heterocycle, which makes them chemically more unstable than flavonoids and their coloration is dependent on the surrounding medium, as far as pH and concentration of SO_2 (sulphur dioxide) are concerned. Anthocyanins have red colour at acidic pH and decolorize when pH rises with a minimum colour around pH 3.2 to 3.5. Anthocyanins have a blue colour at pH values above 4 and at neutral or alkaline pH values their colour is yellow (for further explanations please refer to Ribéreau-Gayon et al. 2004b, pp.192-202). Furthermore anthocyanins are decolorized by sulphurous acid. Copigmentation, formation of anthocyanins-tannin-compounds by weak, non-covalent bonds, between anthocyanins and tannins occur in wine conditions. Condensation, binding reactions involving covalent stable bonds, between anthocyanins and condensed tannin of the grape (procyanidins) occur directly or via ethyl links. These anthocyanin-tannin-compounds can occur in colourless or coloured forms, having bluish, orange or yellow colour depending on their chemical structures. These condensed compounds of anthocyanins and tannins are not decolorized by sulphur dioxide and their concentrations increase during wine storage.

Tannins are by definition phenolic compounds that form stable composites with proteins. The word tannin is derived from “tanner” to tan, which means the preservation of animal hides by treating with tannins or mineral salts. Condensed tannins are found in the skin, the seeds and the stems of grapes and are polymers of 3-flavanols, also called catechins. Red wines are much richer in condensed tannins due to their localization in the grape berry and 1 to 4 g/l are found whereas white must and wines contain around ten times less tannins. (+) catechin and (-) epicatechin are the base units of condensed tannins, also called procyanidins, as these polymers decompose in hot acid media forming cyanidins of red colour. The catechin monomers are too small to form stable (mostly non-covalent) bonds with proteins, but proteins can form stable complexes with catechin dimers.

The structures of condensed tannins (procyanidins) are very complex. The monomers are connected via covalent bonds between the C4 and the C6 or C8- carbon atoms. Ethyl bonds formed by reaction of ethanal (acetaldehyde), out of coupled oxidation between ethanol of the wine and procyanidins, can also link catechin units within procyanidins. A similar type of bonds between catechin units have been also described with other aldehydes. Oligomeric procyanidins are composed of around two to ten monomer units and polymeric procyanidins of more than ten catechin units. Procyanidins are not found in glycosylated forms in wines. Procyanidins are subject of complex oxidation reactions and can scavenge free radicals. The polymers of these reactions have brown colour and precipitate in must or wine.

The degree of procyanidins can be assessed by liquid chromatography after mild acid hydrolysis of the catechin polymers and subsequent reaction with phenylmethanethiol (Rigaud et al. 1991) or phloroglucinol (Kennedy and Jones 2001). This allows distinguishing between terminal and intermediary catechin units and as a result a mean degree of polymerization can be calculated.

Polyphenols in must and wine have a lot of different chemical structures as can be seen in the former passage. The chemical characterization of polymeric procyanidin- and anthocyanin polymers still pose difficulties even with the modern chromatographic and other physico-chemical methods, such as NMR (nuclear magnetic resonance) and mass spectrometry.

Chemical indices have been proposed to estimate the concentrations of different classes of phenolic compounds and those which were used in the studies of this thesis are outlined below.

Absorbance at 280 nm is used to estimate concentrations of total polyphenols in red wines as flavonoid compounds have an absorbance maximum at that wavelength (Somers and Ziemelis 1985). The absorbance spectrum of white musts or wines in UV(ultraviolet light)-domain does not have a sharp absorbance maximum at 280 nm (Somers and Ziemelis 1985), but shows two broad absorbance maxima within the ranges 265-285 nm and 315-325 nm. A part of the absorbance at 280 nm in white musts and wine is caused by proteins naturally present (Somers and Ziemelis 1985). The second peak in UV-absorbance of white musts and wines is caused by non-flavonoid polyphenols, namely esters of derivatives of cinnamic acid with tartaric acid and also glucose. Somers and Ziemelis (1985) propose to characterize polyphenols of white musts and wines by the absorbance at 280 nm and at 320 nm but corrections are made for non-phenolic compounds. They suggest two options and the preferred option would be treating the must or wine sample with a massive dose of PVP (polyvinyl pyrrolidone) of 100 g/l, adsorbing all phenolic compounds, and measure it as a blank against the must or wine. The other option would be subtracting the fixed values determined in their studies, namely 1.4 from the absorbance at 320 nm and 4 of the absorbance at 280 nm. It has to kept in mind that the corrected absorbance measured at 280 nm is not only due to flavonoid compounds in white must or wine but that a part of this absorbance is due to the derivative of cinnamic acids present, which can be estimated to contribute a value of 2/3 of the corrected absorbance at 320 nm to the absorbance at 280 nm.

Total polyphenol-index (absorbance at 280 nm at 10 mm path length) ranged from 27 to 100 (mean of 54) in Australian red wines and the absorbance in white wines from 5 to 15 (mean of 8) (Somers and Ziemelis 1985). Absorbance values at 280 nm of 40 to 70 or 50 to 95 in red wines were found by Vivas et al. (2003) and Iturmendi et al. (2010).

The astringency of tannins in red wine can be estimated by their reaction with “model proteins” and bovine serum albumin (BSA) was used to assess the “tannic power” of tannins of red wine (de Freitas 1995, de Freitas et al. 2003). De Freitas et al (de Freitas,1995; de Freitas et al. 2003) used nephelometry to follow the formation of tannin-BSA-precipitates in model solutions similar to wine.

Kennedy et al. (2006) stated that the absorbance at 280 nm, an index of the concentration of total polyphenols in red wine did not allow statements on wine’s astringency. A “BSA-index” following the

principles of de Freitas (1995) and de Freitas et al. (2003) could however predict astringency of red wines (Kennedy et al. 2006).

The colour intensity of red wines can be estimated by measuring absorbance at 420 nm (absorbance of yellow colour), 520 nm (absorbance of red colour) and 620 nm (absorbance of blue colour) according to Glories (1984). The colour intensity would then be the sum of the absorbance at the three wavelengths. The quotient of absorbance 420 nm / absorbance 520 nm gives an estimation of the hue of the wine. Red wines having a high hue have a more orange to brownish colour and wines with a low hue show a redder colour, but the total colour intensity has also to be considered. Colour intensity values of red wines cover a wide range from 5 found in Pinot noir (Charpentier et al. 2006) to 12 to 14 in Bordeaux wines (Charpentier et al. 2006; Glories 1984) or Spanish red wines (Iturmendi et al. 2010) and 30-50 in some Portuguese wines (Castillo-Sanchez et al. 2008; Cosme et al. 2009).

The concentration of anthocyanins in red wine can be estimated by measuring their red colour (absorbance at 520 nm) in acidic medium and to compare this with the same acidified sample that is decolorized with sulphite. It has to be kept in mind that free anthocyanins and a part of the anthocyanins-tannin compounds are quantified by this method (Glories 1984). PVPP index estimates the proportion of anthocyanins combined with tannins of the whole concentration of anthocyanins measured by decolorization with sulphite (Glories 1984). This method is based on the adsorption of all polphenolic compounds on polyvinyl polypyrrolidone (PVPP) and the subsequent elution of free anthocyanins with an acidified alcoholic solvent. The concentration of free anthocyanins eluted from PVPP and dissolved in model wine is then quantified by decolorization with sulphite (cf. above).

The yellow colour of white must and wine can be estimated by measuring the absorbance at 420 nm, but white must or wine does not have a clear absorbance maximum in light of wavelengths visible by the human eye (Ribéreau-Gayon et al. 2004b pp. 253-255). White wines showed absorbances at 420 nm from 0.05 to 0.1 in case of Pinot blanc wine (Vrhovsek and Wendelin 1998) to 0.3 in a Portuguese white wine (Cosme et al. 2008).

The sensory effect of phenolic compounds on wine, mainly red wine, has been studied by several groups. First, the works of de Freitas (1995), de Freitas et al. (2003) and Kennedy and Jones (2001) using the precipitation reaction between BSA (bovine serum albumin) and polyphenols for modeling astringency have to be mentioned, which have been already detailed. Furthermore phenolic compounds were separated by chromatographic methods and the fractions were evaluated by sensory analysis in the works of Hufnagel and Hofmann (2008), Sun et al. (2013), Vidal et al. (2004) and Weber et al. (2013).

Polymeric proanthocyanidins were described as causing astringency (Hufnagel and Hofmann 2008; Sun et al. 2013; Vidal et al. 2004), whereas Weber et al. (2013) described oligomeric proanthocyanidins as having a higher astringency than polymeric proanthocyanindins and a fraction containing anthocyanins and other phenolic compounds. Hufnagel and Hofmann (2008) described flavan-3-ols and ethylesters of hydroxybenzoic or hydroxycinnamic acids as contributing to bitterness.

Other derivatives of lower molecular weight of hydroxybenzoic and hydroxycinnamic acid were described as astringent as well as glycosides of falvanol and flavonal (Hufnagel and Hofmann 2008). Vidal et al. (2004) pointed out that there are interactions between the alcohol concentration and the perception of bitterness of phenolic compounds and that glycoproteins can diminish the perception of bitterness or astringency of polyphenolic compounds (Vidal et al. 2004).

1.3.3.3 Interactions between proteins and polyphenols

The interaction between proteins and tannins is a key feature in the process of fining and will be outlined in the following. A review on theories of fining wine with proteins can be found in Ribéreau-Gayon et al. (2004b pp. 390-398) and some points of this review will be detailed in the following.

The first studies interpreted the clarification of wine by fining as aggregation of particles having opposite charges. Particles in the wine causing turbidity had a negative charge and gelatine molecules a positive charge as detected by electrophoresis. Their aggregation and at least partial neutralization of charges should be the mechanism underlying clarification (Rüdiger and Mayr 1928 and 1929 as cited by Ribéreau-Gayon et al. 2004b, p. 390).The studies of Ribéreau-Gayon of the 1930's (summarized in Ribéreau-Gayon et al. 2004b, pp. 391 and 392) put up another type of mechanism. Flocculation between fining proteins and tannins of red wine should happen in two stages in that model. First, protein molecules having positive charges in wine conditions should be surrounded by tannin molecules carrying negative charges. That should result in still soluble complexes that are in the second stage discharged by metal cations leading to flocculation and sedimentation.

Modern theories of fining focus more on chemical interactions between wine colloids and proteins of the fining agent. Lagune (1994, as cited in Ribéreau-Gayon et al. 2004b, pp. 392-398) studied the fining of red wine with gelatine. Proteins and tannins carry electric charges on the surface of the molecules under specific conditions (pH, temperature etc.). The sum of positive and negative charges in a solution with charged particles like proteins or tannins or red wine can be characterized by its initial streaming potential and by the charge density of the system (refer to Ribéreau-Gayon et al. 2004b, pp. 392-398 for further explanations). The streaming potential is the potential created by a moving piston in the solution. The charge density is the molar concentration of a polyelectrolyte of opposite charge compared to the initial streaming potential that is needed to completely neutralize this potential. The initial streaming potential of red wines is negative, but not proportional to its tannin concentration as other particles in the red wine like polysaccharides may also carry negative charges. The volume of a gelatine solution (having a positive initial streaming potential) necessary to neutralize the initial streaming potential of a red wine was a lot higher than the gelatine concentrations used for a successful fining/clarification of the corresponding wine. Fining does consequently not rely on the complete neutralization of charges in the wine.

Interactions between proteins and tannins have been extensively studied in the last 40 years and a review is given by Ribéreau-Gayon et al. (2004b, pp.398-402). The nature of binding between proteins and tannins seems to rely mainly on non-covalent bonds such as hydrogen bonding and

hydrophobic binding, but also covalent binding has been reported (as summarized by Ribéreau-Gayon et al. 2004b, pp. 398-401 and Ricardo da Silva et al. 1991). The chemical conformation and concentration of tannin and protein molecules, the proline concentration in the protein molecules and the hydrophobicity of the tannin molecules seem to influence tannin-protein-interactions (as summarized by Ribéreau-Gayon et al. 2004b, pp. 398-401 and Ricardo da Silva et al. 1991).

The glycosylation of proteins can also influence the protein-tannin-interactions (reviewed in Ricardo da Silva et al. 1991). Furthermore factors of the environment of the tannin and protein molecules such as pH, temperature, ionic strength and ethanol concentration seem to influence their interaction (as summarized by Ribéreau-Gayon et al. 2004b, pp. 398-401 and Ricardo da Silva et al. 1991).

Some important studies mainly of the last twenty years will be outlined in the following.

Procyanindin trimers bound more poly-L-proline than dimers and most binding was observed for procyanindin trimers that showed esterification with gallic acid (Ricardo da Silva et al. 1991).

In the latter cases equivalent masses of poly-L-proline and tannin were precipitated and all tannins were precipitated (Ricardo da Silva et al. 1991).The length of proline chains also influenced their binding capacity showing a higher binding efficiency above 19 kDa. The imino acid proline shall preferably form hydrogen bonds with hydroxy rests of the phenolic compounds as it contains an oxygen atom adjacent to secondary amine nitrogen (Ricardo da Silva et al. 1991).

Binding reactions (chemical interactions resulting in release of energy) have been observed for galloylated procyanidin monomers, but not for monomeric catechins, with poly-L-proline (Poncet-Legrand et al. 2007b). This release of energy should be caused by formation of hydrogen bonds, but hydrophobic interactions seemed to be also involved as galloylation increased the hydrophobic character of the procyanidin (Poncet-Legrand et al. 2007b). Gelatine and casein bound also preferably with galloylated procyanidin trimers, but gelatine bound at maximum 30 % of procyanidins and casein 50 % of procyanidins when used in a 3.7 higher concentration than gelatine (Ricardo da Silva et al. 1991). A higher number of o-dihydroxygroups in the molecule of procyanidins shall increase their binding capacity. The lower procyanidin concentrations bound by gelatines and casein compared to L-poly-proline should be partly due to their lower concentrations of proline of at maximum 25 g/100 g in case of gelatine or 12 g/100 g in case of casein (Ricardo da Silva et al. 1991). The proline concentration shall also be an important factor in formation of protein-polyphenol-haze in beverages, as well as the degree of polymerization of procyanidins (as reviewed by Siebert 1999). Hydrophobic interactions and hydrogen bonding seem to be the forces combining haze-active polyphenol-protein aggregates and an increase in pH from 3 to 4 favoured haze formation (as reviewed by Siebert 1999).

An extensive study on binding of procyanidins with gelatine and their hydrolysates did not show an influence of oxygenation or ionic strength on tannin binding (Yokotsuka and Singleton 1987).

Procyanidin dimers and oligomers bound a higher concentration of gelatine molecules of different molecular weights in the range from 70 to 2 kDa when pH value increased from pH 3 to 4 (Yokotsuka, Singleton 1987, 1995) which was also stated by Siebert (1999).

A decrease of temperature during incubation of procyanidin - gelatine mixtures of different molecular masses from 70 Da to 2 kDa from 25 to 15 °C increased procyanidin precipitation (Yokotsuka and Singleton 1987). This behavior should be caused by prevalent hydrogen bonding connecting procyanidins with gelatine molecules.

The molecular weight of gelatine fractions had no influence on their binding capacity with procyanidins. 80 % of the tannins were bound at maximum at equal concentrations of gelatine and tannin (Yokotsuka and Singleton 1987). Precipitation experiments with BSA (bovine serum albumin) and tannins of grape seeds also showed that a complete depletion of the tannins in the mixtures was not possible (Schmauch 2010). Gelatine fractions having molecular masses of 10 to 2 kDa readily precipitated with procyanidin dimers or oligomers (Yokotsuka and Singleton 1987). A complete precipitation of the proteins/peptides was only achieved when gelatine of an average molecular mass of 70 kDa was added to polymeric or oligomeric procyanidins in a ratio of 1:1.

Catechin monomers were not able to precipitate with the gelatine fractions in the study of (Yokotsuka and Singleton 1987), which confirms the statement of Ribéreau-Gayon et al. (2004b).

Glycosylation can also influence the binding capacity of proteins with tannins. An arabinogalactan-protein, a type of protein found in pectin of grapes, showing a low protein concentration of 6 g/100 g and 90 g/100 g of neutral sugars bound procyanidins comparably to gelatines of molecular weights around 10 kDa in the study of Ricardo da Silva et al. (1991). The mannoprotein invertase showed a 10 times lower binding capacity for procyanidins than the non-glycosylated protein BSA (bovine serum albumin) (Rowe et al. 2010).The aggregation of PRP (proline-rich proteins) of human saliva was reduced by their glycosylation and aggregation started at higher proportional ratio of procyanidins to glycosylated PRP. The aggregates were also of smaller size in case of glycosylated PRP (Pascal et al. 2008; Sarni-Manchado et al. 2008). The aggregation of procyanidin molecules can be inhibited by the presence of mannoproteins from yeast (Charpentier et al. 2004; Poncet-Legrand et al. 2007a; Riou et al. 2002). Polysaccharides can inhibit the precipitation of oligomeric procyanidins with the protein BSA and ionic polysaccharides such as xanthan and pectin were more efficient (de Freitas et al. 2003).

Mannoproteins of yeast can also inhibit the formation of haze by wine proteins (Dupin et al. 2000 b; Dupin et al. 2000 a; Waters et al. 1994). This haze formation in wine was attributed to protein-polyphenol interactions by Siebert (1999) as outlined above. On the other hand glycoproteins containing mannose could precipitate tannins of red wine (Gambuti et al. 2012) or possibly precipitate procyanidins and colour pigments of red wine (Guadalupe and Ayestaran 2008).

Negative charge densities in a range of 30 to 120 milli equivalents (meq)/g have been found in condensed tannins consisting of polymerized procyanidins (Lagune 1994 as cited by Ribéreau-Gayon et al. 2004bp. 400), whereas very low, negligible charges have been found on tannins of grape seed at pH of wine (3.5) by Vernhet et al. (1996). The charge density of a protein fining agent, its amino acid composition and its molecular masses determine also its fining capacities (as summarized in Ribéreau-Gayon et al. 2004b, p. 400).

No molar ratio between the concentration of protein fining agent (i.e. gelatine) and the concentration of eliminated tannin was found (Lagune, 1994 as cited by Ribéreau-Gayon et al. 2004b p. 400).
The pH value of the wine influenced the speed of flocculation of fining proteins and more tannin was eliminated at higher pH values. The presence of metal cations was described as indispensable of flocculation between tannins and proteins (all as reviewed by Ribéreau-Gayon et al. 2004b, pp. 400 and 401).
The perception of astringency seems to be related to interactions between human saliva and polyphenols of red wine (de Freitas and Nunez 2001; Schmauch 2010). Saliva has proteins rich in proline called PRP and these PRP are precipitated by procyanidins. The precipitation is already possible with monomers. The intensity of formation of precipitates analyzed as increase in turbidity of procyanidins-PRP-mixtures depended on the stereochemistry of the procyanidins, like position of hydroxyl groups on phenolic rings, type of monomer bonds and galloylation (esterification with gallic acid) (de Freitas and Nunez 2001). The conformation of proteins also influenced the intensity of precipitation. PRP having randomly coiled structures produced more intense turbidity at lower concentrations than α-amylase having a globular structure (de Freitas and Nunez 2001). Bovine serum albumin (BSA) did not bind procyanidin dimers and trimers at pH 5 (de Freitas and Nunez 2001), but did bind to commercial tannins of grape seed. The maximum amount of binding and precipitation was observed at pH 3.5 by nephelometry when BSA was in F-form, in which a part of the interdomain helices are disrupted catalyzed by acid pH (Nakamura et al. 1997). High binding activity between BSA and tannins was still observed at pH 5, a pH near pH of mucus in oral cavity (de Freitas, Nunez 2001) at which BSA is at its isoelectric point (Schmauch 2010). Different red wines precipitated all proteins found in human saliva and Schmauch (2010) raised the question if the perception of astringency was only related to complete precipitation of saliva proteins resulting in a lack of lubrication of the oral cavity. He put up the hypothesis that direct interactions between proteins of mucous membranes of the oral cavity may also be a cause of the perception of astringency (Schmauch 2010). The presence of polysaccharides, mainly ionic ones like xanthan and pectin, diminish reactivity of procyanidin oligomers with BSA at pH similar to saliva, which could also modulate perception of astringency (de Freitas et al. 2003). Mannoproteins seem also to reduce astringency of red wine (Escot et al. 2001) and can reduce bitterness probably caused by polyphenols in mixture with arabinogalactan proteins in wine-like conditions (Vidal et al. 2004).

1.3.3.4 Selected studies on fining of must and wine and characterization of protein fining agents

The studies shown in this passage used mainly the protein fining agents gelatine, casein, isinglass and yeast protein extracts (YPE) also examined in the research of this thesis.
The study of Ricardo da Silva et al. (1991) showed that fining red wine of the variety Mourvèdre with the same concentrations of gelatine or casein used in the binding study with procyanidins resulted in no decrease in concentrations of procyanidin dimers and trimers. The same fining agents bound

however these phenolic compounds in synthetic wine solution (cf. above), which may be due to the fact that more reactive polymeric procyanidins or anthocyanins compounds “protected” the procyanidin di- and trimers. It can be concluded that interaction studies between procyanidins and proteins in wine-like conditions are useful for the study of chemical interactions but cannot be a model for the reactions in wine in any case. The colour intensity of this Mourvèdre wine diminished slightly by around 5 % as well as the index of total polyphenols (absorbance at 280 nm) (Ricardo da Silva et al. 1991).Gelatine of mean molecular weights between 20 and 10 kDa reduced turbidity of Pinot noir red wine from 70 NTU (nephelometric turbidity units), a noticeable turbidity, to 30 NTU, a still slight turbidity, when used at 8 g/hL (Marchal et al. 2002a). The sensory character of the wine was however not changed by this potential reduction in tannin concentration (Marchal et al. 2002a).

Gelatines of different mean molecular masses ranging from 10 to 66 kDA bound preferably proanthocyanidins of red wine and their concentration was reduced at maximum by 9 % (Sarni-Manchado et al. 1999). Gelatine of a mean molecular mass of 25 kDa showed the highest effect and the mean degree of polymerisation of tannins precipitated by gelatine was higher than that of the wine and galloylated tannins were in particular precipitated (Sarni-Manchado et al. 1999).The authors pointed out that soluble protein-tannin complexes were formed, mainly when fining was done with gelatine of high molecular weight. A liquid gelatine preparation of a mean molecular mass of 25 kDa and two fractions of this gelatine of 16 and 190 kDa did not bind monomeric proanthocyanidins, but condensed tannins, i.e. proanthocyanidins to 10 to 15 %. The original gelatine had the highest effect (Maury et al. 2001) and precipitated mainly tannins of high molecular mass, as well as the gelatine fractions. The gelatine fraction of lower molecular mass of 16 kDa bound tannins of a higher mean degree of polymerization than the original gelatine and the high molecular mass fraction. Furthermore galloylated tannins were preferentially bound to gelatine molecules. The concentration of tannins of red wines after fining was lower after fining with original gelatine and its two fractions, but only two of four wines showed a lower mean degree of polymerization after fining with the gelatine fraction of low molecular mass. A part of the wines was tasted after fining with the gelatine fractions and astringency was decreased, which was related with a decrease in total proanthocyanidins in both wines. A decrease of the mean degree of polymerization reduced astringency only in one of two wines fined with the gelatine fraction of lower molecular weight of 16 kDa.

Gelatine at a concentration until 10 g/hl, casein until a concentration of 60 g/hl and isinglass at a concentration until 2 g/hl did not decrease the concentrations of total polyphenols, phenolic acids and catechins in white or red wine in the study of Fischerleitner et al. (2003).

Highly hydrolyzed gelatine with proteins of masses below 14 kDa reduced tannin fractions of mean degree of polymerization of 1.5, 3.4 and 4.9 of a red wine more or as only one when compared to a less hydrolyzed gelatine used at the same concentration (Cosme et al. 2009). This more hydrolyzed gelatine also reduced colour intensity of the red wine. The mean degree of polymerization of the

corresponding red wine was not reduced by fining with gelatine. Castillo-Sanchez et al. (2008) reported that fining red wines with 20 g/hl gelatine reduced their colour intensity.

First studies on yeast protein extracts (YPE) showed that YPE at concentrations between 20 and 50 g/hl clarified red wines to a similar extent than fining with liquid or solid gelatine preparations of concentrations of 6 g/hl or 30 to 100 ml/hl (Charpentier et al. 2006; Iturmendi et al. 2012).

The colour intensity of red wines of Burgundy or Bordeaux was reduced by YPE in a way comparable to fining with gelatine (Charpentier et al. 2006).

Patatin, a glycoprotein out of potato, was used for fining and did not reduce colour intensity of red wine at the same concentration as gelatine, which caused a loss of colour (Gambuti et al. 2012). On the other hand both fining agents reduced concentrations of total polyphenols and tannins, which was related with a decrease in astringency.

Fining with casein, isinglass or gelatine did not reduce the tendency of browning of white wine, a phenomenon that should be at least partly due to polyphenol oxidation, as stated in the studies of Fischerleitner et al. (2003) and Vrhovsek and Wendelin (1998).Vrhovsek and Wendelin (1998) also found that concentrations of esters of tartaric acid with cinnamic acids were not reduced by treatment with casein or gelatine. Some types of gelatine, isinglass and casein were however reported to decrease the concentrations of flavanol monomers and procyanidin oligomers and polymers in the study of Cosme et al. (2008). Furthermore the mean degree of polymerization of wine's procyanidins was decreased after fining with casein, isinglass or gelatine. The turbidity of white must or wine could be reduced by fining with isinglass and casein as found by Cosme et al. (2008) and Marchal et al. (2002b). Caseins reduced also the yellow colour of white wine as stated by Cosme et al. (2008) and Vrhovsek and Wendelin (1998).

Only three studies were found that evaluated the sensory effect of fining (Gambuti et al. 2012; Marchal et al. 2002a; Maury et al. 2001 cf. above). Most of the articles cited in this section/passage stated that fining agents precipitated preferably oligomeric and polymeric proanthocyanidins. These proanthocyanidins were described as participating in the sensory impression of astringency as outlined by Gambuti et al .(2012), Hufnagel and Hofmann (2008), Marchal et al. (2002a), Maury et al. (2001), Sun et al.(2013) and Vidal et al. (2004). Fining could thus influence wine's astringency. It is also important to mention that wine colour and limpidity can be influenced by fining as shown in this section and these parameters are also part of the sensory perception of wines.

Protein fining agents should be characterized in a physico-chemical way to allow the winemakers to obtain effective fining agents and to avoid that their use could introduce material to wine that could be detrimental in any way to human health. Consequently, the International Organization of Vine and Wine (OIV) set up maximum concentrations of minerals, heavy metals and microorganisms in gelatine, casein and isinglass preparations, mostly to avoid the commercialization of products having negative influence on human health (cf. resolutions in International Oenological Codex of International Organization of Vine and Wine, 2012). On the other hand their protein concentration is

only delimited by their total nitrogen concentration after a mineralization of organic nitrogen compounds. This “nitrogen minimum“is 130 g/kg in case of casein, 140 g/kg in case of gelatine and isinglass. Yeast protein extracts (YPE) are recommended as fining agents by the OIV (OIV-OENO 416-2011 and 417-2011) and are permitted in the European Union (regulation EC No 144/2013) at concentration until 30 g/hl in must white and rosé wine and until 60 g/hl in red wine. The OIV (International Organization of Wine and Vine) also specified these preparations (OIV-OENO 452-2012). The YPE should contain at minimum of 500 g/kg of proteins quantified by the recommended method of Lowry et al. (1951). Furthermore 50 % of these proteins should have a molecular mass above 15 kDa as detected by size exclusion chromatography or polyacrylamide-gel-electrophoresis using sodium dodecyl sulphate derivatization.

Different parameters were evaluated to define protein fining agents and their effectiveness, e.g. their amino acid composition, distribution of molecular masses, superficial charge density and isoelectric point.

The amino acid composition of protein fining agents allows statements on their suitability as fining agents. Oenological gelatine of lower molecular masses of 13 to 9 kDa showed a concentration of total amio acids after hydrolysis of proteins of 40 to 60 g/100g and proline/hydroxyproline, asparagine / aspartic acid, glutamine / glutamic acid, as well as glycine, alanine and arginine were the predominant amino acids (Ricardo da Silva et al. 1991). A gelatine of cattle bone was reported to have proline and hydroxyproline, glycine and alanine as predominant amino acid (Yokotsuka and Singleton 1987) and the same gelatine composition was found by Maury et al. (2001). Casein showed a molecular mass of 24 kDa and was composed of 90 g/100 g of amino acids of which proline, glutamine and glutamic acid, leucine, lysine, threonine, serine, and histidine dominated (Ricardo da Silva et al. 1991). The importance of the proline concentration of a protein on its interaction with tannins was outlined in the previous section.

The molecular masses of proteins in fining agents based on plant proteins can have an influence on their binding reactions with polyphenols in red wines and highly proteolysed protein products had generally a lower effect on polyphenolic composition of the red wines (Tschiersch et al. 2010).

The study of Tschiersch et al. (2010) also demonstrated that the origin of the plant protein clearly influenced its binding with polyphenols. Wheat proteins diminished mostly colour intensity of red wines, whereas rice and potato proteins bound preferably procyanidins. Gelatines are produced by partial hydrolysis of collagen of skin or bones of different animals like cattle, pig or fish and their degree of hydrolysis determines the distribution of molecular masses of the products (resolution Oeno 13/2003 of International Organization of Vine and Wine 2012; Ribéreau-Gayon et al. 2004b, pp.403-410). Gelatines intended for fining of must and wine with various molecular weight distributions of around 100 kDa, above 43 kDa, below 43 kDa and below 14 kDa were described (Cosme et al. 2008; Marchal et al. 2002a; Marchal et al. 2002b; Ricardo da Silva et al. 1991). Gelatines of a higher degree of hydrolysis do no more form gels in aqueous solution and are soluble in cold water or used as

stabilized liquid solution for fining products (resolution Oeno 13/2003 of the International Organization of Vine and Wine; Ribéreau-Gayon et al. 2004b, pp.403-410).

The molecular mass distribution of gelatine preparations seem to be important for the precipitation of tannin-fining protein-particles, but had no influence on tannin-protein-ratio in the particles in model wine solution (Yokotsuka and Singleton 1987). The influence of mean molecular weight of enological gelatines on binding capacity with different tannin fractions of red wine was stated by Maury et al. (2001) and Sarni-Manchado et al. (1999). Later studies of Cosme et al. (2008 and 2009) confirmed that gelatines having the same isoelectric point but different distributions of molecular masses bound different concentrations of procyanidins monomers, dimers, oligo- and polymers in red and in white wine. Caseins used for fining were mainly composed of proteins having molecular masses around 30 kDa (Cosme et al. 2008; Marchal et al. 2002a; Marchal et al. 2002b; Ricardo da Silva et al. 1991).

Different isinglass preparations differed in their molecular mass distribution showing only masses below 20 kDa or also masses above 94 kDa and between 94 and 43 kDa (Cosme et al. 2008).

Yeast protein extracts had molecular masses below 10 kDa in the studies of Iturmendi et al. (2010) and Iturmendi et al. (2012).

The isoelectric point of a preparation of fining proteins determines the point when no net charges are still present and could allow predictions of the possible sign (positive or negative) of charge density of this protein preparation at wine pH. On the other hand the solubility of an uncharged protein molecule would be lower in aqueous conditions. Gelatines of different molecular mass distributions had isoelectric points between 4.2 to 4.7 (Cosme et al. 2008; Iturmendi et al. 2010) and a lower isoelectric point tended to be related with lower charge densities (Cosme et al. 2008) at wine-like pH of 3.4. The same trend was observed for different casein and isinglass preparations, which had isoelectric points around 4.5 except one isinglass preparation of swim bladder (pI of 6.5). Yeast protein extracts were reported to have isoelectric points between 1.4 and 2.4 and thus having negative charge densities in wine-like conditions.

Charge densities of protein fining agents could influence their reactivity with tannins and other charged wine constituents, which would result in a different flocculation and sedimentation behavior.

The charge densities of different gelatines were positive and ranged between 1.3 and 0.05 meq (milli equivalents)/ g (Lagune, Glories 1996) or between 0.1 and 0.7 meq/g (Cosme et al. 2008). The differences of charge densities of the gelatine preparation could not predict their reaction with different procyanidins fractions (Cosme et al. 2008). Casein preparations and one isinglass preparation of fish skin had charge densities of 0.05 to 0.1 meq/g, whereas isinglass of swim bladder had higher charge densities of 0.4 meq/g (Cosme et al. 2008).

Unfortunately, no study was found that analyzed amino acid composition, distribution of molecular masses, isoelectric point and charge densities in protein fining agents together. Such a study would allow conclusions of the influence of these “protein parameters” on wine’s colour, limpidity and

polyphenol composition after fining. Thus final remarks about physico-chemical characters of protein fining agents and their impact on wine's composition are difficult.

1.3.4 Use of yeast autolysates or extracts during winemaking and their (potential) sensory effects

Pozo-Bayon et al. (2009b) reviewed the use of inactive dry yeast preparations in winemaking. They grouped different yeast products, i.e. inactive yeast, yeast autolysates, yeast cell walls and yeast extracts into the term inactive dry yeast preparations (IDY). They classified these IDY preparations in categories, such as products favouring the development and fermentation capacity of wine yeasts or lactic acid bacteria performing malolactic fermentation and "wine quality" enhancers.

There is scientific evidence that IDY preparations can favour the onset and completeness of alcoholic and malolactic fermentation (as reviewed by Pozo-Bayón et al. 2009b) as they provide nutrients, like organic nitrogen compounds, minerals and vitamins to yeast and bacteria performing the fermentation, or as they adsorb compounds inhibiting yeast and bacteria activity and proliferation such as some fatty acids, e.g. octanoic acid. Different effects of inactive yeast preparations on wine's chemical composition and organoleptic characteristics are mentioned by the producers as reported by Pozo-Bayón et al. (2009b) and as observed by the author of this thesis. A selection of some effects will be detailed in the following. One is an antioxidant effect which could be due to the presence of reduced cysteine groups in proteins and peptides of the cell walls, but also of the soluble fraction of inactive yeast as found by Tirelli et al. (2010). Oxygen can also be absorbed by dead yeast cells as observed in yeast lees (Fornairon et al. 1999 and Fornairon-Bonnefond, Salmon 2003). Furthermore mannoproteins can be released by inactive yeast preparations (Comuzzo et al. 2012) and can interact with wine's polyphenols by inhibiting tannin aggregation and by reducing tannins' astringency or bitterness as outlined in the section 1.3.3.3. Mannoproteins and nucleotides released from inactive yeasts could influence the perception of wine's aroma profile as outlined in the section 1.3.1.6. Yeast cell walls bound wine aroma compounds such as higher alcohols like methyl butanol, esters and the norisoprenoid ß-ionone (Lubbers et al. 1994a). Yeast cell walls could also bind aroma compounds that cause off flavours in wine such as volatile phenols (Pradelles et al. 2008) or thiols causing reductive notes in wine (Lavigne 1996 and Vasserot et al. 2003). A study of inactive yeast preparations (Pozo-Bayón et al. 2009a) confirmed that the volatility of esters of wine was increased by a short contact time of 3 days with inactive dry yeast preparations (IDY), which was attributed to interaction with soluble compounds of IDY. More hydrophobic aroma compounds like ethylhexanoate and ethyl phenylacetate were on the other hand reduced in volatility in contact with IDY, which was attributed to a retention effect probably caused by insoluble compounds of IDY. Furthermore the capacity of yeast lees to adsorb polyphenol compounds was outlined in the section 1.3.1.6.

The mycotoxin ochratoxin A produced by mould fungi that are also found on grapes can be adsorbed by yeast cells (Garcia-Moruno et al. 2005).

On the other hand IDY preparations contain a lot of aroma-active compounds and those at high concentrations were mainly short chain fatty acids such as acetic and butanoic acid, 2-methylpropanoic and 3-methylbutanoic acid. These fatty acids were described with sensory impressions such as “cheese” and “solvent”, which could have a negative impact on wine aroma if they would appear in wine at concentrations above odour threshold (Comuzzo et al. 2006; Comuzzo et al. 2012). The treatment of white wines with IDY could have a positive effect on full bodied more neutral wines such as Pinot gris by addding complex “yeasty” aroma notes, but it had negative effects on aroma of white wines of aromatic varieties such as Traminer or Sauvignon Blanc (Comuzzo et al. 2006). Yeast extracts are widely used in the food industry to confer meaty aroma notes to different preparations. Such yeast extracts contain furan derivatives, methylbutanal and methional, that have high odour activity values and aroma impressions of roast bread or meat (Münch and Schieberle 1998b; Mahadevan and Farmer 2006).

The use of inactive yeast preparations during wine making is recommended for some product categories by OIV and the following preparations are specified. “Inactive yeasts” are used as yeast nutrients and for fining of wine, e.g. to reduce toxic ochratoxin A, “yeast autolysates” are used as yeast nutrients and “yeast hulls” are used to favour alcoholic fermentation by adsorption of yeast-inhibiting fatty acids. Furthermore mannoproteins are recommended for use in wine to stabilize wine against precipitation of instable proteins or tartrates, as well as yeast protein extracts for fining (cf. resolutions OIV-OENO 459-2013, OIV-OENO 496-2013, OIV-OENO 497-201, OIV-OENO 452-2012 and 26/2004).

It has also to be kept in mind that the European Union only allows the use of yeast hulls, of yeast lees of production of dry wines and of yeast mannoproteins according to regulation No 606/2009.

Yeast protein extracts for fining of must and wine are also permitted to use as outlined in regulation No 144/2013 during winemaking and for wine treatment

The main topic of the studies of this thesis was the development and evaluation of yeast protein extracts (YPE) used for fining of must and wine. The yeast proteins precipitate in the wine during fining and thus the potential main influence of YPE on wine’s aroma profile could derive from the introduction of aroma compounds as found in IDY and commercial yeast extract (cf. above) to wine. This could negatively modify wine aroma if the aroma compounds of YPE would be present above odour thresholds in wine after fining.

2 Thesis Part I: Mutagenesis and selection of yeast strains

2.1 Material and methods

2.1.1 Chemical substances

All substances and compounds used were of analytical grade unless otherwise specified.

Water purified in a Genpure-CAD-apparatus of TKA (part of Thermo Electron LED, Niederelbert, Germany) was used for all analyses. Methanol, sodium hydroxide, acetic acid, phosphoric acid > 85 %, Tris (Tris (hydroximethyl) aminomethane), Tris hydrochloride, glycine, ammonium sulphate, 5-bromo-4-chloro-3-indolyl phosphate (BCIP) disodium salt and erythrosin B sodium salt were from Carl Roth (Karlsruhe, Germany). Glucose monohydrate, hydrochloric acid 37 % fuming, sodium dodecyl sulphate (SDS > 85 %), glycerol anhydrous, copper sulphate pentahydrate, sulphuric acid, sodium potassium tartrate tetrahydrate, starch, sodium thiosulphate, sodium carbonate, Folin Ciocalteu's phenol reagent were delivered by Merck (Darmstadt, Germany). Potassium dihydrogen phosphate, Coomassie Brilliant Blue R-250 (Sigma reference number 27816), Coomassie Brilliant Blue G-250 (Sigma reference number 27815), bromophenol blue (Sigma reference number B 0126), bovine serum albumin (BSA, Sigma reference number A 7906) and DL- dithiothreitol (DTT) were obtained from Sigma-Aldrich Chemie (Munich, Germany). Bacto ™ is a trademark of Becton, Dickinson & Company (BD, Heidelberg, Germany). Agar was obtained from Becton, Dickinson & Company (BD, Heidelberg, Germany).

2.1.2 Media and sterile solutions

YEPD used during mutagenesis and initial yeast selection:

The medium was composed of 10 g/l Bacto ™ yeast extract, 10 g/l Bacto ™ peptone, 20 g/l glucose, 20 g/l agar, if medium was solid, and was sterilized 20 min at 120°C.

YEPD used during second yeast selection:

The medium was composed of 10 g/l yeast extract (Fermtech, Merck, Darmstadt, Germany), 20 g/l peptone from pancreatic digest (Roth, Karlsruhe, Germany), 20 g/l glucose, 25 g/l agar, if medium was solid. pH of the medium was adjusted between 6.5 and 7 and it was sterilized 20 min at 120°C.

Medium used for screening of autolysis:

Solid YEPD was used and before pouring the plates 40 mg/l BCIP (5-bromo-4-chloro-3-indolyl phosphate) disodium salt were added according to Gonzalez et al. (2003) and Molero et al. (1993).

BCIP was used as a substrate of alkaline phosphatase as described by these authors. The release of alkaline phosphatase was regarded as an indicator of cell autolysis as already pointed out by Cabib, and Duran (1975) and Molero et al. (1993).

Medium used for screening of reduced viability:

Solid YEPD was used and 15 ml of a solution of Erythrosin B prepared according to Bonneu et al. (1991) were added before pouring the plates. Erythrosin B can only migrate in cells having membrane damages and will stain colonies having damaged cells pink to red depending on total viability of the colonies.

EP2 media:

A specific type of molasses called EP2 was used. It was provided by a yeast producer cooperating with Sofralab (Epernay, France). The medium contained 167 ml/l EP2 and had a pH of 5.5, which was adjusted with 1M HCl. If solid medium was required 25 g/l of agar were added and in all cases the solution was sterilized 20 min at 120°C.

Sodium chloride solution:

Distilled water with 9 g/l NaCl was sterilized 20 min at 120°C.

Phosphate buffered saline solution (PBS):

8 g/l NaCl, 0.2 g/l KCl, 1.44 g/l of $NaHPO_4$ and 0.24 g/l of KH_2PO_4 were dissolved in distilled water, pH was adjusted to 7.4 and sterilized 20 min at 120°C.

2.1.3 Analysis of EP2

Sugars were analysed according to Rebelein (Rebelein 1973).

Nitrogen was measured in form of ammonium by flow injection analysis in a Foss FIASTAR 5000 apparatus (Foss, Relingen, Germany) with a method provided by Foss (based on colorimetry) after mineralization based on Schaller (1988, p.363 and 364).

2.1.4 pH measurements

A pH-meter of WTW (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) was used.

2.1.5 Spectophotometry

Measurements were taken in a Unicam UV 300 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany).

2.1.6 Mutagenesis and first selection of yeast strains

A commercial yeast strain of Oenofrance (Bordeaux, France) of the species *Saccharomyces cerevisiae* was used. The yeast was cultivated ovenight in YEPD liquid medium at 27°C in a shaking incubator at 100 rpm (rotations per min). Cell concentration was then determined in a Thoma hemocytometer and 7 x 10^4 cells were spread on YEPD plates. The plates were irradiated by UV-light to reduce the cell viability to 10 to 1 % and incubated at 20°C until colonies appeared. YEPD plates that presented between 50 and 300 colonies were replicated by using sterile velvet onto YEPD plates containing BCIP. The BCIP plates were cultivated for 24 h at 20°C to allow the replicated colonies to grow on and then the mutants were exposed to heat shock of 48 hours at 37°C. Colonies that stained blue

during (24h) or after (48h) the heat shock were selected. These mutants were examined once again on solid YEPD medium enriched with BCIP as described above and strains becoming blue after 24 h of heat shock were retained.

2.1.7 Yeast strain conservation

The yeasts were re-cultivated on YEPD plates to have fresh single colonies, YEPD liquid medium was inoculated out of the single colonies and incubated at 27°C under shaking until visual growth was observed. Purity of yeast strains was verified under the microscope and cells out of 1.5 ml medium were harvested by centrifugation, suspended in 1 ml sterile PBS containing 30 % glycerol, shock-frozen in liquid nitrogen and conserved at -80°C.

2.1.8 Second selection of yeast strains

2.1.8.1 Autolysate production

Yeast strains were cultivated on solid EP2-medium at 27°C. 10 ml EP2 liquid in culture tubes (diameter 16 mm, length 160 mm) were inoculated out of cell material of the plate of the corresponding strain and cultivated at 27°C on a rotatory incubator at 40 rpm.

Yeast growth was monitored by measuring absorbance at 600 nm. The kinetic was repeated once for each strain and a second time if the results could not be repeated.

Yeast strains were cultivated under the same conditions as above until cultures were in the middle of the exponential growth phase and yeast biomass was harvested by centrifugation, washed three times in 5 ml 0.9% NaCl-solution and suspended in 2 ml sterile distilled water. Autolysis of the yeast strains was then induced by incubation at 37°C for 24 h in a shaker at 100 rpm. Cell concentration was measured before and after autolysis in the yeasts suspended in water in a Thoma hemocytometer. Yeast cells were separated from the autolysate after 24 h and the autolysate was deep-frozen at -20°C.Two or three autolysates per strain were produced. Three autolysates were made, when the results were not reproducible.

2.1.8.2 Morphological examination of yeast cells during/after autolysis

Samples of cells were taken after 3 hours and at the end of autolysis and observed under the microscope (Biozero of Keyence, Neu-Isenburg, Germany).

2.1.8.3 Protein analysis in the autolysates

Proteins were quantified in the autolysates by the method of Lowry et al. (1951) and bovine serum albumin (BSA) was used as standard. All autolysates were measured in duplicate.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to obtain qualitative and quantitative data of the proteins present in the autolysates. The analysis was performed on a Mini PROTEAN- 3-Cell of Bio-Rad Laboratories (Munich, Germany). The procedure was based

on Laemmli (1970) and precast gels (Mini-PROTEAN TGX-gels of Bio-Rad) with a polyacrylamide gradient of 4 to 20% were used. Autolysates were lyophilised in an Alpha 1-2 apparatus of Martin Christ (Osterode, Germany) before analysis. The lyophilised autolysates were dissolved in sample buffer (pH 6.8, 62.5 mM Tris-HCl, 250 ml/l glycerol, 20 g/l SDS, 20 mM DTT and 125 mg/l bromophenol blue) and heated 5 min at 95°C. 15 µl sample per gel well were applied and SDS-PAGE molecular weight standards of Bio-Rad (reference number 161-0317) were dissolved in sample buffer to have a concentration of 1 µg of the individual proteins per well and treated as the samples. Running buffer for electrophoresis was prepared according to Laemmli (1970) and Electrophoresis was done at a voltage of 150 V until bromophenol blue left gel at ambient temperature. All autolysates still available after the measurement with the Lowry method were evaluated. The staining of the proteins was done with Coomassie Brilliant Blue according to the recommendations of Bio-Rad. Gels were fixed and simultaneously stained for 40 min in an aqueous solution containing 400 ml/l methanol and 100 m/l acetic acid and 1 g/l Comassie Brilliant Blue R 250. The same solution without Comassie Brilliant Blue was used for destaining the gels. Gels were documented in an in an Alpha Imager ® EC of Alpha Innotech (now part of ProteinSimple, Santa Clara, USA). Molecular mass and mass of protein bands of the autolysates was determined by densitometry using the software LabImage (Kapellan Bio-Imaging Solutions, Leipzig, Germany). The BSA-band of the molecular mass standard mixture served as reference for mass analysis by densitometry.

2.1.8.4 Phenotypic characterization of the strains after heat shock

The yeasts of all strains were put in strips on YEPD containing BCIP or Erythrosin B (see above), cultivated for 2 to 3 days at 27°C and then exposed to heat shock for 48 h at 37°C. This was done 15 months after the beginning of the second selection step of the strains. Yeasts from cultures on agar plates that had been re-inoculated every four to six weeks and stocked at 4°C for 15 months and cultures out of cryo-conservation were used to be able to obtain an indication of the stability of these phenotypic traits. Colonies were examined before, during and after heat shock every 24 hours and each yeast strain was tested in triplicate.

2.1.9 Autolysate production at pilot scale in the lab

EP2-medium was prepared as outlined above. Yeast strains were cultivated on solid EP2 medium, then 50 ml liquid EP2-medium in 100 ml Erlenmeyer flak was inoculated with three loops of biomass and cultivated at 27 °C under shaking of 80 rpm until stationary phase was reached (after approximately 42 h). Growth was monitored by measuring the absorbance at 600 nm (cf. above).The cultures were then used to inoculate 1l of EP2 medium, which was also incubated at 27°C and 80 rpm. Growth was again observed. The first cultures were submitted to autolysis when stationary phase was reached, then cultures were prepared under the same conditions but cultivation was stopped in the middle of the exponential growth phase as done during yeast selection. Biomass was separated by centrifugation at the convenient moment, washed three times with water and suspended in 100 ml of water. Autolysis

took place in 300 ml Erlenmeyer flasks at 37°C for 24 h under shaking of 80 rpm. Cells were quantified in a Thoma hemocytometer before and after autolysis. After autolysis cells were separated by centrifugation from the autolysate and proteins in the extract were precipitated with 4 volumes of ice cold ethanol at 4°C for 24 h. The precipitate was separated from the ethanolic solution by centrifugation and the pellet was dried under nitrogen. Extracts were stored at -20°C.

Finally one autolysate per strain was produced out of a culture stopped at the beginning of stationary phase and two were made out of cultures stopped at mid-exponential phase.

The protein extracts were analysed in duplicate by SDS PAGE as outlined above, but these extracts had a low solubility in water. Consequently the samples were dissolved in 2 steps in two solutions finally resulting in the sample buffer as outlined above. 1 mg of extract was first dissolved in 0.380 ml of an aqueous solution of 0,166 M Tris-HCl pH 6.8, 53 g/l SDS and 0.05 M DTT incubated 10 min at 95°C and left over night at room temperature. Then 0.62 ml/sample of an aqueous solution containing 400 ml/l glycerol and 200 mg/l bromophenol blue were added. The samples were heated once again at 95°C for 10 min and after cooling down 15 µl of sample were put on the gel. Molecular weight markers were prepared in the same way than the samples and 1 µg of the individual proteins were put on the gel. Gels were stained by colloidal Coomassie Brilliant Blue according to Neuhoff et al. (1988) modified. Gels were fixed in a solution of 400 ml/l ethanol and 100 ml/l acetic acid, washed two times 10 min in water and stained over night or until 24h in a solution of 200 ml/l ethanol, 80 g/l ammonium sulphate, 16 g/l phosphoric acid and 800 mg/l Coomassie Brilliant Blue G 250. Destaining was performed in 10 ml/l acetic acid in water.

2.2 Results

2.2.1 Mutagenesis and first selection of mutants

335 mutants were selected and tested once again on YEPD-BCIP-medium. 50 strains were retained out of the mutants and conserved at -80°C.

2.2.2 Second selection of yeast strains

The 50 yeast strains chosen after mutagenesis and the mother strain were cultivated in EP2-medium having a much higher sugar concentration (144 g/l sugars after inversion), a lower nitrogen concentration (1 g/l) and a lower pH (5.5) than YEPD. This medium was recommended by the yeast producer cooperating with Sofralab (Epernay, France). The cells were grown at 27°C in EP2 medium and were then exposed to a combined shock of heat and change in osmotic pressure by incubation in distilled water at 37°C. 33 of the 50 mutant strains examined showed morphological abnormalities (cf. figure 1 and annex) during and after autolysis in a reproducible way. Figure 1 shows a microscopic picture of strain 19 after 24 hours of autolysis. Cells having several vesicles instead of the typical big vacuole, burst cells, cells with plasmolysis and cells with damages in the cell wall can be seen.

15 out of the 50 strains released more than 500 mg/l proteins (as BSA-standard) quantified according to Lowry when cell concentration in autolysate was set to be at 10^8 cells/ml (cf. annex). Normally cell concentrations during autolysis were between 1x and 3x 10^8 cells / ml. It has to be remarked that in 8 of the 50 mutant strains the difference or standard deviation between the protein concentration of the replicated autolysates was higher than 30 %. 13 of the 50 strains showed abnormal cell morphology and released more than 500 mg/l proteins (cf. above) in a reproducible way and among those the strains releasing the highest protein concentrations were chosen. Finally 8 strains remained which are described in table1.

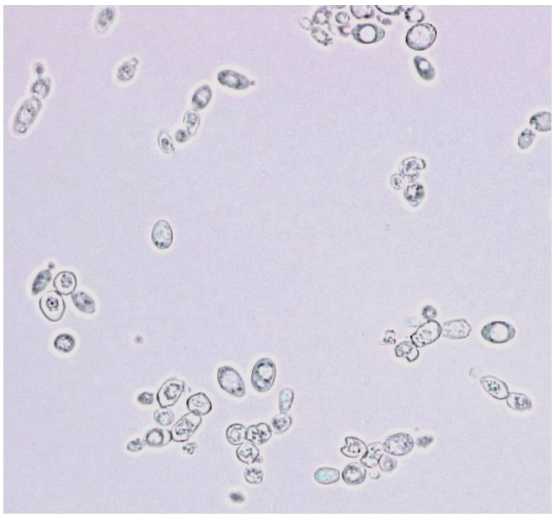

Figure 1: Strain 19 showing morphological abnormalities (enlargement 400)

Table 1: Description of the nine chosen mutant strains

Strain	**Morphology at the end of autolysis**	**mg/l proteins in autolysate ***		**Protein profile SDS PAGE (Zones of molecular mass [kDa])**
		Mean	Standard deviation	
1 (mother strain)	Some cells with small vesicles	402	192	55-50 40 30 20-10
2	Some cells with small vesicles	608	51	50-40 30-25 20 15-3
4	Many cells with deformed cell walls, cells with vesicles	725	167	80 50-40 30 20
14	Deformed cells with many vesicles	732		50 30 20 10-5
19	Cells partly deformed or with small vesicles	799		60-50 40-35 30 15-10
20	Deformed cells, sometimes with vesicles	602	62	50-40 30 20

*protein concentration calculated for an autolysate of a cell concentration of 10^8 cells/ml

Standard deviation is given, when three autolysates were available, otherwise mean is given.

Table 1: continued

Strain	**Morphology at the end of autolysis**	**mg/l proteins in autolysate ***		**Protein profile SDS PAGE (Zones of molecular mass [kDa])**
		Mean	Standard deviation	
21	Cells with vesicles	659	200	50-45 40 30 20
26	Deformed cells	796	183	50 40 30
30	Deformed cells with many vesicles	939		50 40 30 20

The qualitative protein profile of the nine strains showed high similarities presenting fractions of molecular weight between 80 and 10 kDa (kilo Dalton) and mostly having more pronounced fractions between 30 and 50 kDa (cf. table 1). The same amount of protein (220 µg quantified according to Lowry et al. (1951)) was put into the wells of the electrophoresis gel, but the SDS-PAGE profiles pointed out that the concentration of proteins detected by Coomassie Blue differed clearly (cf. figure 2).

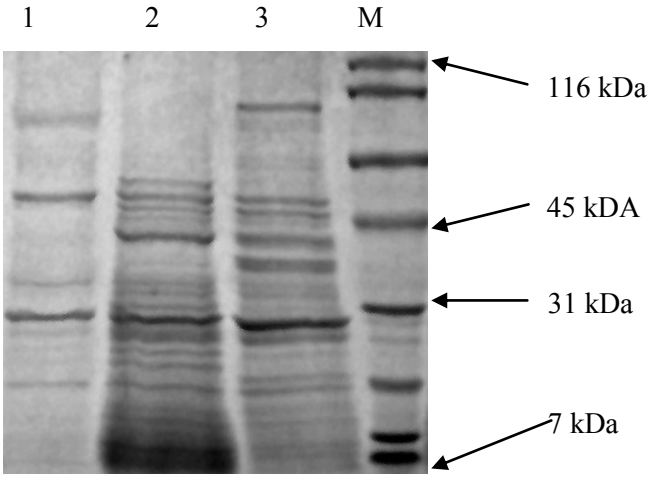

Figure 2: Detail of a SDS-PAGE gel showing autolysate samples of strain 1, 2, 4 (samples 1 to 3) and a molecular mass marker (M)

A densitometric quantification of proteins on SDS-PAGE was tried in case of strains that showed morphological abnormalities and figure 3 shows a comparison between protein quantification by the Lowry method and by densitometry of SDS-PAGE gels for the autolysates. Proteins were quantified in at least two autolysate samples per strain except strain 30 (only one autolysate) by SDS PAGE and densitometry. The reproducibility of the densitometric data of the SDS-PAGE gels was low when protein concentrations in the different autolysates per strain were compared.11 out of the 33 strains examined had a standard deviation or difference between the protein values lower than 30%.
The correlation between the measurements with both methods for the same autolysate was weak (cf. figure 3; $R^2 = 0.192$) and as a result protein release could not be used as single selection criterion.

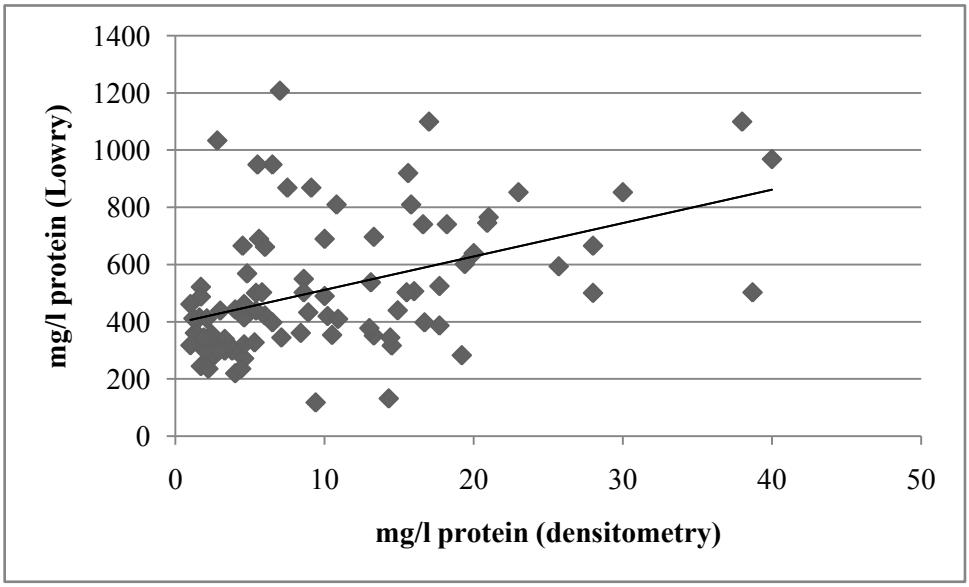

Figure 3: Comparison between protein quantification methods used in autolysates (Results are expressed for an autolysate having 10^8 cells/ml)

The autolytic behaviour and the reduced viability during/after heat shock of the 50 strains used to produce the autolysates were examined. Cultures that were preserved at 4°C on YEPD-agar plates for 15 month (cf. annex) with re-inoculation every four to six weeks or preserved at -80°C were tested. The aim of this testing was to screen the strains for the genetic stability of these two phenotypic traits.
Strain 45 and 51 showed reduced viability and release of alkaline phosphatase in a stable way as cultures of both conservation methods gave a positive result (cf. also annex).
Figures 4 and 5 show examples of the detection of release of alkaline phosphatase by BCIP and of low viability by erythrosin B of yeasts on the plates. Strain 45 was finally chosen as the protein release during autolysis was higher than for strain 51. Strain 31 and 34 showed only reproducibly low viability after heat shock, but in case of 34 the protein release was slightly higher and more reproducible. Strain 34 was thus selected. Finally strain 19 was also taken, because its protein release was high in both quantification methods. The data are presented in table 2.

Table 2: Characterization of the chosen yeast strains

Strain	**Morphology after autolysis**	**Protein concentration [mg/l per 10 ^{8} cells/ml]**		**Qualitative protein profile (zones of molecular mass)**	**Low viability after heat shock**	**Release of alkaline phosphatase after heat shock**
		Lowry	SDS-Page densitometry	SDS-PAGE		
1 (mother strain)	Abnormal	402 ± 192	7.6 ± 5.2	40, 30	no	no
19	Abnormal	799	24.6 ± 4.8	55-45, 30, below 7	no	no
34	Abnormal	536 ± 102	9.1 ± 5.5	40, 25-30, 10	yes	no
45	Abnormal	353 ± 97	10.6	50, 30, 20-10	yes	yes

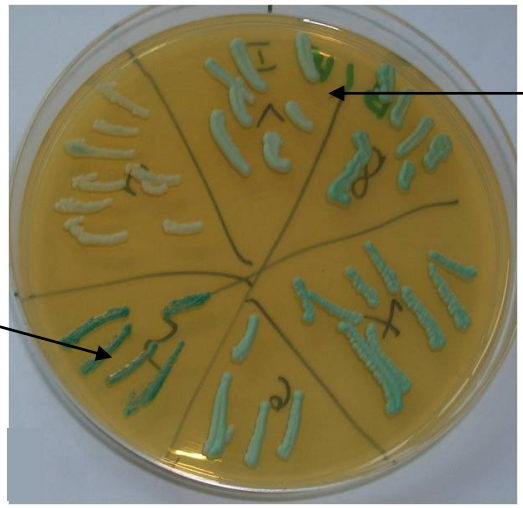

Figure 4: Yeast strains on BCIP medium after 48 h of heat shock (six strains in six sectors)

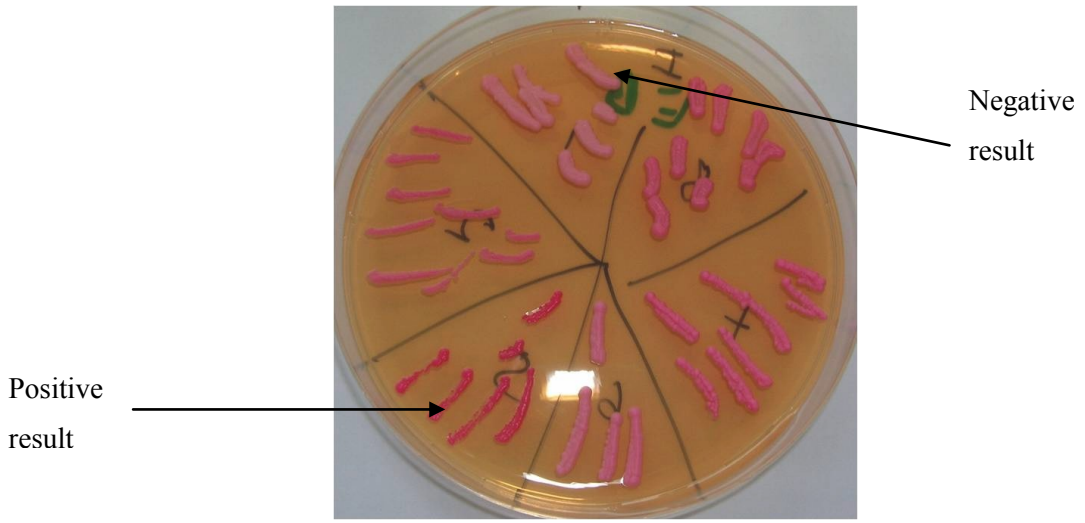

Figure 5: Yeast strains on erythrosine B medium after 48 h of heat shock (six strains in six sectors)

2.2.3 Autolysate of pilot production

Another batch of EP2 had to be used as molasses of the first batch was no more available. The final medium had thus a different composition than the medium used for the second step of the yeast selection. It had a total concentration of sugars of 125 g/l and 2.7 g/l of organic nitrogen and ammonium (instead of 144 g/l sugar and 1 g/l nitrogen of the medium used during the yeast selection). The protein composition of the extracts of yeast cultures submitted to autolysis in the middle of the exponential growth phase and thus produced analogous to the autolysates of the yeast characterization can be seen in figure 6. The SDS-PAGE profile of the mother strain and the strains 19, 34 and 45 showed all similar profiles. The autolysates made during the second yeast selection of strain 1 (mother strain) and 34 did not show protein fractions in a mass range above 40 kDa, detected in the extracts of the pilot production. The yield of extract out of cultures stopped at mid exponential growth phase was fluctuating ranging from 10 mg to 115 mg per 1l of culture and having mainly concentrations of 30 to 50 mg/l culture. That was limiting the use in fining experiments as at least 400 mg per strain would have been necessary.

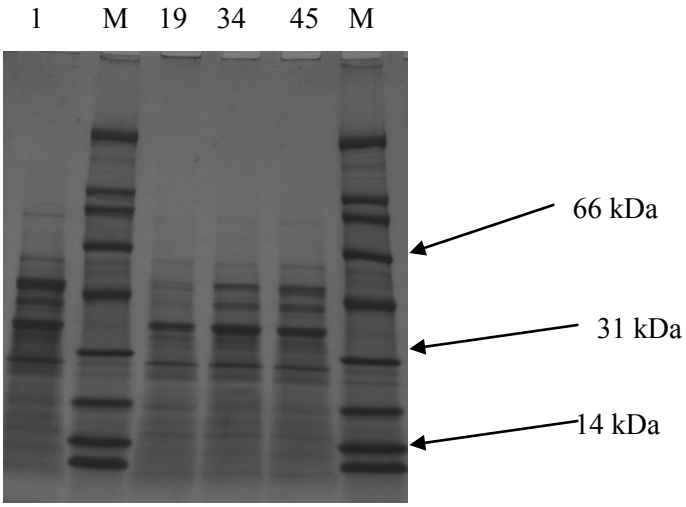

Figure 6: SDS-PAGE profile of extracts of pilot production
(M- molecular marker, 1 etc. numbers of the strains)

2.3 Discussion

The aim of this study was to select yeast mutants that autolysed during heat shock.

The release of alkaline phosphatase was used as indicator of autolysis as done by Cabib and Duran (1975), Giovani, and Rosi (2007), Gonzalez et al. (2003) and Molero et al. (1993). BCIP was used as substrate of alkaline phosphatase in solid YEPD medium. The selection of yeast strains having a positive result on BCIP-medium yielded many strains at first (335) and still 50 strains after a second screening on the same medium. There may be several reasons why so many strains were found. Earlier studies (Giovani and Rosi 2007; Gonzalez et al. 2003) using the same way of mutagenesis and selection of yeast applied a lower radiation intensity to the cells. As a result higher survival rates after UV-treatment were observed what is possibly linked with a lower number of mutations within the genome of the yeast cells. Furthermore a difficulty of the screening on BCIP-medium was that the staining of the colonies was gradual depending on how many cells within a colony were in autolysis and thus releasing phosphatase. This problem of background staining was already described by Gonzalez et al. (2003).

Abnormal morphology of yeast mutant cells in autolysis was observed in 66 % of the selected strains. Such abnormalities, like cells with small vesicles that could be autophagsomes (Martinez-Rodriguez et al. 2001a), and cells with plasmolysis, were also observed in the studies of Giovani and Rosi (2007) and Gonzalez et al. (2003) and in a work on yeast autolysis in sparkling wine or sparkling wine like conditions (Martinez-Rodriguez et al. 2001a). Striking morphology could only be one of the indicators of “interesting” mutants as the mother strain also showed cells with small vesicles at the end of autolysis, but in a small proportion. It was possible to select 8 mutants that had a striking morphology and released high protein concentrations detected by the method of Lowry et al. (1951).

The analysis of autolysates of these mutants on SDS-PAGE gels showed that a high protein release observed with the method of Lowry et al. (1951) did not coincide with a high protein concentration detected by densitometry of protein bands stained with Coomassie Blue. That can be due to different staining principles of both protein quantification methods. The Folin reagent used in the method of Lowry et al. (1951) is quite specific to residues of aromatic amino acids in proteins and nucleic acids can interfere in the detection. Nucleic acids have been detected in yeast autolysates (Hernawan and Fleet 1995; Kollar et al. 1993; Vosti and Joslyn 1954; Zhao and Fleet 2003, 2005), but nucleotides that did not interfere with the reagents of Lowry et al. (1951) prevailed (Zhao and Fleet 2003, 2005) among the degradation products of cell’s DNA and RNA. Coomassie Brilliant Blue is mainly specific for residues of arginine in proteins and polypeptides of a molecular mass higher than 3000 Da (Compton, Jones 1985; Sedmak, Grossberg 1977). Furthermore the protein quantification by the method of Lowry and by SDS-PAGE and densitometry of the extracts of small scale autolysis was referred to the cell concentration counted in the hemocytometer. This could cause problems as cells of different strains seemed to differ slightly in cell diameter (results not shown), which would mean a difference in cell mass put into autolysis if same cell number was applied. It might have been more

precise to measure the cell dry mass that was put to autolysis in each case, but there was not enough cell mass to perform this. Polypeptides seem to constitute an important part of the nitrogen compounds released during autolysis in a wine-like medium (Martinez-Rodriguez and Polo 2000; Martinez-Rodriguez et al. 2001b; Perrot et al. 2002; Guilloux-Benatier and Chassagne 2003). Polypeptides could also be a part of the nitrogen compounds released by the yeast strains of this study during autolysis. These compounds could react with the reagents of the Lowry method and be detected as proteins. Polypeptides below 7 kDa will not be separated and retained on the SDS-PAGE gels and can thus not be detected by this method. That could partly explain the differences in protein concentrations observed by the two quantification methods used herein.

The yeast strains selected in the first cycle (selection of eight mutants) and second cycle (final selection of three mutants) of this work as well as the mother strain showed defined protein fractions of molecular masses between 55 and 10 kDa in both cases. It should be pointed out that the protein profile of the autolysate of small scale autolysis (second selection cycle) and of pilot production were similar. That is striking as the concentration of protein put on SDS-gel measured according to Lowry is in case of the autolysate of small scale production higher (220 µg protein/well) than in the autolysate of pilot scale production (15 µg precipitate per well). This could be explained partly by the problem of the protein quantification according to Lowry as mentioned above.

Proteins of the autolysates of the three strains and the mother strain of the pilot production were precipitated by ethanol and not concentrated by lyophilisation due to practical limitations. That could modify the protein composition, which was however not detected on the SDS-PAGE gels in this study. A study on blood platelets confirmed that ethanol precipitation did not modify qualitative presence of protein fractions of different molecular masses on SDS-PAGE gels (Zellner et al. 2005).

Unfortunately the protein precipitates of the four autolysates of the pilot production showed a low solubility when treated as the lyophilized autolysates of the small scale autolysis. Consequently, the proteins had to be dissolved in different conditions as stated in material and method. That could modify the qualitative composition of proteins of different molecular mass, but no alteration of the proteins used in the standard for detection of molecular masses, which was treated as the samples, was found. The proteolytic activity in the yeast cells of the strains chosen in this work seemed to be restricted during autolysis as protein fractions of defined masses were found. On the other hand the temperature conditions of autolysis used were close to those ideal for proteolysis in the yeast cell (Hernawan and Fleet 1995; Kollar et al. 1993; Vosti and Joslyn 1954). Other conditions to which the yeast cells were exposed during autolysis in this study, e.g. heat, starvation could also increase cell's overall proteolytic activity according to Achstetter and Wolf (1985) Hilt and Wolf (1992), Sorokin et al. (2009) and van den Hazel et al. (1996). The washing of the yeast cells before inducing autolysis by heat shock and by change of osmotic pressure as done in this study will deplete the mineral concentrations of the autolysis medium surrounding the yeast cells and perhaps partly in the cells themselves when cell membrane selectivity exists no more. This fact could inhibit the activity of

metalloproteases, which constitute a part of yeast cell's proteolytic activity (Achstetter and Wolf 1985). Some protein fractions detected by SDS-PAGE may be a least partially glycosylated. Glycoproteins could be released from the yeast cells during autolysis as they are present in different cell compartments like the vacuole and the cell wall as reviewed by Achstetter and Wolf (1985) and Klis et al. (2002). Changes in cell walls of yeasts in autolysis as observed by Charpentier et al. (1986), Martinez-Rodriguez et al. (2001b) and Troton et al. (1989) and also in this study could lead to a release of glucanes or glycosylated proteins in the autolysis medium. Saccharides were indeed detected in yeast extracts produced by autolysis in the studies of Hernawan and Fleet (1995) and Kollar et al. (1993). These authors did not investigate if glycoproteins were part of the saccharides which they quantified. Yeast mutants showing autolysis during heat shock also released more glycoproteins than the mother strain in the work of Giovani and Rosi (2007). Glycosylation may confer more resistance to a protein against enzymatic break down by rendering the sites of attack less accessible to the proteases. A higher resistance of glycoproteins against proteolysis was shown in the studies of Walsh and Chapman (1991) in case of desmosomes in epidermis of pig ear and of Puccia and Travassos (1991) in case of pathogenic fungus *Paracoccidioides brasiliensis*. The detection of glycoproteins in the autolysates of the yeast strains selected in this work was not yet possible in this step of the work of the thesis.

The test of stability of the autolytic phenotype as detected by the release of alkaline phosphatase on the BCIP-medium showed that at maximum 10 % of the strains showed a "very positive" reaction after one year of culture on agar plates. That could be due to the fact that *Saccharomyces* strains are naturally not haploid and as a result UV-mutagenesis could only modify some of all the allels of a corresponding gene. Giovani and Rosi (2007) used haploid yeast spores for UV-mutagenesis to overcome that problem. That was not done in the present study as the mother strain showed a high sensitivity to UV radiation, which was a sign of profound mutations in the cell genome and as in the first screening steps many mutants showing the autolytic phenotype could be selected. The gradual development of blue colour by alkaline phosphatase as mentioned above constituted another difficulty when judging the stability of the autolytic trait of the yeast mutants. The screening of low viability of the yeast mutants according to Bonneu et al. (1991) was integrated in the final steps of mutant selection of this study. Cell death precedes cell autolysis as stated by Babayan and Bezrukov (1985). The coincidence of increase of cell mortality with cell autolysis was confirmed by Vosti and Joslyn (1954) and Hernawan and Fleet (1995). Bonneu et al. (1991) described no problem of background staining at the erythrosine B concentration used in this work and stated that a dark pink staining of a yeast colony on erythrosine B medium was related to mortality level of 50 to 60 %. Strains 34 and 45 finally selected in the present study showed dark pink staining and stability of this trait.

3 Thesis Part II: Fining of must and wines with yeast protein extracts

3.1 Material and methods

3.1.1 Chemical substances

All substances and compounds used were of analytical grade unless otherwise specified.
Water purified in a Genpure-CAD-apparatus of TKA (part of Thermo Electron LED, Niederelbert, Germany) was used for all analyses. Ethanol and sodium hydroxide were from Carl Roth (Karlsruhe, Germany). Sulphuric acid, hydrochloric acid 37 % fuming and sodium disulphite were delivered by Merck (Darmstadt, Germany). Polyvinyl-polypyrrolidone (PVPP) was purchased at Applichem (Darmstadt, Germany). L-tartaric acid and bovine serum albumin (BSA, Sigma reference number A 7906), were obtained from Sigma-Aldrich Chemie (Munich, Germany).

3.1.2 General wine analysis

Concentrations of alcohol and reducing sugars were determined by FTIR (Fourier Transform Infrared Spectroscopy) according to Patz et al. (1999) with a WineScan^{TM} FT 2 of FOSS (Relingen, Germany).
pH was measured by potentiometry in a pH-meter of WTW (Wissenschaftlich-Technische Werkstätten, Weilheim, Germany). Organic acids were quantified by HPLC (High Performance Liquid Chromatography) according to Schneider et al. (1987) as modified by Sponholz et al. (unpublished). An Agilent 1100 Series system (Agilent Technologies, Böblingen, Germany) equipped with an Allure Organic Acids column of Restek (Bad Homburg, Germany) and a multi-wavelength-detector was used. 5 µl of sample were injected and elution was performed at a flow rate of 0.6 ml in isocratic mode with 5 ml/l ethanol and 0.139 ml/l H_2SO_4 at 46°C and acids were detected at 210 nm.

3.1.3 Spectrophotometric measurements

These analyses were performed in a Unicam UV 300 spectrophotometer (Thermo Fisher Scientific, Dreieich, Germany).

3.1.4 Colour measurements of white wines and musts

The spectrum of light at wavelengths visible by the human eye shows no characteristic maximum in case of white wines and musts. The absorption of light at 420 nm gives yet an estimation of the intensity of the yellow colour of white wines (Ribéreau-Gayon et al. 2004b pp.253-255) and will be expressed as absorbance at 1 cm path length.

3.1.5 Colour measurements of red wines

The colour parameters according to Glories (1984) were determined by measuring absorbance at the wavelengths of 420 nm, 520 nm and 620 nm at 1 mm path length.
Colour intensity is the sum of absorbance 420 nm, 520 nm and 620 nm, which will be calculated and expressed for a wavelength of 1 cm.

Hue is the quotient of 420 nm / 520 nm expressed as abstract index and gives an indication of the nuance of colour of the wine. A high hue characterizes wines with a brownish-red colour, a low hue characterizes wines with an intensely red to bluish-red colour.

PVPP-index was determined according to Glories (1984) and is the proportion of anthocyanins combined with tannins of all the anthocyanins quantified according to Ribéreau-Gayon and Stonestreet (1965).This method is based on bleaching anthocyanins with sulphur dioxide.

$$\text{PVPP-index} = \frac{\text{(Concentration of total anthocyanins) – (Concentration of free anthocyanins)}}{\text{Concentration of total anthocyanins}}$$

3.1.6 Polyphenol measurements of white wines and musts

Absorbance at 280 nm, the maximum absorbance of polyphenols of flavonoid type, and absorbance at 320 nm, characteristic of derivatives of cinnamic acid, was determined as recommended by Somers and Ziemelis (1985). It has to be mentioned that derivatives of cinnamic acid also absorb light at 280 nm. Must or wines were diluted to have absorbance values at 1 cm path length of maximum 1. No corrections for non-phenolic compounds contributing to absorbance at the wavelengths 280 nm and 320 nm were made and values corrected for dilution and expressed for a path length of 1 cm are given.

3.1.7 Polyphenol measurements of red wines

Absorbance at 280 nm was used as index of total polyphenols as proposed by Somers and Ziemelis (1985). Polyphenols of flavonoid type have the absorption maxima around 280 nm and are predominant in red wines (Somers and Ziemelis 1985). Red wines were diluted with water to have an absorbance below 1 at 280 nm at 1 cm path length, but the index is corrected for the dilution.

Reactivity of polyphenols with proteins was estimated by the BSA-index based on the studies of de Freitas (1995) and de Freitas et al. (2003) using BSA (bovine serum albumin) as reference. Red wine was diluted to 1/50 with a synthetic wine solution (120 ml/l ethanol, 5 g/l tartaric acid, pH 3.2) and a solution of 0.4 g/l BSA was added to a final concentration of 30 mg/l. After 45 minutes the turbidity as NTU (nephelometric turbidity units) was measured in a nephelometer (Nephla of Hach Lange, Düsseldorf, Germany). The diluted wine served as blank

$$\text{BSA-index} = \frac{\text{(NTU of diluted wine with BSA) – (NTU of diluted wine)}}{0.4}$$

3.1.8 Turbidity of musts and wines

All turbidity measurements were performed in the nephelometer mentioned above.

3.1.9 Sensory analysis

Red wine samples were tasted four to eight weeks after the fining by panellists experienced in wine tasting. Triangle test was used to discriminate the wines fined with different fining agents from a non-fined control sample. The test was evaluated according to DIN 10951.

The wines were in some cases tasted by experienced panellists without doing discriminative triangle test, but recording their remarks.

3.1.10 Fining experiments

The fining experiments were done in six cycles cf. below. Fining products used are described in table 3. All products were provided by Oenofrance (Bordeaux, France; part of the Sofralab corporation, Epernay, France), except yeast products 1 and 2. Remarks concerning the strains are outlined below (cf. cycle 2).

Table 3: Products used for fining in all 6 cycles (YPE- yeast protein extract)

Product	**Description**	**Form**	**Used in cycles**
YPE 1	YPE useable for fining	Powder	1 to 5
YPE 2	Commercial yeast extract	Powder	1
YPE 3	Commercial yeast extract	Powder	1
YPE 4	Commercial yeast extract	Powder	1
Tannin	Tannin out of chestnut	Powder	1 to 5
Isinglass		Flakes	1 and 2
Yeast product 1	Partly soluble yeast product	Powder	1
Yeast product 2	Yeast cell wall product	Powder	1
Gelatine	out of pork skin, enzymatically hydrolyzed 10 – 12 % dry mass	Liquid	1 to 6
YPE A1	First production method from strain A	Powder	2 and 3
YPE B1	First production method from strain B	Powder	2
YPE C1	First production method from strain C	Powder	2
Casein	soluble	Powder	2
YPE A 2.1.	Second production method from strain A, 1st charge	Powder	3
YE A 2.2.	Second production method from strain A, 2nd charge	Powder	3 and 4
YPE B2	Second production method from strain B	Powder	3

Table 3: continued

Product	Description	Form	Used in cycles
YPE C2	Second production method from strain C	Powder	3
YPE A3	Third production method from strain A	Powder	4 and 5
YPE B3	Third production method from strain B	Powder	4
YPE C3	Third production method from strain C	Powder	4
Silica gel	20 to 31 % of dry mass SiO_2	Suspension	5
YPE A4	4^{th} production method from strain A; 2 % dry mass	Liquid	5
YPE 5		Powder	5
YPE 6	4 % dry mass	Liquid	5
YPE 7		Powder	5
YPE 8	YPE produced at industrial scale and commercialized	Powder	6

3.1.10.1 Cycle 1

The experiments were carried out with musts and wines of the harvest 2009.

At that moment no protein extracts of the three yeast strains selected in the first part of the work could be produced as yeast selection was not yet finished.

3.1.10.2 White must of variety Arnsburger (cycle 1)

The must was treated with 80 mg/l of SO_2 (sulphur dioxide), stored 20 h at 17 to 20 °C to allow pectin hydrolysis by endogenous enzymes and racked of the lees formed after this storage. Fining was performed at 20 °C was performed after racking. All fining products were supplied by Oenofrance (Bordeaux, France). Four yeast protein extracts (YPE 1 to 4) and isinglass were used. YPE 1 represented a yeast extract developed by Oenofrance that had already proven to be an alternative fining product. The production process of YPE 1 was long and complex and the product contained essentially polypeptides of masses below 7 kDa (cf. part III of the thesis). YPE 2, 3 and 4 were commercial yeast extracts provided by a company cooperating with Sofralab/Oenofrance. The YPE

were used in combination with a tannin preparation out of chestnut (tannin) (cf. details in table 4). Isinglass was used as reference fining product. The control treatment did not receive any fining agent.
The YPE and tannin were dissolved in water at a concentration of 20 g/l and tannin was always added to the must before the protein fining agent. Isinglass was prepared according to the instructions of Oenofrance and a solution of a concentration of 12.5 g/l was used.
The experiment was performed in measuring cylinders of PMP (polymethyl pentene) of a volume of 250 ml at 20°C and fining was observed for two days. Each variant was repeated once and must was racked from the fining lees after two days and before doing the analyses.

Table 4: Fining scheme of must of cycle 1

Variant	**Tannin concentration [g/hl]**	**Concentration of protein product [g/hl]**
YPE 1	10	10
YPE 2 or 3 or 4	10	5
YPE 2 or 3 or 4	10	10
YPE 2 or 3 or 4	10	20
Isinglass	None	2.5
Control	None	None

3.1.10.3 Cuvee of white wines (cycle 1)

The concentration of free SO_2 of the wine was adjusted to 45 mg/l before fining to avoid oxidation of wine during fining. The same fining scheme was used as for the white must, but the variants:

- Yeast product 1 at 20 g/hl
- Gelatine at 100 ml/hl

were added. The fining was performed at 20 °C. Yeast product 1 was from an alternative producer of wine fining agents and should clarify white wines. The gelatine used was in liquid form
(100 to 120 g/l dry mass) and derived from pork skin. Gelatine and yeast product 1 were used in combination with 10 g/hl tannin (cf. YPE). The gelatine was applied without dilution in water and yeast product 1 was prepared in the same way as the YPE. The experiment was performed in measuring cylinders of a volume of 250 ml (cf. trials with must) and variants were repeated once.
The fining was observed for 5 days, as clarification was not finished after 2 days and wine was then racked from the fining lees and analysed.

3.1.10.4 Fining of red wines (cycle 1)

A red wine of the variety Pinot noir and one of the variety Cabernet Sauvignon were used. Both wines had performed malolactic fermentation. Experiments were carried out in plastic cylinders (cf. above) with one repetition to determine the optimum concentration for an experiment in 1.5 l bottles

(explanation under results). These experiments were performed for two days and at a temperature of 20°C. Fining schemes are described in tables 5, 6 and 7. YPE 1, 2, 3 and 4 already described above were used dissolved in water at a concentration of 50 g/l. Yeast product 1 already described and yeast product 2 of the same producer were used in the experiments in 1.5 l bottles at a concentration of 20 g/hl. Yeast product 2 was a preparation of yeast cell walls that should clarify red wines. The gelatine used already in the former fining experiments served as reference product for fining of red wine. The final experiment in 1.5 l bottles was done in triplicate at 20°C and fining was observed for three days because of the bigger height of the bottles compared to the measuring cylinders and wine was racked from the lees before the analyses.

Table 5: Fining scheme of red wines – experiments in cylinders of cycle 1

Variant	**Concentration of protein product [g/hl] or [ml/hl]**
YPE 1 30	30 g/hl
YPE 1 50	50 g/hl
YPE 2 or 3 or 4 20	20 g/hl
YPE 2 or 3or 4 30	30 g/hl
YPE 2 or 3 or 4 50	50 g/hl
Gelatine	100 ml/hl (as in liquid form)
Control	None

Table 6: Fining scheme of Pinot noir – final experiment in bottles – cycle 1

Variant	**Concentration of protein product [g/hl] or [ml/hl]**
YPE 1	30 g/hl
YPE 3	30 g/hl
YPE 4	50 g/hl
Yeast product 1	20 g/hl
Yeast product 2	20 g/hl
Gelatine	100 ml/hl
Control	None

Table 7: Fining scheme of Cabernet Sauvignon – final experiment in bottles – cycle 1

Variant	**Concentration of protein product [g/hl] or [ml/hl]**
YPE 1	50 g/hl
YPE 3	50 g/hl
Gelatine	100 ml/hl
Control	None

3.1.10.5 Cycle 2

The experiments were done with musts and wine of vintage 2010.

The three yeast strains selected in part 1 of the experiments of the thesis were used to produce YPE at pilot scale at an external company cooperating with Sofralab. The selected yeast strains 19, 34 and 45 will be coded A, B, C and the YPE of this first production cycle will then be:

A1 (of strain 19), B1 (of strain 34) and C1 (of strain 45)

3.1.10.6 Experiment with Riesling must (cycle 2)

Whole bunches of grapes were crushed and pressed in order to obtain a must rich in polyphenols.

80 mg/l of SO_2 were added to the must after pressing and must was stored for 20 hours at 17°C to allow pectin hydrolysis by endogenous enzymes. The must was racked from these first lees and fined.

Isinglass and soluble casein, a mixture of 80 – 85 % of mass of casein and 15-20 % of mass of potassium carbonate, were provided by Oenofrance and used as references. Furthermore YPE 1 (cf. cycle1), YPE A1 and B1 were used. The YPE and casein were dissolved in water at 50 g/hl.

Isinglass was prepared according to the supplier and used in solution of 6.25 g/l.

The concentrations used are reported in table 8 and fining was performed at 20°C in triplicate and observed for two days. YPE C1 was delivered too late to integrate it in must fining.

Table 8: Fining of Riesling must (cycle 2)

Variant	**Concentration of protein product [g/hl] or [ml/hl]**
Isinglass	2.5 g/hl
Casein 25	25 g/hl
Casein 40	40 g/hl
YPE 1 or A1 or B1 10	10 g/hl
YPE1 or A1 or B1 30	30 g/hl
Control	None

3.1.10.7 Experiment with Riesling wine (cycle 2)

The must already used in the fining experiment with Riesling must was racked from its lees after 20 hours of static sedimentation in presence of 80 mg/l SO_2. Fermentation was initiated by inoculation with commercial yeast strain Levuline CHP (a strain of *Saccharomyces cerevsisiae* of Oenofrance, Bordeaux, France) at 20 g/hl as outlined by the supplier. Three days after inoculation with yeast 30 g/hl diammonium phosphate and 60 mg/l thiamine were added to facilitate onset of fermentation by giving supplementary nutrient and vitamine. Seven days after yeast inoculation fermentation had not started, but it could then be started by a second inoculation with the same yeast strain at 30 g/hl. Yeasts were adapted two days to the Riesling must in two steps starting with a water must mixture of 1:1 for one day and a mixture of 1:3 for one day resulting in a volume of 5 % of total must volume before the second inoculation. Alcoholic fermentation was completed after 18 days and wine was racked from yeast lees and treated with SO_2 to obtain a concentration of free SO_2 between 30 mg/l and 45 mg/l. Fining was performed five days thereafter and under the same conditions as in the must in triplicate. The same protein products and concentrations were used as in the must experiment and YPE C1 was also used at 10 and 30 g/hl. The time of fining was 3 days to allow a complete settlement of the lees.

3.1.10.8 Experiment with a cuvee of German red wines (cycle2)

A cuvee of different red vine varieties of the Department of Grapevine Breeding of Hochschule Geisenheim University was used. The wine was dry and had completed malolactic fermentation.

The YPE A1, B1 and C1 of the three selected strains, YPE 1 and gelatine as reference (cf. cycle1) were used. All YPE were dissolved in water at a concentration of 50 g/l. The liquid gelatine (cf. cycle1) was used as supplied. The experiment was done in triplicate in the plastic cylinders already used in the other experiments at 20°C and fining was observed for two days. The fining scheme is described in table 9.

Table 9: Fining of red wine cuvee (cycle 2)

Variant	**Concentration of protein product [g/hl] or [ml/hl]**
Gelatine	100 ml/hl
YPE 1 or A1 or B1 or C1 30	30 g/hl
YPE1 or A1 or B1 or C1 50	50 g/hl
Control	none

3.1.10.9 Cycle 3: Fining of red wines:

The YPE A1, B1 and C1 had not yet shown suitability as fining agents (cf. thesis part II results of cycle 2). The three yeast strains selected in part I of the thesis were used to manufacture again YPE at pilot scale but with a different production technique at an external company cooperating with Sofralab. These YPE were named:

A 2.1. and A 2.2., B 2 and C 2,

the letters A etc. being the identity of the strains like in cycle 2 and the number 2 signifies the second production cycle. Two charges of YPE of strain A, i.e. 2.1. and 2.2 were produced.

These YPE were tested in fining of a cuvee of red wines which was composed of 80 % of the red wine used in cycle 2 and of 20 % of red wines of vintage 2009. This wine could be fined with the gelatine serving also as reference treatment in the former fining cycles. YPE 1 and YPE A1 already used in cycles 1 and 2 were again tested in this wine.YPE and gelatine were also combined with the tannin already used in cycle 1. If tannin was used for fining, it would be added to the wine before gelatine or YPE. The fining was done in measuring cylinders of glass with a volume of 250 ml in triplicate at 20°C. Tannin and YPE were dissolved in water at 50 g/l and gelatine was used as supplied.

Four experiments had to be performed due to the high number of fining variants. A control variant was integrated in all experiments to judge a possible evolution of the wine.

The first experiment (YPE at 30 g/hl) was observed for two days before racking, but in the later experiments fining was terminated after three days. Two days of fining proved to be a too short period to allow complete sedimentation of the precipitate of fining. The concentrations of all fining agents and their combinations are described in table 10. YPE A1 and A2.1. were also used in a concentration of 70 g/hl combined with tannin as the variants with a concentration of 50 g/h YPE did not show formation of a deposit in the majority of the replicates.

The YPE allowing successful clarification of wine were then used in a final experiment in the same conditions than the other experiments of this cycle.

The following YPE were selected:

YPE 1 at 30 g/hl, YPE A 2.2. at 30 g/hl, YPE B 2 at 30 g/hl and YPE C 2 at 30 g/h.l

Table 10: Fining of red wine cuvee in cycle 3

Variant	**Concentration of tannin [g/hl]**	**Concentration of protein product [g/hl] or [ml/hl]**	**Abbreviation**
Gelatine	None	100 ml/hl	Gelatine
Gelatine T	5 g/hl	100 ml/hl	Gelatine T
YPE 1or YPE A 1 YPE A 2.1. YPE A 2.2. YPE B 2 YPE C 2	None	30 g/hl	YPE 1 etc. 30
YPE 1or YPE A 1 YPE A 2.1. YPE A 2.2. YPE B 2 YPE C 2	15 g/hl	30 g/hl	YPE 1 etc. 30T
YPE 1or YPE A 1 YPE A 2.1. YPE A 2.2. YPE B 2 YPE C 2	None	50 g/hl	YPE 1 etc. 50
YPE 1or YPE A 1 YPE A 2.1. YPE A 2.2. YPE B 2 YPE C 2	15 g/hl	50 g/hl	YPE 1 etc. 50T
YPE A1 or YPE A2.1.	15 g/hl	70 g/hl	YPE A1 etc. 70T
Control	None	None	None

3.1.10.10 Cycle 4: Fining of red wines:

Two red wines were used in this cycle. One was of the vine variety Syrah and one of the variety Rondo. Both wines had completed alcoholic and malolactic fermentation.

YPE 1 that proved to be an effective fining agent in former experiments with red wine did not precipitate in the red wine of Rondo without a preliminary treatment with a pectolytic enzyme preparation. That was why the red wine of Rondo was treated with a pectolytic enzyme at the highest concentration recommended by the supplier (Oenofrance, Bordeaux, France) for three days before fining. A new production cycle of YPE at pilot scale with the three yeast strains selected in part I was done by a manufacturing technique different from that of the YPE A 2 etc. This new technique allowed production of YPE A3, B3 and C3 with proteins of higher molecular mass than that found in the YPE of the strains produced before (cf. part III of this thesis). Gelatine was again used as reference and YPE 1, YPE A 2.2. and A3 were used in both wines in the concentration reported in table 10.The wine of Syrah was also fined with YPE B3 and C3, but these extracts had an inferior fining performance and were thus not used in the wine of Rondo treated later. Furthermore the mass of YPE B3 and C3 was also too small to test it completely in a second wine. Fining took two days in case of fining with gelatine and Oenolysat and three days in case of the other YPE as in every case at least 12 hours without visual decrease in turbidity and increase in the volume of fining lees was waited for. Fining was performed in glass cylinders of a volume of 100 ml at 20°C in triplicate and fining scheme is described in table11. Tannin and the YPE were dissolved in water at 50 g/l and gelatine was used as supplied. Four experiments were done for Syrah and 2 for Rondo and every experiment contained a control variant.

Table 11: Fining of red wines in cycle 4

Variant	**Concentration of tannin [g/hl]**	**Concentration of protein product [g/hl] or [ml/hl]**
Gelatine	None	100 ml/hl
Gelatine T	5 g/hl	100 ml/hl
YPE 30	None	30 g/hl
YPE 30T	15 g/hl	30 g/hl
YPE 50	None	50 g/hl
YPE 50T	15 g/hl	50 g/hl
YPE 70T	15 g/hl	70 g/hl
Control	None	None

3.1.10.11 Cycle 5: Fining of red wine cuvee

A cuvee of four red wines of the four varieties Syrah, Pinot noir, Rondo and Bolero was used.

Fining took place in glass cylinders of a volume of 100 ml already used in cycle 4 and was done in triplicate. Fining was observed and performed for three days at 20°C and wine was then racked.

The gelatine used in the former cycles served as reference.YPE 1 and YPE A3 that proved to be successful in red wine clarification were again used. New EPL of other yeast strains and production technologies provided by the yeast company cooperating with Sofralab were now also tested.

They will be named:

YPE A 4 (4th production cycle of YPE of strain A), YPE 5, YPE 6, YPE 7

Gelatine and the YPE were tested pure, combined with the tannin (T) used in all former cycles and were also combined with the fining aid silica gel (Si) at 30 % m/v of Oenofrance (Bordeaux, France) as described in table 11. Tannin and the YPE were dissolved in water at 20 g/l. Gelatine and silica gel were used as supplied. The fining was done with two repetitions of each variant (table 12) in six experiments and every experiment contained a control variant. The six experiments were done in cylinders measuring 100 ml at 20°C for three days.

Table 12: Fining experiments of in measuring cylinders red wines in cycle 5

Variant	**Concentration of tannin [g/hl]**	**Concentration of Silica gel [ml/hl]**	**Concentration of protein product [g/hl] or [ml/hl]**
Gelatine	None	None	100 ml/hl
Gelatine T	5 g/hl	None	100 ml/hl
Gelatine Si	None	40 ml/hl	100 ml/hl
YPE 30	None	None	30 g/hl
YPE 30T	15 g/hl	None	30 g/hl
YPE 30 Si	None	40 ml/hl	30 g/hl
YPE 50	None	None	50 g/hl
YPE 50 T	15 g/hl	None	50 g/hl
YPE 50 Si	None	40 ml/hl	50 g/hl
YPE 70 T	15 g/hl	None	70 g/hl
YPE 70 Si	None	40 ml/hl	70 g/hl
Control	None	None	None

Gelatine and all YPE were also tested in trials of bigger volumes for sensory analyses and finer chemical analyses. The lowest concentration of gelatine or YPE allowing a successful clarification was chosen. The fining aids tannin and silica gel were not retained as they did not improve speed of

clarification and limpidity of the wines after fining. Gelatine and the YPE 1, A3, 5 and 6 as well as a control were used for fining red wine in carboys of 5l in triplicate for three days at 20°C (cf. table 13) YPE 7 arrived later as the other YPE and was tested in a trial in bottles of a volume of 0.75 l in triplicate at 20°C. In fact the wine volume left was in fact too small to perform an experiment in carboys. In that latter trial gelatine and a control were also integrated (cf. table 14).

Table 13: Fining scheme of experiment in carboys (cycle 5)

Fining product	**Concentration**
Gelatine	100 ml/hl
YPE 1	30 g/hl
YPE A 3	50 g/hl
YPE 5	30 g/hl
YPE 6	30 g/hl
Control	None

Table 14: Fining scheme of experiment in 0.75 l bottles (cycle 5)

Fining product	**Concentration**
Gelatine	100 ml/hl
YPE 7	30 g/hl
Control	None

3.1.10.12 Cycle 6: Fining experiment with commercial YPE 8

The research of this thesis took part in the development of the commercial YPE 8 commercialized by Oenofrance (Bordeaux, France). This product was tested without fining aids in the following concentrations:

5 g/hl, 10 g/hl, 20 g/hl and 40 g/hl

in red wines of three vintages (2011, 2012 and 2013). The red wines were fined after different periods of storage, after 18 month in case of Dornfelder of 2011, 6 month in case of cuvee of vintage 2012 and after 2 month of storage in case of cuvee 2013. All wines were fined after malolactic fermentation and microbial and chemical stabilization with SO_2. Gelatine at 100 ml/hl served as a reference and a control variant was also integrated in every experiment. The fining trials were performed in triplicate in 100 ml cylinders of glass at 20 °C for three days. YPE was dissolved at 20 g/l in water, gelatine was used as supplied. An experiment in carboys of 10 l was also performed with the variants control, YPE 8 at 20 g/hl and at 40 g/hl with cuvee 2012 and with 10 g/hl YPE 8, 100 ml/hl gelatine and control with cuvee 2013.

3.2 Results

3.2.1 Fining experiments of cycle 1

3.2.1.1 Fining of white must of the variety Arnsburger

The must had a density of 1077 g/l, contained 180 g/l reducing sugars and presented a pH of 3.2.
Isinglass, used as a reference fining agent, was the only variant showing immediate formation of visible flakes. All variants in which YPE were used for fining and the control variant showed flocculation and settlement of lees at 2 hours of fining. The volumes of lees at the end of fining (after 48 hours) were 1.6 ml/100 ml in all variants except must fined with isinglass (2.4 ml/100 ml).
The turbidity of the must fined with the YPE was not decreased compared to the control (values of 67 to 69 NTU) and must treated with isinglass had 53 to 58 NTU. Clarification of the must was thus not increased by fining with YPE. Fining with YPE which was combined with a preliminary addition of the tannin preparation increased absorbance at 420 nm (specific of yellow colour) by 10 % compared to the control (absorbance at 420 nm was 0.21). Absorbance at 280 nm (specific of aromatic rings) was increased by combined fining with tannin and YPE by 15 to 30 % compared to the control (absorbance at 280 nm of 6). It has to be mentioned, that the indices of polyphenols in white must, white and red wine (absorbance at 280 nm and 320 nm) will usually have values much higher than 1. The absorbance was thus measured in diluted samples and values were corrected for the dilution.

3.2.1.2 Fining of a cuvee of white wine

The wine had an alcohol concentration of 13.2 ml/100 ml, 5 g/l of residual reducing sugars and a pH 3.3. The clarification of the wine was slow and gradual showing more limpid zones on top of the cylinders. No visual improvement as judged by eye was observed between 4 and 5 days and consequently wine was racked from the lees after 5 days of fining. Wine fined with isinglass showed a formation of visible flakes after 4 hours and wine treated with combinations of tannin and gelatine or of tannin and YPE1 also showed flocculation observable by eye after 9 hours. Settlement of lees was observed at 9 hours of fining in the variants isinglass, gelatine combined with tannin, YPE 1 and tannin and YPE 3 and 4 in all concentrations when combined with tannin. 0.8 ml/100 ml of lees were found at the end of fining (after 5 days) in all variants including the control. Turbidity of the control wine was 15 to 19 NTU. A clear wine will have a turbidity of below 10 NTU. Fining with YPE 3 at 5 g/hl or 20 g/hl combined with tannin or YPE 4 at 20 g/hl combined with tannin diminished the turbidity of the wine below 10 NTU.
Absorbance at 420 nm (specific of yellow colour) of the wine was not influenced by fining.
The absorbance at 320 nm was increased by fining with tannin and gelatine, with tannin and YPE 1,2, 3 or 4 or with tannin and yeast product 1 compared to the control (absorbance at 320 nm of 4.8).
The absorbance at 280 nm (specific of aromatic rings also found in all polyphenols) was increased by fining with tannin and gelatin or with tannin and YPE 1, 2, 3 or 4 or with tannin and yeast product 1

by 10 to 20 % compared to the control (absorbance at 280 nm of 7.8 to 8).

3.2.1.3 Fining experiments with red wine of Pinot noir

The wine had an alcohol concentration of 11.6 ml/100 ml, a pH of 3.7 and it was dry, having 3 g/l of reducing sugars. Malolactic fermentation was finished as no more malic acid was detectable.

The YPE concentration of 20 g/hl proofed to be too low for reliable fining and is not shown herein.

Gelatine formed flakes in the wine after 30 min and started sedimentation at 1 hour.

YPE 1 was the only yeast extract that formed flakes observable by eye in the wine when used at 50 g/hl. YPE 1 and 3 showed precipitation of probably proteins of the YPE with wine tannins at both concentrations.YPE 4 formed only a precipitate during fining when applied at 50 g/hl.

All variants using YPE that showed precipitation improved limpidity of the wine (figure 7), but the performance of gelatine was not reached.

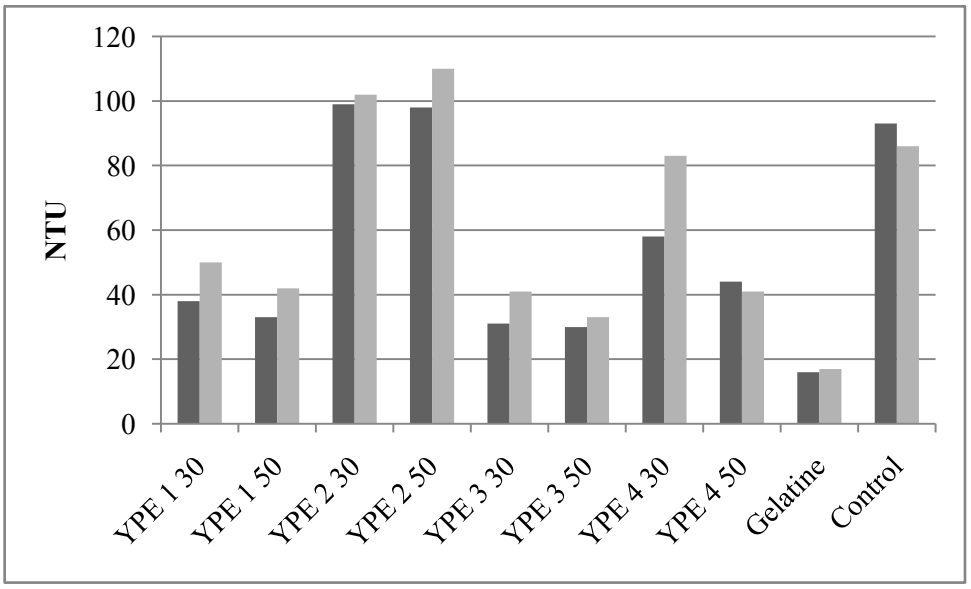

Figure 7: Turbidity in Pinot noir wine after fining – experiment in cylinders (Each column is the value of one of the duplicates)

The variants YPE 1 at 30 g/hl, YPE 3 at 30 g/hl and YPE 4 at 50 g/hl were chosen for the experiments in 1.5 l bottles. Yeast product 1 and 2 at 20 g/hl, gelatine at 100 ml/hl and controls were integrated in this experiment. Formation of visible flakes could not be observed in any of the variants.

All fining variants showed formation of lees whereas no sedimentation was observed in the control variant (cf. table 15). Fining with gelatine, which served as reference fining product, resulted in the wine with the highest limpidity (figure 8) and YPE 3 and 4 clearly clarified the Pinot noir wine when standard deviations are considered.

Table 15: Fining parameters of the Pinot noir wine after fining – Experiment in 1.5 l bottles (YP is Yeast product)

Variant	Sedimentation observed at hour	Lees at end of fining [ml/100 ml]	Visual aspect at end of fining
YPE 1	8	1	More limpid than control
YPE 3	22	0.8	
YPE 4	22	0.3	
YP 1	8	0.3	Turbid
YP 2	2	0.5	More limpid than control
Gelatine	2	2	
Control	Never	None	Turbid

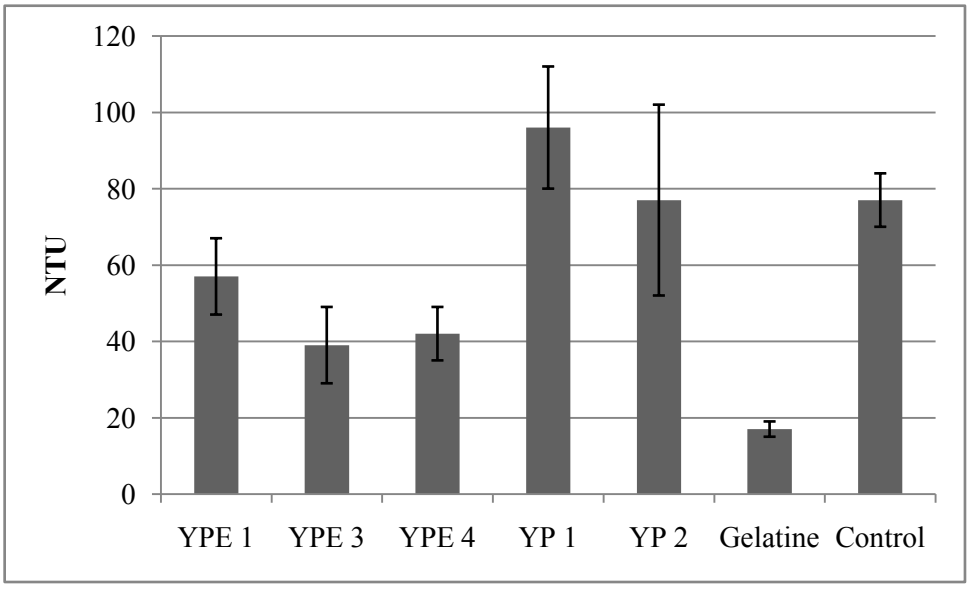

Figure 8: Turbidity of the Pinot noir wine after fining - experiment in 1.5 l bottles (bars indicate standard deviation above and below the mean shown in the columns)

The colour intensity (figure 9) of the wine fined with YPE or gelatine diminished by 5 to 10 % compared to the control variant. The hue (figure 9) of the wine, indicating the nuance of the red colour of the wine, remained nearly unchanged by the fining taking into account the control value. An increase in hue would result in a more brownish red colour and a decrease of the hue would result in a more intense red colour or a more bluish wine colour. The maximum reduction of the hue was observed in wine fined with gelatine, which was 3 % of the value of the control. PVPP-index of the wines (figure 10), estimating the proportion (of 100 parts of total anthocyanins) of anthocyanins combined with tannins, was increased after fining. It has to be remarked that only two samples of the three per fining variant could be analyzed due to time limitations. All control samples were analyzed and had a mean of 40 and a standard deviation of 2. An increase of PVPP index is related with a

higher proportion of anthocyanins combined with tannins which can be related with a higher stability of the red pigments in the wine during storage.

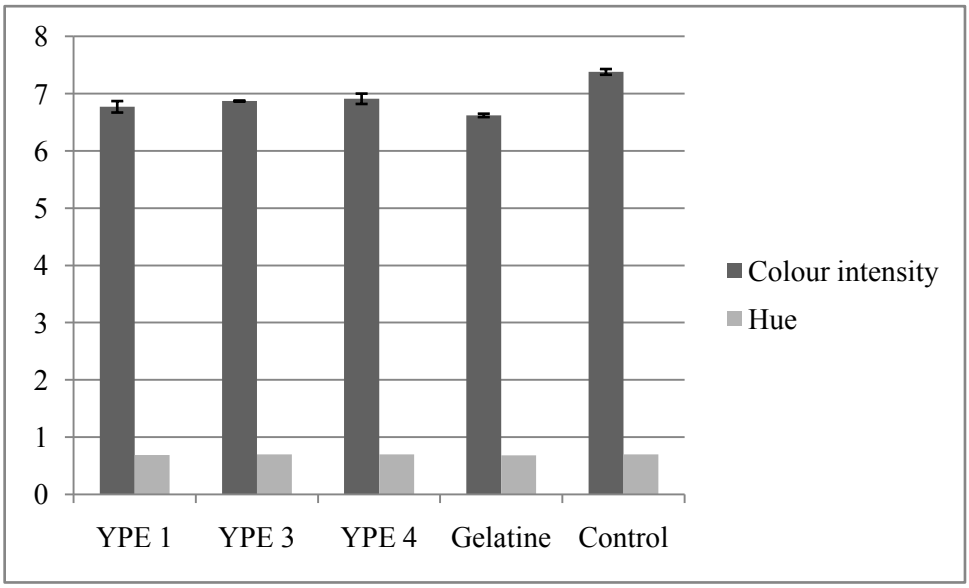

Figure 9: Colour intensity and hue of Pinot noir wines after fining – experiment in 1.5 l bottles (bars indicate standard deviation above and below the mean shown in the columns)

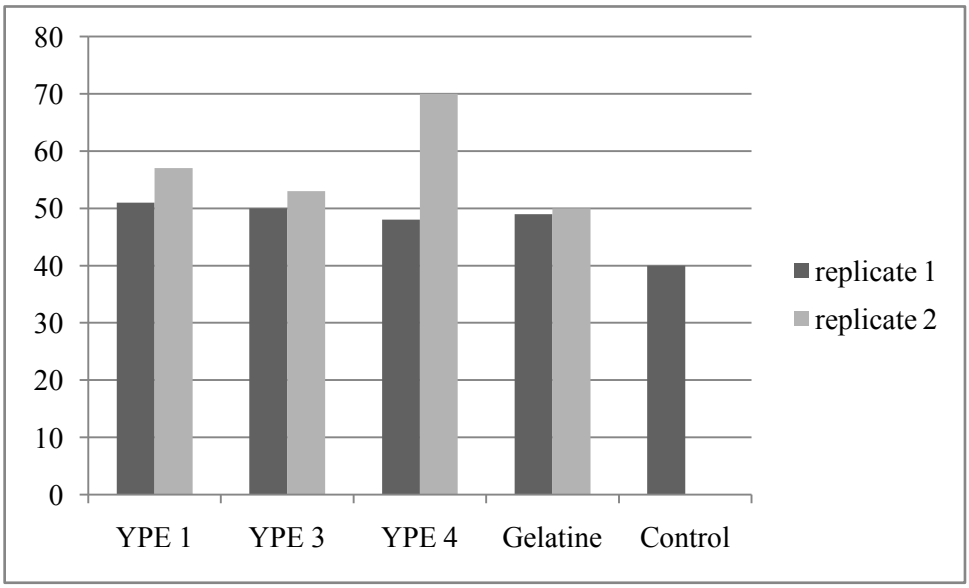

Figure 10: PVPP-index of Pinot noir wine after fining – experiment in 1.5 l bottles

Fining Pinot noir wine with YPE 1 and 3 diminished the absorbance at 280 nm, which is regarded as an index of the concentration of total polyphenols in the wine (cf. figure 11).The values have to be measured in dilute samples due to the high absorbance of red wines at 280 nm, but are given in the form corrected for the dilution resulting in values bigger than 1.

The BSA-index of the wines after fining remained unchanged considering the high standard deviation within the control samples. This index reflects the reactivity of polyphenols with the protein BSA (measured by the formation of turbidity), what is regarded as an indication of wine astringency (cf. figure 11)

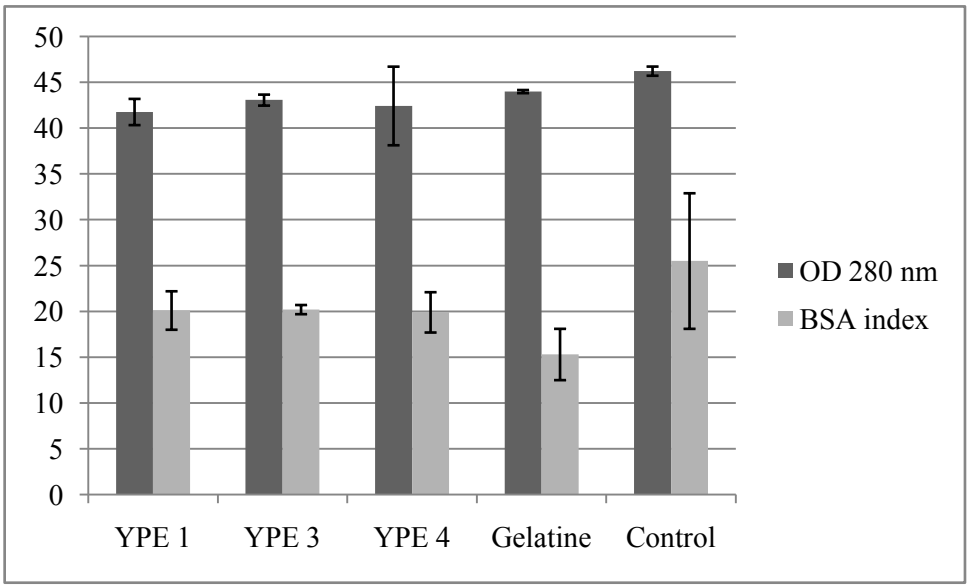

Figure 11: Absorbance at 280 nm and BSA index in Pinot noir wines after fining - experiment in 1.5 l bottles (bars indicate standard deviation above and below the mean shown in the columns)

Fifteen experienced tasters examined the control wine and the wines fined with YPE 1, 2 and 4 and gelatine. Only wine fined with YPE 3 was distinguished from the control at $p = 0.99$ according to DIN 10951 in a triangle test. The descriptors “red berries” “baker’s yeast” and” reductive notes” chosen before the tasting by some persons with big experience in wine tasting did not show differences between the variants YPE 3 and control when standard deviations were also considered.

3.2.1.4 Fining experiments with red wine of Cabernet Sauvignon

This wine had an alcohol concentration of 14.8 ml/100 ml, pH of 3.9, had made malolactic fermentation and was dry having only 3.5 g/l of residual reducing sugars

YPE 1, 2, 3 and 4 formed a precipitate in the wine in all tested concentrations in the preliminary trial in cylinders. Yet, only fining with YPE 1 and YPE 3 at 50 g/hl or with gelatin allowed a clarification of the wine to a level of 40 NTU or below. The control variant had a turbidity of 140 NTU, so 40 NTU meant a clear effect of clarification, but a level of below 10 NTU, typical of a very limpid wine was not reached (cf. figure 12).

The variants YPE 1 at 50 g/hl, YPE 3 at 50 g/hl, gelatine at 100 ml/hl and control were chosen for the experiment in 1.5 l bottles.

Yeast products YP 1 and YP 2 were not examined in the wine of Cabernet Sauvignon as their clarifying effect was not satisfying in the experiment with red wine of Pinot noir.

The fining experiment in 1.5 l bottles was observed for three days. All variants including the control showed formation of lees during fining. The volume of lees at the end of fining was 3 ml/100 ml in the control wine, 4 ml/100 ml in wine fined with YPE and 5 ml/100 ml in wine fined with gelatine.

The flocculation of any of the fining products could not be observed by eye in the Cabernet Sauvignon wine, which had a very dark red-blue colour. The fining variants showed clearly a faster onset of sedimentation of lees at 10 min than the control in which lees started only to settle after 1 hour.

The turbidity of wine was diminished by the fining. It has to be remarked that the control variant showed also a much lower turbidity than in the preliminary experiment in cylinders due to natural wine clarification (cf. figure 13).

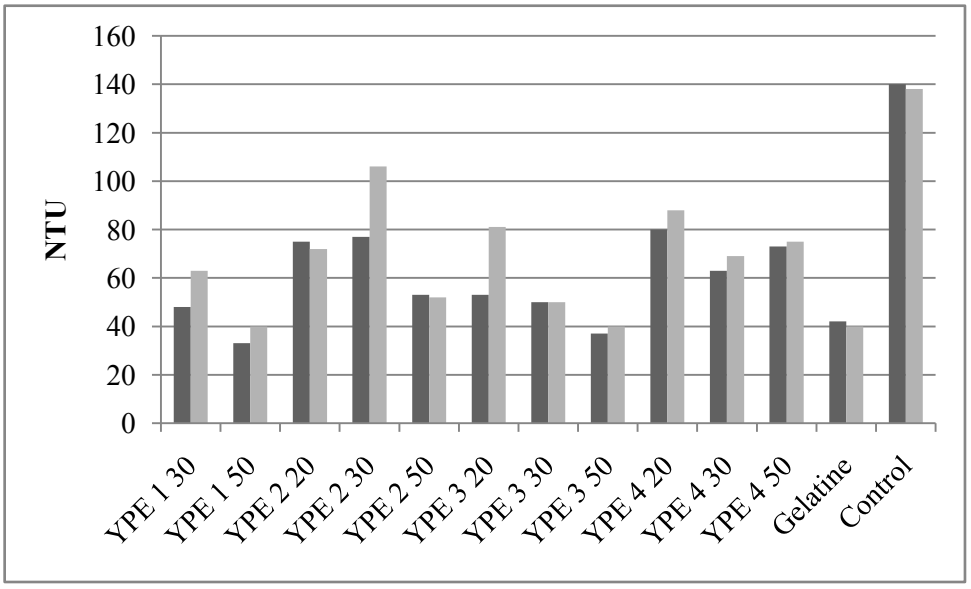

Figure 12: Turbidity in Cabernet Sauvignon wine after fining – experiment in cylinders (Each column is the value of one of the duplicates)

The colour intensity of the Cabernet Sauvignon wine was diminished by 10 % by fining with YPE 1 or 3. The hue remained unchanged by the fining with all products (cf. figure 14) and standard deviation in case of hue was below 1 % of the mean in all variants considering the standard deviations. The PVPP index did not show differences between the fining variants and the control in the experiment in 1.5 l bottles (cf. figure 15).

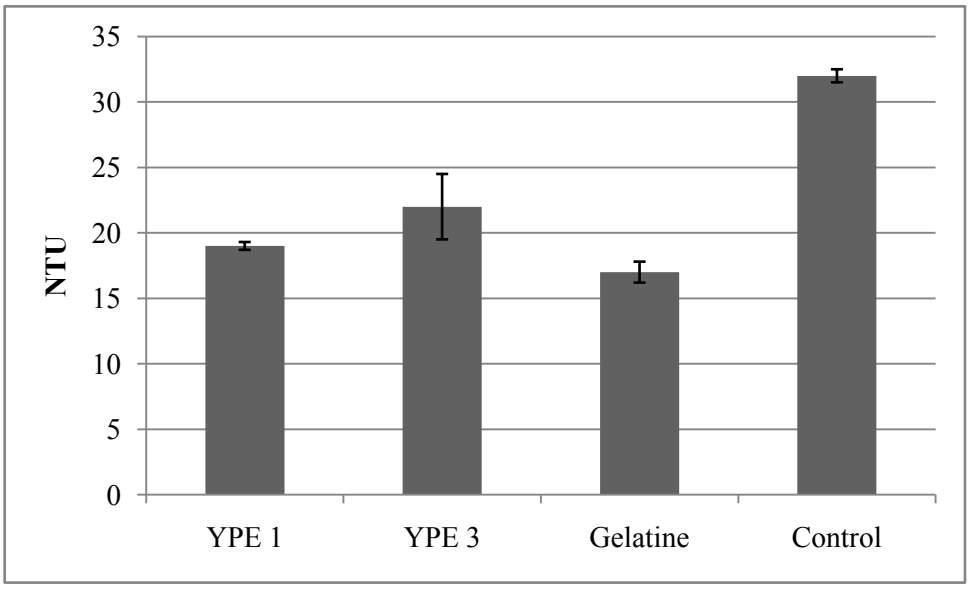

Figure 13: Turbidity in Cabernet Sauvignon wine after fining – experiment in 1.5 l bottles (bars indicate standard deviation above and below the mean shown in the columns)

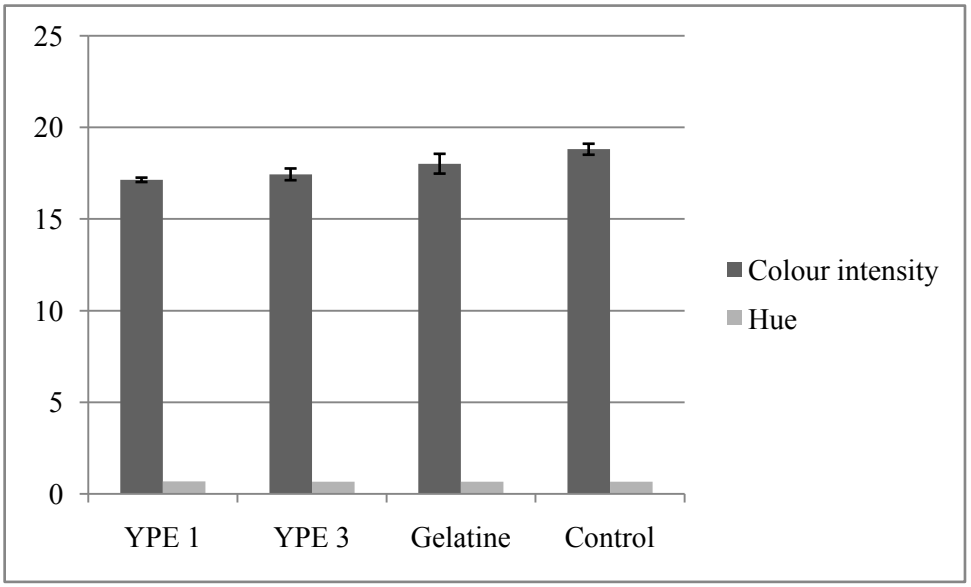

Figure 14: Colour intensity and hue in Cabernet Sauvignon wine after fining – experiment in 1.5 l bottles (bars indicate standard deviation above and below the mean shown in the columns)

Fining the Cabernet Sauvignon with YPE 1 or 3 or gelatine did not change the index of total polyphenols estimated by absorbance at 280 nm (cf. figure 16) taking into account standard deviations. BSA index reflecting wine's astringency was not changed either by the fining procedures (cf. figure 16) regarding the standard deviations.

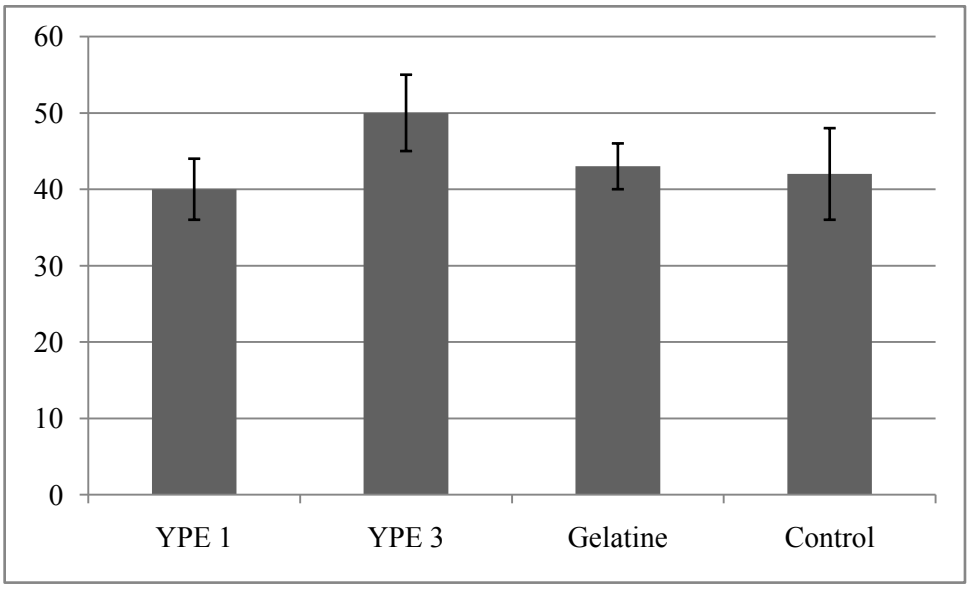

Figure 15: PVPP-index of Cabernet Sauvignon wine after fining – experiment in 1.5 l bottles (bars indicate standard deviation above and below the mean shown in the columns)

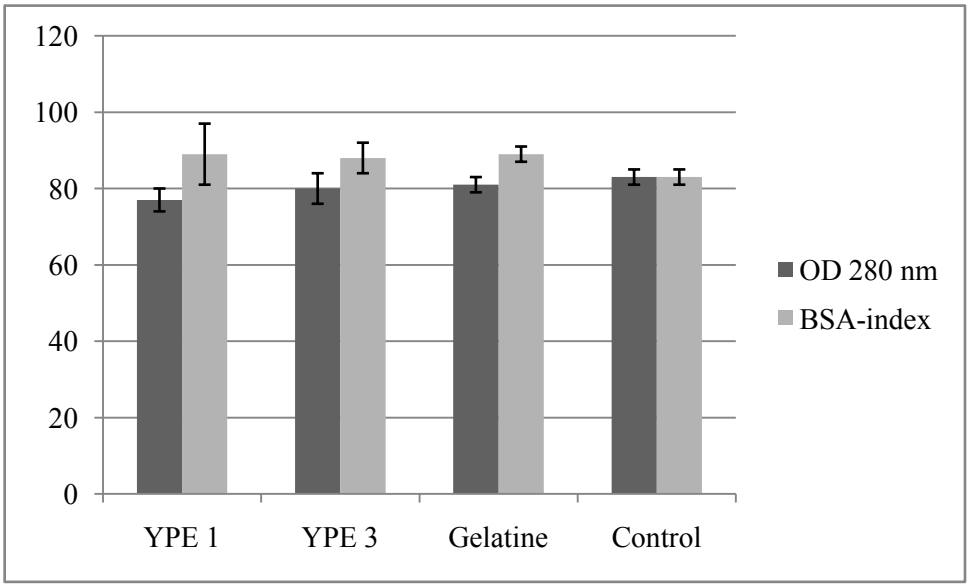

Figure 16: Absorbance at 280 nm and BSA index in Cabernet Sauvignon wine after fining - experiment in 1.5 l bottles
(bars indicate standard deviation above and below the mean shown in the columns)

12 experienced tasters examined the wine of the different fining variants and the control variant 4 weeks after the fining. The tasters could not distinguish the wine of the different variants from the control variant in triangle tests, which were evaluated according to DIN 10951.
It can be summarized that the YPE used in cycle 1 showed the most interesting fining abilities in case of red wines.

3.2.2 Fining experiment of cycle 2

3.2.2.1 Experiment with Riesling must (cycle 2)

The first YPE of the three strains A, B and C were available in that cycle of fining experiments as outlined in material and methods.

The must had a density of 1086 g/l, 185.2 g/l of reducing sugars and a pH of 2.8. The pH was unusually low, which was a characteristic of vintage 2010 in the Rheingau area.

It was tried to increase the polyphenol concentration of the must (cf. material and methods), but the values were in the same range than in a Riesling must of 2009 (results not shown) produced under standard conditions.

The initial turbidity was 150 NTU, a value already acceptable for grape must used for wine production (cf. discussion). It has to be remarked that YPE C1 was not yet available for the must experiment.

Isinglass and casein showed flocculation in the first 15 minutes of fining and flakes and formation of lees were also observed in the control variant after 4 hours. The turbidity of the control must was 8 NTU after 48 hours of fining, which characterizes a limpid commercial grape juice. None of the fining variants improved natural must clarification taking into consideration standard deviation in the variants. Fining must with YPE B1 even increased wine turbidity to 20 NTU.

Absorbance at 420 nm (specific of yellow colour) was increased when must was fined with YPE B1 at 30 g/hl by 40 % (0.155 instead of 0.110).

Indices of total polyphenols (absorbance at 280 nm) and of derivatives of cinnamic acid (absorbance at 320 nm) were diminished by 10 % when must was fined with casein.

3.2.2.2 Experiment with Riesling wine (cycle 2)

The wine was made from the juice used also in the must experiment (cf. material and methods) and had, 11.5 ml/100 ml alcohol, a pH of 2.9 and 6 g/l of reducing sugars, characterizing a dry wine.

The pH of this Riesling wine was unusually low which was already found in the must experiment.

Casein was the only fining product showing immediate flocculation in the wine and isinglass started flocculation at 4 hours of fining. The clarification of the wine was gradual and zones of higher limpidity were first found on top of the measuring cylinders. Lees were formed in all variants including the control within the 3 days of fining. The turbidity at the end of fining was 23 NTU in the control and only isinglass allowed a successful clarification resulting in a limpid wine of a turbidity of 5 NTU. Casein and YPE B1 showed even a higher turbidity at the end of fining than the control variant.

Absorbance at 420 nm (specific of yellow colour) was increased when the wine was fined with YPE B1 at both concentrations of 10 g/hl and 30 g/hl by 50% or 150 %. The absorbance at 420 nm of the control variant was 0.06 and it reached 0.170 in the variant YPE B1 at 30 g/hl.

Casein diminished slightly the indices of total polyphenols (absorbance at 280 nm) and of derivatives of cinnamic acid (absorbance at 320 nm). The other fining variants did not change these indices.

3.2.2.3 Experiment with cuvee of red wine (cycle 2)

Lees were observed at 1.5 hours in red wine fined with gelatine or YPE 1. The control variant and the wine fined with the other YPE showed lees after 6 hours. The variants gelatine and YPE 1 were also the only ones which improved wine turbidity during fining (cf. figure 17) taking also into consideration the standard deviations.

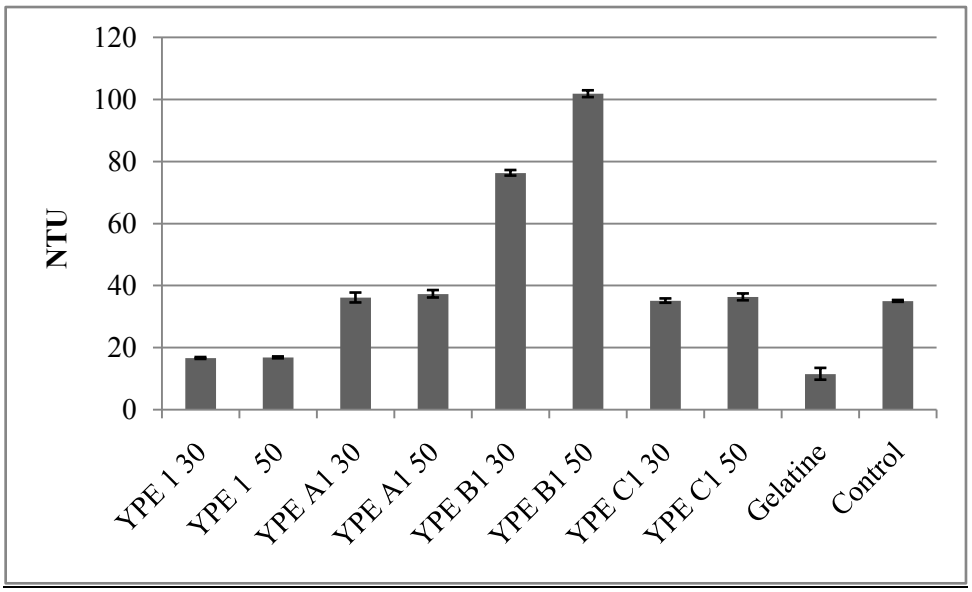

Figure 17: Turbidity in red wine cuvee after fining – experiment of cycle 2

Regarding all fining experiments done in cycle 2 it can be concluded that the YPE A1, B1 or C1 did not show a potential to be used as alternative fining agents.

3.2.3 Fining experiments of cycle 3: fining of a cuvee of red wines

The YPE A 1, B1 and C1 had not yet proofed to be efficient fining agents. New protein extracts of the yeast strains A, B and C were thus produced. The experiments were restricted to red wines and in cycle 3 a cuvee of red wines of 2009 (20 %) and 2010 (80 %) was used. This red wine cuvee had an alcohol concentration of 13.2 ml/100 ml, was dry (1 g/l of reducing sugars), had completed malolactic fermentation (no malic acid anymore detectable) and had a pH of 3.5. YPE 1 that proofed to be interesting yeast fining agent in red wine in cycles 1 and 2 and YPE A 1 were both integrated in experiments of cycle 3. Figure 18 shows that YPE 1 and A 2.2. decreased wine's turbidity during fining in all concentrations also combined with tannin. YPE B2 and C2 only clarified the red wine successfully during fining when applied without tannin. YPE A1 and A 2.1. did not show precipitation in all concentrations applied for fining of red wine and were thus not further retained. In all cases standard deviations were taken into account. Gelatine used alone at 100 ml/hl or combined with 5 g/l tannin successfully clarified the red wine. The wine had turbidity values between 3 and 5 after fining with gelatine (results not shown).

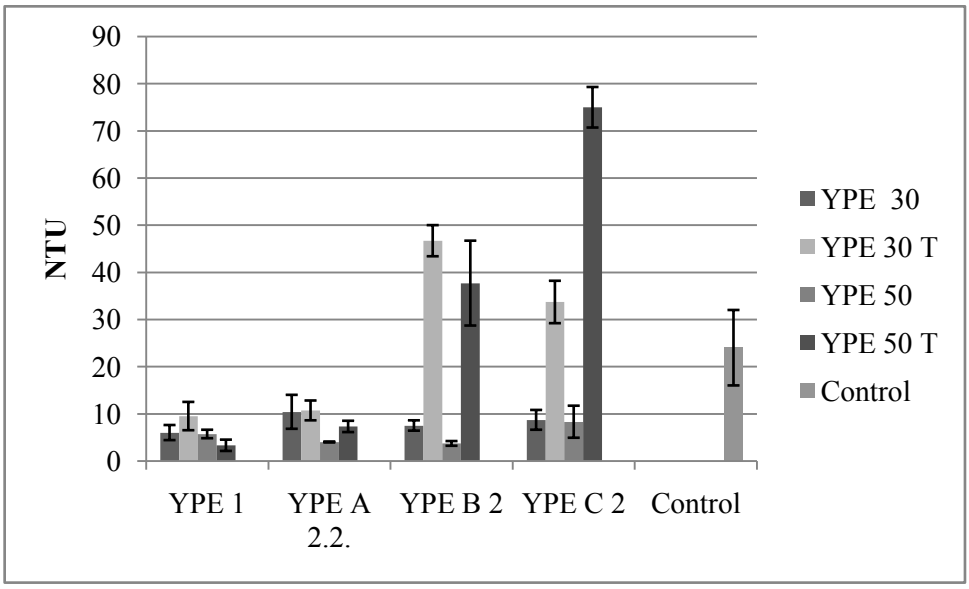

Figure 18: Turbidity after fining of red wine with YPE of cycle 3
(bars show standard deviation above and below the means;
30 etc. are the concentrations in g/hl;
T is used for fining with combination of YPE and tannin)

YPE 1, A 2.2., B 2 and C 2 at 30 g/hl as well as gelatine and a control variant were integrated in a final fining experiment under the same conditions than the preliminary experiments. Figure 19 shows the turbidities in the red wines after fining. The control wine had a much lower turbidity in this experiment than in the former experiments of cycle 3 due to natural clarification. Gelatine, YPE 1 and most replicates of YPE 2.2. showed flocculation within 6 hours. Lees and flocculation were not observed until 20 hours of fining (cf. table 16) in the other variants. Volume of lees was higher after fining red wine with gelatine than with YPE.

Table 16: Cycle 3 final experiment: Beginning of flocculation and lees after fining of red wine

Variant	Beginning of flocculation at hour	Lees after fining in ml/100 ml
YPE 1	1.5	1.6
YPE A 2.2.	6 20 (1/3 cases)	1.6
YPE B2	20	1.6
YPE C2	28	1.6
Gelatine	0.5	2.8
Control	never	None

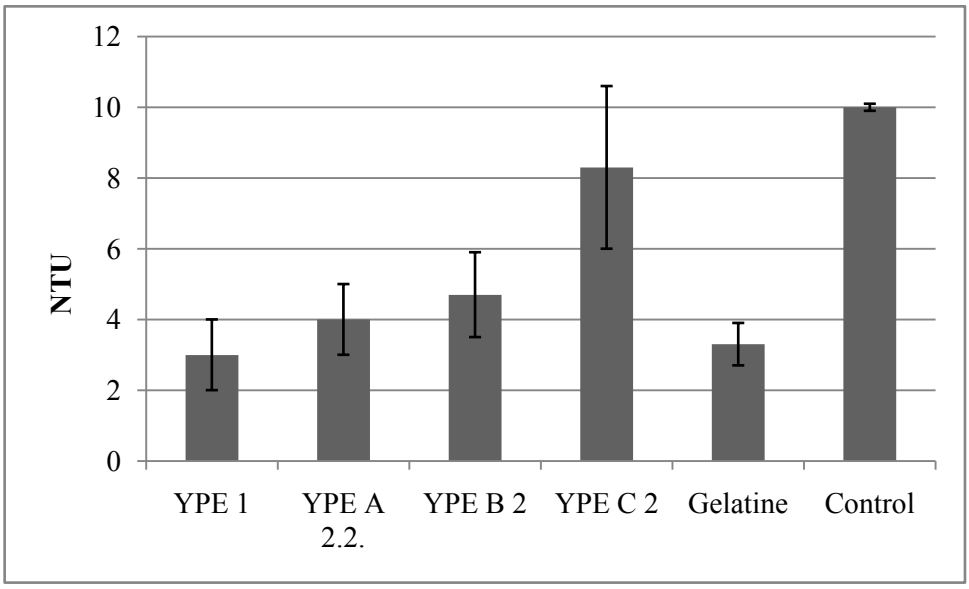

Figure 19 : Turbidity after fining in red wine of final experiment of cycle 3 (bars show standard deviation above and below the means)

The colour intensity of the wine was slightly diminished when the red wine was fined with gelatine or YPE 1 by 5 to 8 %. Hue of the wines remained unchanged by the fining (cf. figure 20).
The index of total polyphenols was not changed in the red wine by fining with YPE, but decreased by 5 % when red wine was fined with gelatine (cf. figure 21). Fining the red wine with gelatine reduced the BSA index of the wine, reflecting wine astringency, by 15 %. No change of BSA index was observed in red wine after fining with YPE (cf. figure 22) considering standard deviations.

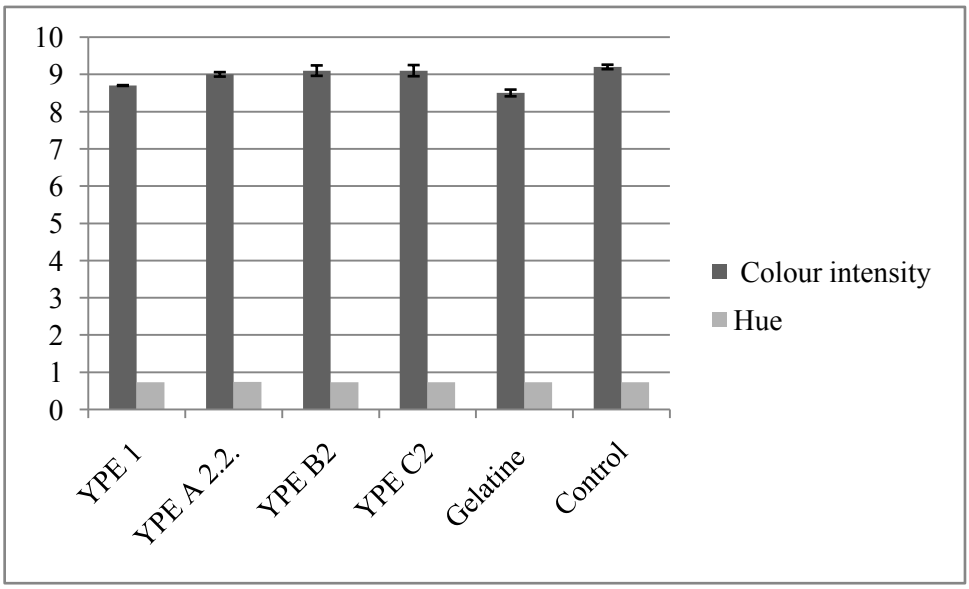

Figure 20: Colour parameters in red wines after fining in final experiment of cycle 3 (bars show standard deviation above and below the means)

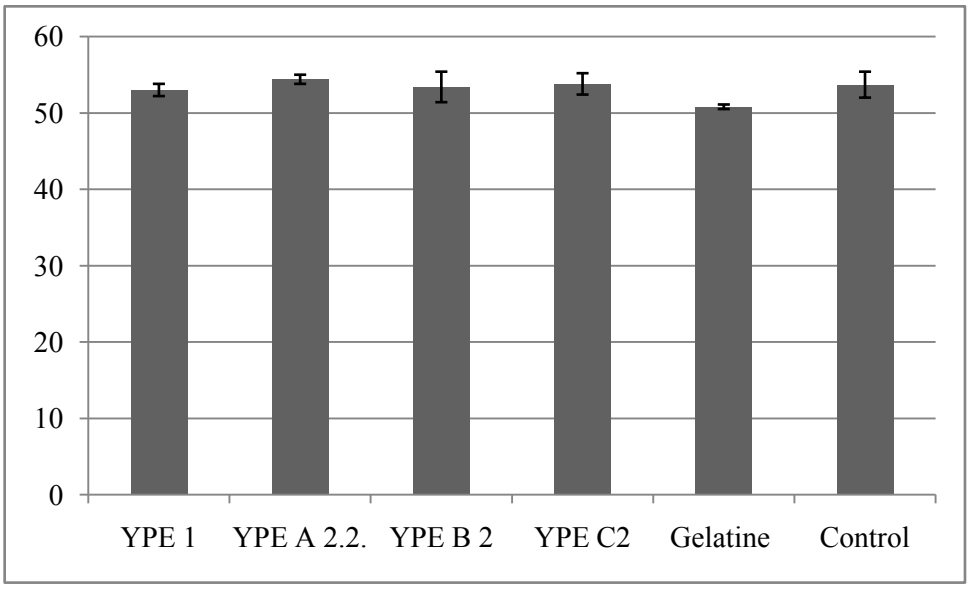

Figure 21: Index of total polyphenols in red wines after fining in final experiment of cycle 3 (bars show standard deviation above and below the means)

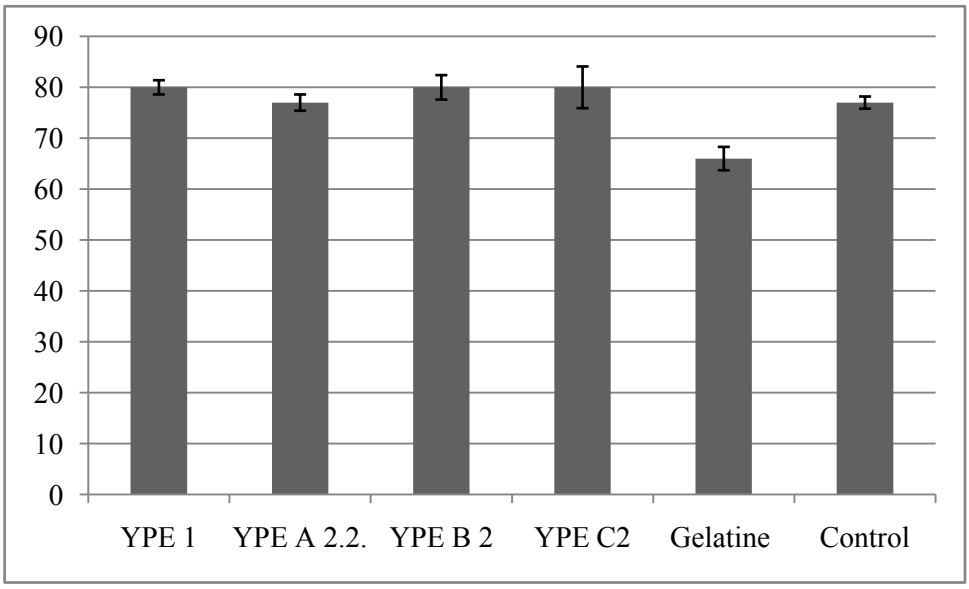

Figure 22: BSA index in red wines after fining in final experiment of cycle 3 (bars show standard deviation above and below the means)

3.2.4 Fining experiments of cycle 4

3.2.4.1 Fining of red wine of Syrah

Red wines of the varieties Syrah and Rondo were used. In the following the results of the Syrah wine are shown. YPE 1, A 2.2., A3, B3 and C3 were tested in the red wine of Syrah of which the characteristics are given in table 17. It was a dry red wine which had finished malolactic fermentation.

Gelatine used as fining agent alone or combined with tannin was integrated in this fining cycle as reference treatment. Fining Syrah red wine with gelatine also combined with tannin diminished wine turbidity to a level at which wine could be described as limpid (NTU below 10, cf. figure 23).

Fining Syrah wine with the YPE 1, A 2.2., A3, B3 and C3 also combined with tannin did not result in a clarification comparable to that of gelatine (cf. figure 24), but YPE 1 and A3 used without tannin at concentrations of 30 or 50 g/hl resulted in a wine as limpid or slightly more limpid than the control wine. The same was observed when Syrah wine was fined with YPE B3 or YPE C3 at 50 g/hl.

YPE 1, A 2.2., B3 and C3 increased turbidity of Syrah wine during fining when applied combined with tannin, which was not the case in YPE A3 when concentration of YPE was below 50 g/hl combined with tannin.

Table 17: Chemical characteristics of red wine of Syrah

Alcohol	14 ml/100 ml
Reducing sugars	2.7 g/l
Tartaric acid	2.5 g/l
Malic acid	not detected
Lactic acid	1.2 g/l
pH	3.8

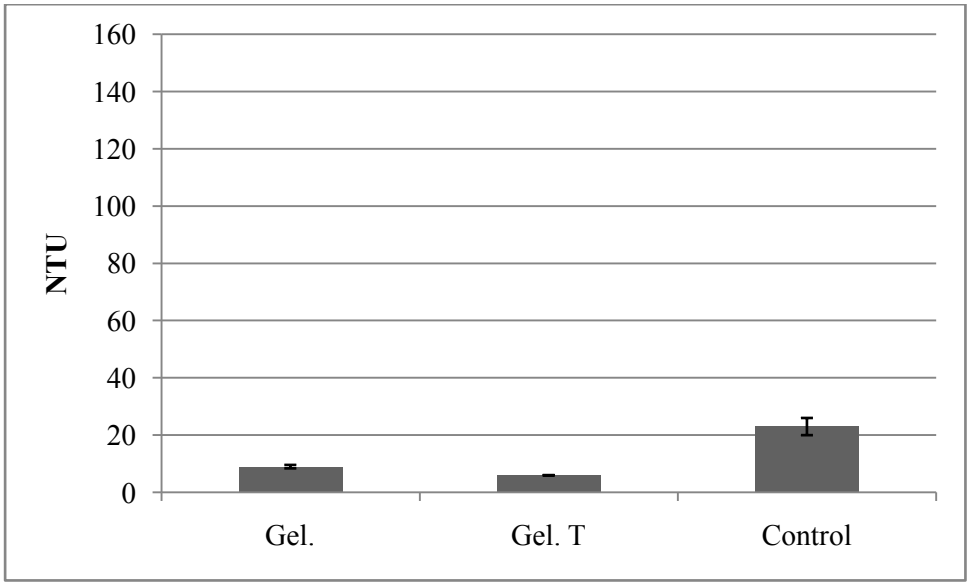

Figure 23: Turbidity after fining in Syrah wine of cycle 4: Fining with gelatine ("T" stands for "combined with tannins"; bars show standard deviation above and below the means)

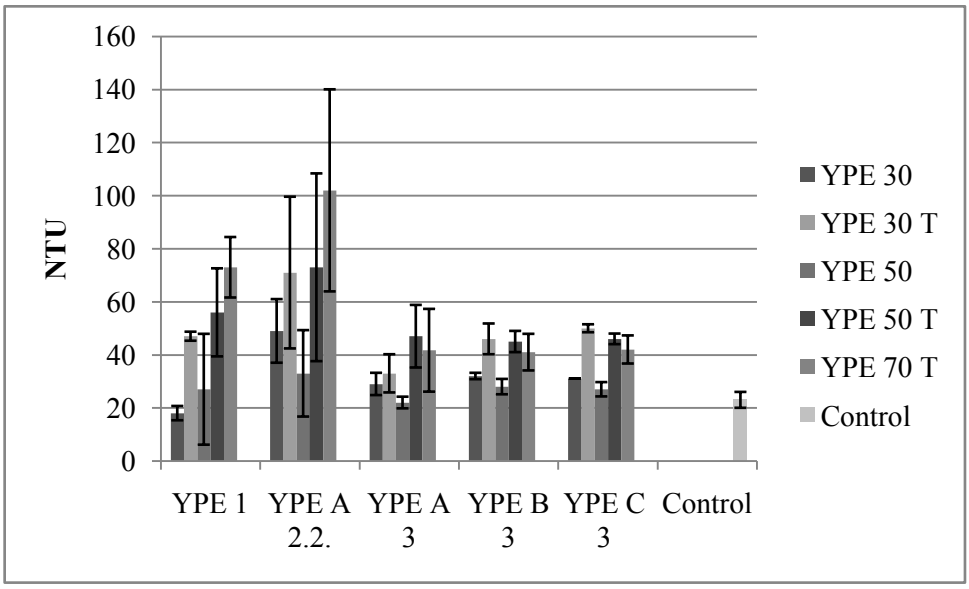

Figure 24: Turbidity after fining Syrah wine of cycle 4: Fining with YPE ("T" stands for "combined with tannins" also in the following figures of cycle 4; bars show standard deviation above and below the means)

The precipitation of the fining agent in the wine and the onset and speed of sedimentation of the precipitate are important parameters in fining besides the clarification capacity, which was measured by nephelometry in NTU. Visible flakes were formed when gelatine was used in all concentrations and combinations, when YPE 1 was used combined with tannin or pure at 50 g/hl and when YPE A3 was used at 70 g/hl combined with tannin. The other YPE used for fining of the Syrah wine did not show visible flocculation as the precipitated fining agent formed too small particles to be detected by eye, but caused visible turbidity. The beginning of sedimentation of the YPE and gelatine is documented in table 18. Gelatine was clearly the fining agent with the fastest onset of sedimentation of the fining lees (0.2 to 0.5 hours). It was followed by YPE 1 which started sedimentation of the fining lees at 1.5 to 6 hours in all variants. YPE A 2.2. and B3 precipitated in the Syrah wine in all concentrations and combinations, but the fining lees started sedimentation much later than in case of YPE 1 at 20 to 24 hours. YPE A3 did not precipitate in Syrah wine at 30 g/hl, but when applied at 30 g/hl combined with tannin or at higher concentrations there was a tendency that onset of sedimentation was equal or faster than in case of YPE A 2.2., B3 and C3.The volume of lees was 1 ml/100 ml or below 1 ml/100 ml in all fining variants with YPE and 2 ml/100 ml when Syrah wine was fined with gelatine. The control variants showed a precipitate of below 1 ml/100 ml in 3 of 18 repetitions.

Fining Syrah wine with YPE or gelatine did not influence colour intensity (mean: 11.6; standard deviation: 0.8), hue (control: mean 0.63; standard deviation: 0.002) and index of total polyphenols (O.D at 280 nm; mean of control: 60; standard deviation 3) of the wine when standard deviations were taken into account. An exception was that colour intensity was diminished when fining wit YPE 1 at 30 g/hl or gelatine, both combined with tannin, was performed (cf. figure 25).

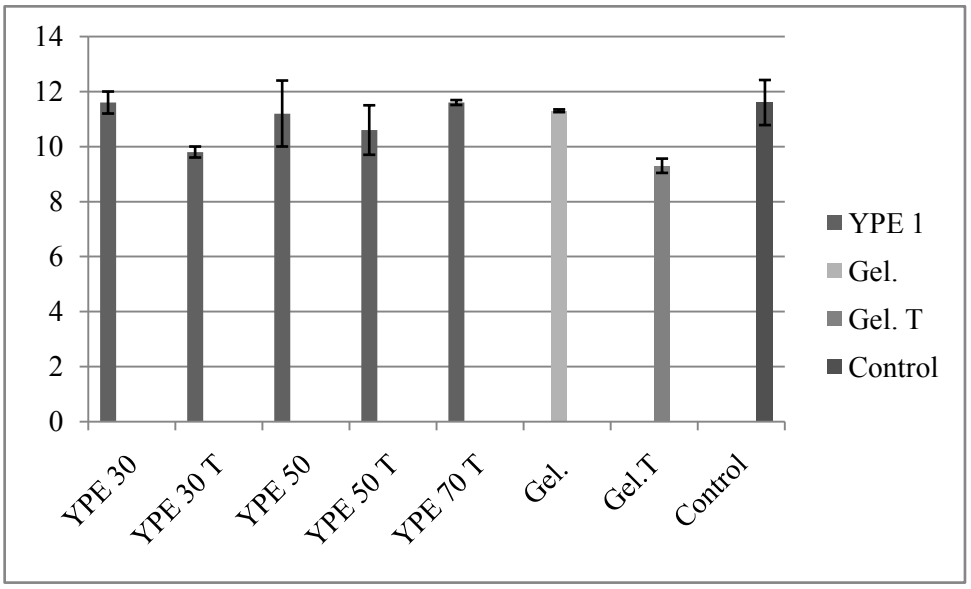

Figure 25: Colour intensity in Syrah wine of cycle 4 after fining with gelatine or YPE 1 (bars show standard deviation above and below the means)

Table 18: Sedimentation of fining lees in Syrah wine of cycle 4

Fining agent	**Variant**	**Sedimentation observed**	**Beginning of sedimentation at hour**
YPE 1	30 g/hl	In all variants and all repetitions	4 to 6
	30 g/hl plus tannin		6
	50 g/hl		1.5 to 4
	50 g/hl plus tannin		4
	70 g/hl plus tannin		2
YPE A 2.2.	30 g/hl	In all variants and all repetitions	**31 to 68**
	30 g/hl plus tannin		**28 to 44**
	50 g/hl		22 to 24
	50 g/hl plus tannin		19 to 28
	70 g/hl plus tannin		24 to 32
YPE A3	30 g/hl	**no**	**none 50 % or 44 to 52**
	30 g/hl plus tannin	In all repetitions	19 to 24
	50 g/hl		22 to 28
	50 g/hl plus tannin		19
	70 g/hl plus tannin		8

Table 18: continued

Fining agent	Variant	Sedimentation observed	Beginning of sedimentation at hour
YPE B 3	30 g/hl	In all variants and repetitions	**48**
	30 g/hl plus tannin		29
	50 g/hl		**45**
	50 g/hl plus tannin		24
	70 g/hl plus tannin		24
YPE C3	30 g/hl	**no**	**none**
	30 g/hl plus tannin	In all repetitions	29
	50 g/hl		**56**
	50 g/hl plus tannin		29
	70 g/hl plus tannin		29
Gelatine	100 ml/hl	In both variants and all repetitions	0.2
	100 ml/hl plus tannin		0.5
Control	Control	In 3 of 18 cases	**45**

3.2.4.2 Fining of red wine of Rondo

Red wine of Rondo was treated with pectolytic enzymes as pectin compounds inhibited precipitation of proteins of the fining agents gelatine and YPE 1 in a preliminary experiment.

The Rondo wine was dry and had completed malolactic fermentation like the Syrah wine and alcohol concentration and pH of both red wines were also similar (cf. table 19).

Table 19: Chemical characteristics of red wine of Rondo

Alcohol	14 ml/100 ml
Reducing sugars	1.8 g/l
Tartaric acid	2.2 g/l
Malic acid	Not detected
Lactic acid	1.4 g/l
pH	3.9

The Rondo wine could be clarified successfully with gelatine like the Syrah wine (cf. figure 26). Fining Rondo wine with YPE 1 and A3 could diminish wine's turbidity in all variants, but to a lower extent than gelatine (cf. figure 27).

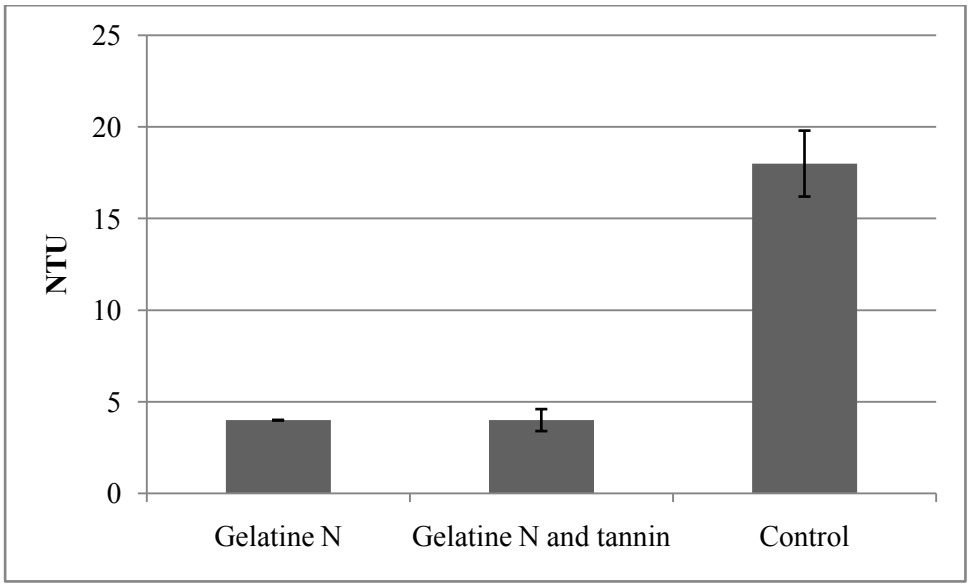

Figure 26: Turbidity after fining in Rondo wine of cycle 4: Fining with gelatine (bars show standard deviation above and below the means)

All fining agents used in red wine of Rondo precipitated in the wine, but in 50 % of the control samples natural lees deposited. Flocculation could be observed by eye in the variants treated with gelatine and YPE 1. Gelatine showed the fastest formation of flakes and of sediment of lees, followed by YPE. The time situation was comparable to the observations made in the red wine of Syrah (cf. table 20). The sedimentation of lees was complete and faster in the variants YPE A2.2. and A3 at 30 g/hl also combined with tannin than in the Syrah wine.

Volume of lees was below 1 ml/100 ml in case of the control variants, 1 ml/100 ml when Rondo wine was fined with YPE and 3 ml/100 ml when Rondo wine was fined with gelatine.

Colour intensity (control had a mean of 18 and a standard deviation of 1) and hue (control had a mean of 0.64 and a standard deviation of 0.01) of the Rondo wine was not influenced by the fining variants taking into account the standard deviations.

Polyphenol index (control had a mean of 100 and a standard deviation of 3) was not changed by the fining variants taking into consideration the standard deviations.

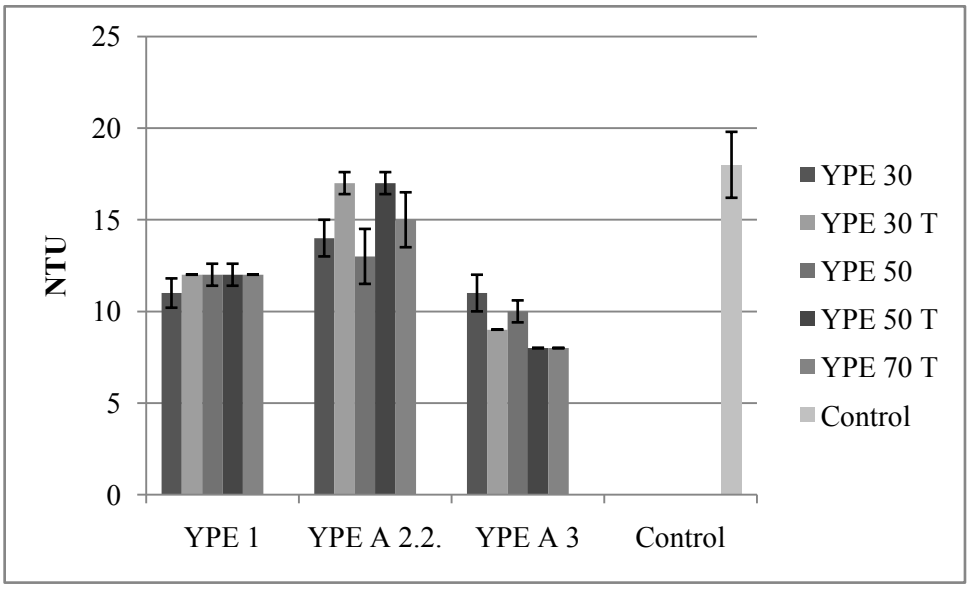

Figure 27: Turbidity after fining in Rondo wine of cycle 4: Fining with YPE (bars show standard deviation above and below the means)

Table 20: Sedimentation of fining lees in Rondo wine of cycle 4

Fining agent	**Variant**	**Sedimentation observed**	**Beginning of sedimentation at hours**
YPE 1	30 g/hl	In all variants and repetitions	8
	30 g/hl plus tannin		6
	50 g/hl		6
	50 g/hl plus tannin		4
	70 g/hl plus tannin		4
YPE A2.2.	30 g/hl	In all variants and repetitions	24
	30 g/hl plus tannin		21
	50 g/hl		18
	50 g/hl plus tannin		18
	70 g/hl plus tannin		18
YPE A3	30 g/hl	In all variants and repetitions	32
	30 g/hl plus tannin		21
	50 g/hl		21
	50 g/hl plus tannin		21
	70 g/hl plus tannin		8

Table 20: continued

Fining agent	Variant	Sedimentation observed	Beginning of sedimentation at hours
Gelatine	100 ml/hl	In all repetitions	0.5
	100 ml/hl plus tannin	In all repetitions	0.5
No	Control	In 3 of 6 repetitions	70

3.2.5 Fining experiments of cycle 5

3.2.5.1 Fining of a red wine cuvee at small scale

A red wine cuvee of the four varieties Pinot noir, Syrah, Bolero and Rondo in equal parts, was used for all experiments of that cycle. This cuvee consisted of dry red wines having completed malolactic fermentation (cf. table 21).

The experiments with YPE 7 could not be done in the same period than those with YPE A4, YPE 5 and YPE 6 as this product was only available later. Consequently YPE 7 was tested in a second time and gelatine and YPE 1 at 30 g/hl were integrated in these experiments.

Besides the YPE A4, 5 and 6, YPE 1 and A3 that proved to be interesting fining agents in former cycles were tested in the first set of experiments of cycle 5 and gelatine served as reference product. The fining agents were also combined with tannin or silica sol to facilitate precipitation and complete sedimentation of proteins of the fining agents.

Table 21: Chemical characteristics of the cuvee of red wines

Alcohol	13.6 ml/100 ml
Reducing sugars	0.7 g/l
Tartaric acid	1.7 g/l
Malic acid	not detected
Lactic acid	2.3 g/l
pH	3.7

The red wine in the control variant was not limpid as its turbidity was over 10 NTU and the reference product gelatine used without fining aid successfully clarified the wine until a limpid state (figure 28). YPE 1, 5 and 6 used without the fining aids tannin or silica sol and at the lowest concentration of 30 g/hl successfully clarified the wine. YPE A3 showed an inferior clarification performance but clarified the wine when used without fining aid at a concentration of 50 g/hl. YPE A4 could not reduce the turbidity of the wine in any concentration and combination with fining aids tested. It has to be remarked that fining the cuvee with gelatine or YPE 1, A3, 5 and 6 resulted in a limpid wine with a turbidity measured by nephelometry of below 10 NTU (cf. figure 29).

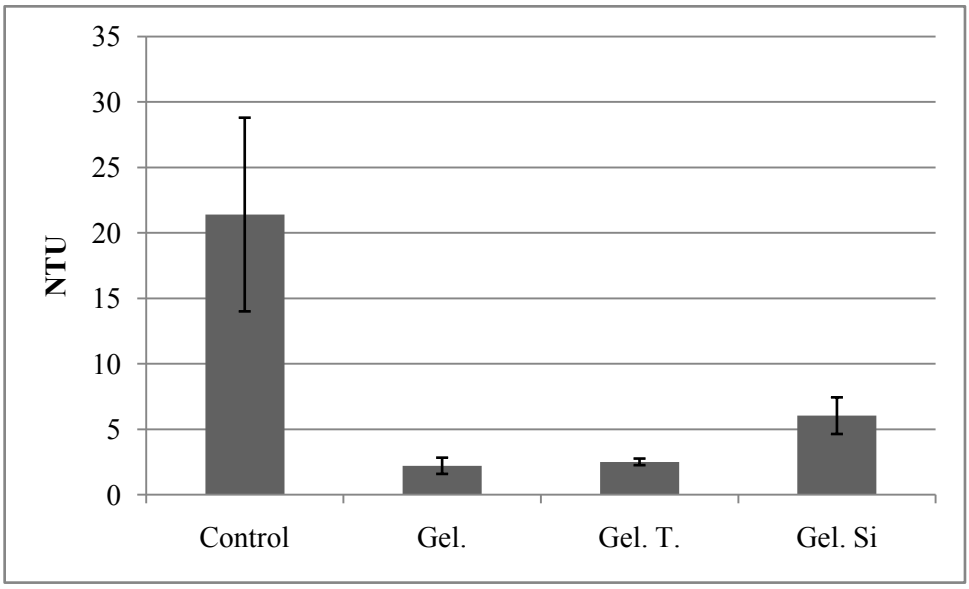

Figure 28: Turbidity after fining in red wine cuvee of cycle 5: Gelatine
("T" and "Si" stand for "combined with tannin" or "silica sol" respectively in all figures of cycle 5; bars show standard deviation above and below the means)

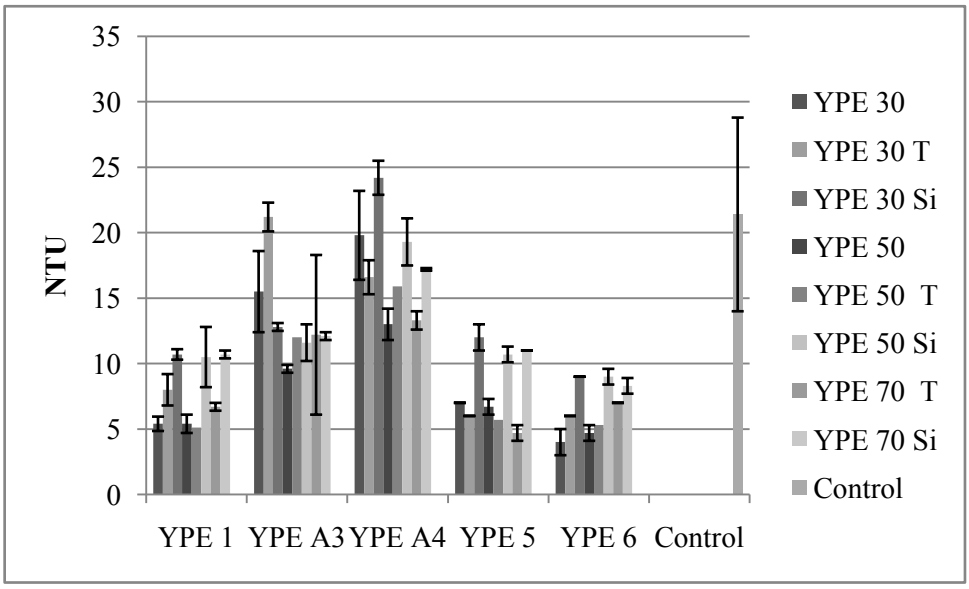

Figure 29: Turbidity after fining in red wine cuvee of cycle 5: YPE 1, A3, A4, 5 or 6
(bars show standard deviation above and below the means)

Table 22 shows that gelatine was as in former cycles of experiments the fining agent that had the fastest onset of flocculation and sedimentation and started formation of lees at 1 hour.

The formation of flakes could be observed by eye during fining in case of all variants fined with gelatine, YPE 1, YPE 6 and YPE A3 at 50 g/hl and 70 g/hl combined with silica sol and at 70 g/hl combined with tannin. A precipitate and lees were never the less formed in all other variants of fining with YPE. YPE 1 and 6 were the YPE that showed the fastest beginning of sedimentation of fining lees when used at low concentration without fining aids. YPE A3 had also a rapid beginning of

sedimentation of lees when used with fining aids at 50 g/hl plus silica sol and at 70 g/hl. YPE A4 did not clarify the wine, but had (probably) proteins that precipitated in the red wine. The beginning of sedimentation of lees was however slow and particles in the wine causing turbidity did not aggregate with the precipitated compounds of the fining agent.

Table 22: Sedimentation of fining lees in case of gelatine, YPE A3, A4, 5, 6 and control variant in red wine cuvee of cycle 5

Fining agent	**Variant**	**Sedimentation observed**	**Beginning of sedimentation at hours**
YPE 1	30 g/hl	In all variants and repetitions	6
	30 g/hl plus tannin		4
	30 g/hl plus silica sol		6
	50 g/hl		4
	50 g/hl plus tannin		1.5 to 4
	50 g/hl plus silica sol		4
	70 g/hl plus tannin		2
	70 g/hl plus silica sol		2
YPE A 3	30 g/hl	In all variants and repetitions	44
	30 g/hl plus tannin		24
	30 g/hl plus silica sol		21
	50 g/hl		21
	50 g/hl plus tannin		21
	50 g/hl plus silica sol		6
	70 g/hl plus tannin		4-8
	70 g/hl plus silica sol		6
YPE A4	30 g/hl	In all variants and repetitions	57
	30 g/hl plus tannin		28
	30 g/hl plus silica sol		52
	50 g/hl		45
	50 g/hl plus tannin		21
	50 g/hl plus silica sol		45
	70 g/hl plus tannin		21
	70 g/hl plus silica sol		45

Table 22: continued

Fining agent	Variant	Sedimentation observed	Beginning of sedimentation at hours
YPE 5	30 g/hl	In all variants and repetitions	20
	30 g/hl plus tannin		6-8
	30 g/hl plus silica sol		20
	50 g/hl		4
	50 g/hl plus tannin		4
	50 g/hl plus silica sol		2
	70 g/hl plus tannin		4
	70 g/hl plus silica sol		4
YPE 6	30 g/hl	In all variants and repetitions	4
	30 g/hl plus tannin		4
	30 g/hl plus silica sol		4
	50 g/hl		1.5 to 2
	50 g/hl plus tannin		1.5
	50 g/hl plus silica sol		1.5 to 2
	70 g/hl plus tannin		1 to 1.5
	70 g/hl plus silica sol		1
Gelatine	100 ml/hl	In all variants and repetitions	0.5 to 1
	100 ml/hl plus tannin		0.5
	100 ml/hl plus silica sol		0.5
None	Control	never	never

YPE 7 arrived 4 months after the other YPE of this cycle as already outlined. The red wine cuvee had become clear at that time by auto-clarification and showed a limpidity of 5.5 (with a standard deviation of 1.1). YPE 1 at 30 g/hl and gelatine also combined with tannin or silica sol flocculated and settled in the red wine, but the limpidity was as in the control or only slightly improved in case of YPE 1 (NTU of 3.3; cf. all figure 30). YPE 7 formed visible flakes in all variants and showed a beginning of flocculation and sedimentation that was similar to that of the gelatine variants and faster than in case of YPE 1 at 30 g/hl (visible flocculation only observed in two of 6 repetitions; cf. table 23). Fining the red wine with YPE 7 increased slightly the limpidity measured by nephelometry in case of all concentrations, but only used alone or in combination with tannin (cf. figure 30).

Table 23: Sedimentation of fining lees in case of gelatine, YPE 1, 7 and control variant in red wine cuvee of cycle 5

Fining agent	Variant	Sedimentation observed	Beginning of sedimentation at hours
YPE 1	30 g/hl	In all repetitions	8
YPE 7	30 g/hl	In all variants and repetitions	2
	30 g/hl plus tannin		2
	30 g/hl plus silica sol		2 to 4
	50 g/hl		1.5
	50 g/hl plus tannin		1.5
	50 g/hl plus silica sol		1.5
	70 g/hl plus tannin		1
	70 g/hl plus silica sol		1 to 1.5
Gelatine	100 ml/hl	In all variants and repetitions	2
	100 ml/hl plus tannin		2
	100 ml/hl plus silica sol		2
None	Control	never	no

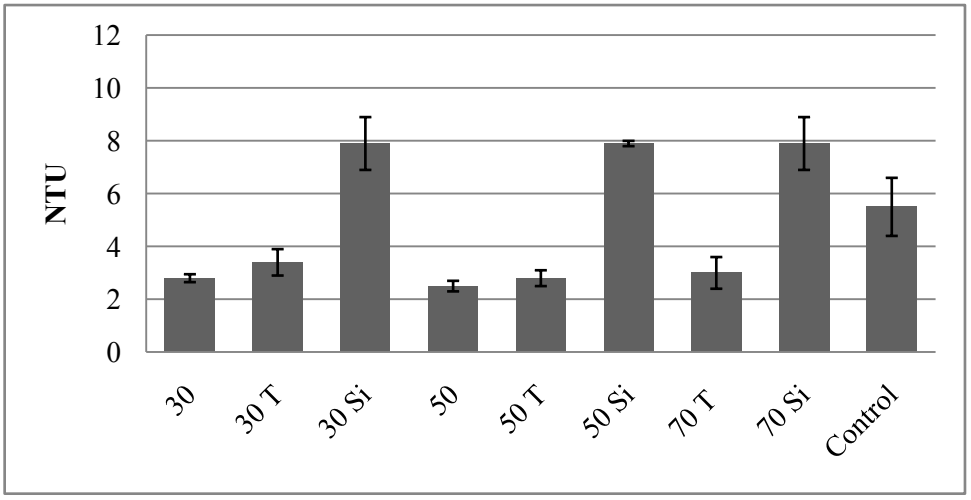

Figure 30: Turbidity after fining in red wine cuvee of cycle 5: YPE 7
(bars show standard deviation above and below the means)

3.2.5.2 Fining of red wine cuvee in bigger volumes

YPE 1, A3, 5 and 6 as well as the reference gelatine were used in a fining experiment in carboys of 5 l to have a wine volume big enough for more detailed polyphenol analysis and a sensory evaluation. Gelatine was used at a concentration of 100 ml/hl and the YPE at 30 g/hl except YPE A3 which was used at 50 g/hl.

YPE 7 was in a later fining experiment also tested in a volume of 0.75 l in bottles, as the rest of the red wine cuvee was too small to perform the assay in carboys. As result of this wine fined with YPE 7 could not be tasted. Gelatine was again used at 100 ml/hl and YPE 7 at 30 g/hl.

The wine in the control variant in the experiment in carboys had the same turbidity expressed in NTU as in the small scale fining experiment of a volume of 100 ml. Gelatine and the YPE used at the ideal concentration selected of the small scale trial also clarified successfully the wine in this experiment (figure 31).The height of lees was estimated in the carboys with a ruler and later transformed to volumes. Gelatine, YPE 1 and YPE 6 had a volume of lees of 4 to 6 ml/100 ml. YPE A3 and 5 formed a smaller volume of lees of 2 ml/100 ml.

YPE 7 at 30 g/hl and gelatine also precipitated and settled in the red wine cuvee, but the control was already limpid (NTU below 10) before fining and consequently no visible clarification could be observed (figure 32). The volume of lees estimated in the bottles as in the carboys was around 1 mL/100 ml in case of gelatine and YPE 7.

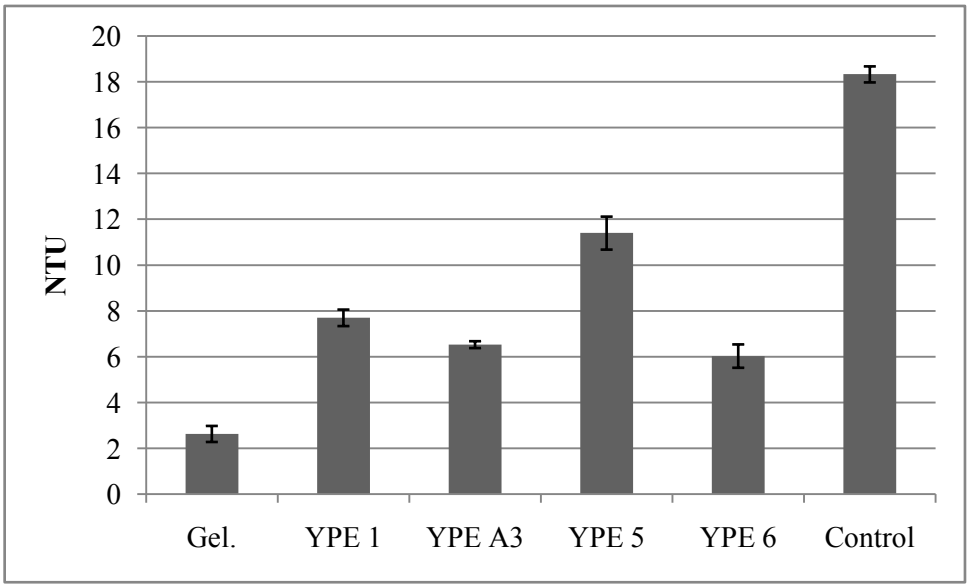

Figure 31: Turbidity in wine after fining in experiment in carboys of cycle 5 (bars show standard deviation above and below the means)

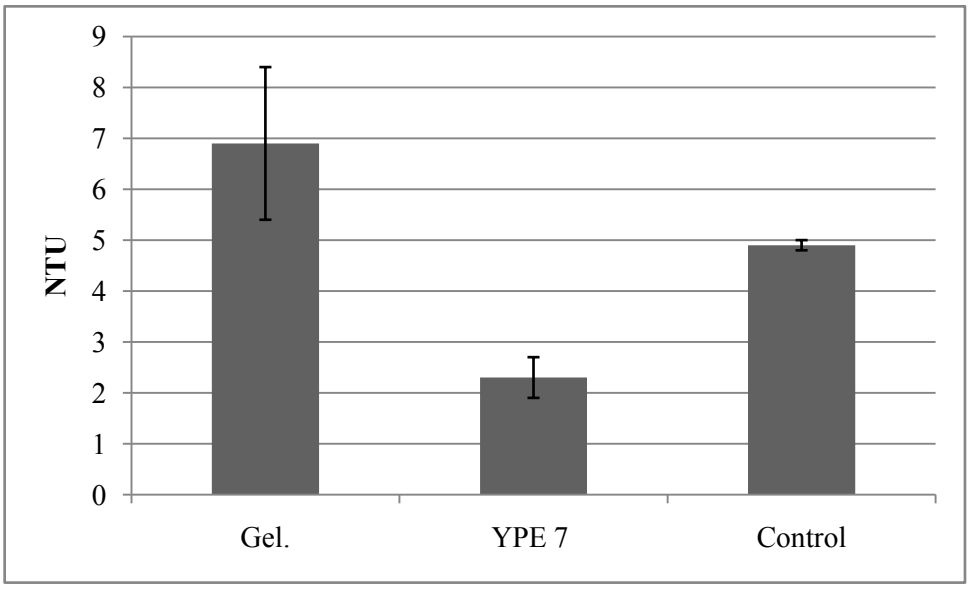

Figure 32: Turbidity in wine after fining with YPE 7 in experiment in bottes of cycle 5 (bars show standard deviation above and below the means)

The colour intensity diminished by maximum 10 % when wine was fined with gelatine or YPE and the global effect of the YPE was comparable to that of gelatine (figures 33, 34).

Hue of the red wine cuvee was not influenced when wine was fined with gelatine, YPE 1, A3, 5, 6 or increased by 2 % taking into account the standard deviations. PVPP- index was increased when red wine was fined with YPE 6 in the experiment in carboys, whereas the other fining treatments did not influence this parameter considering standard deviations (figure 35). YPE 7 and gelatine slightly decreased PVPP-index in experiment in bottles (figure 36). PVPP-index indicates the proportion of anthocyanins combined with other tannins and these pigments are regarded as more stable during wine storage.

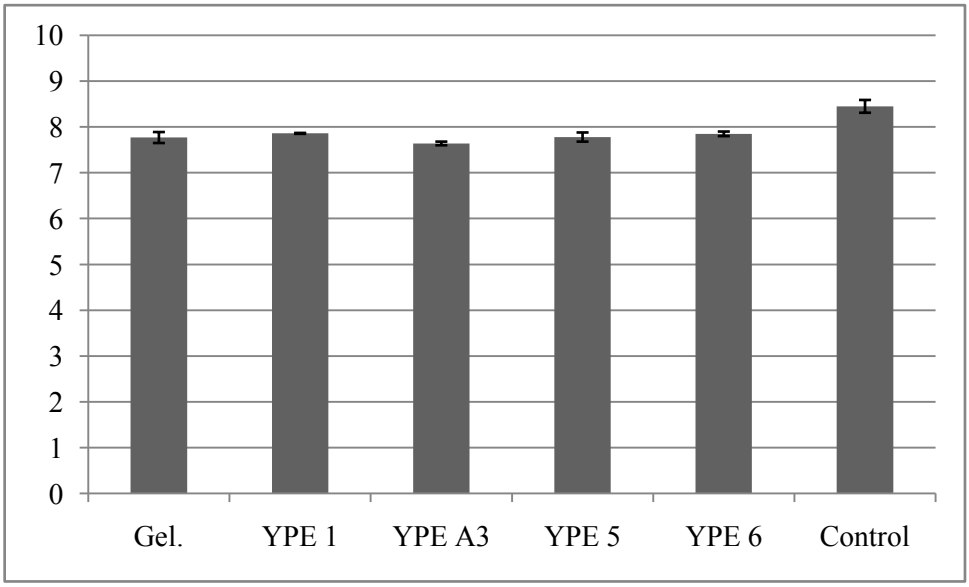

Figure 33: Colour intensity in wine after fining in experiment in carboys of cycle 5 (bars show standard deviation above and below the means)

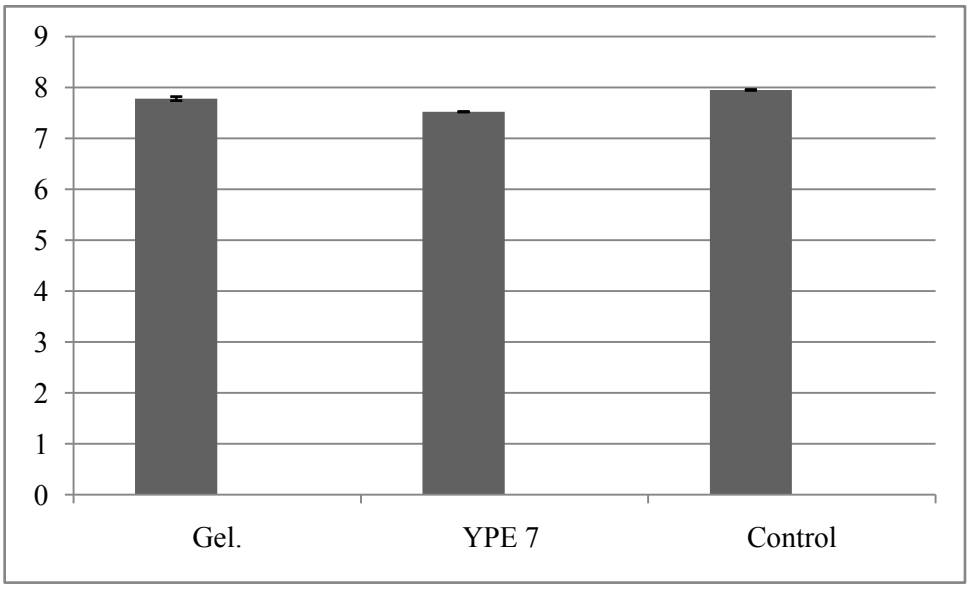

Figure 34: Colour intensity wine after fining with YPE 7 in experiment in bottles of cycle 5 (bars show standard deviation above and below the means).

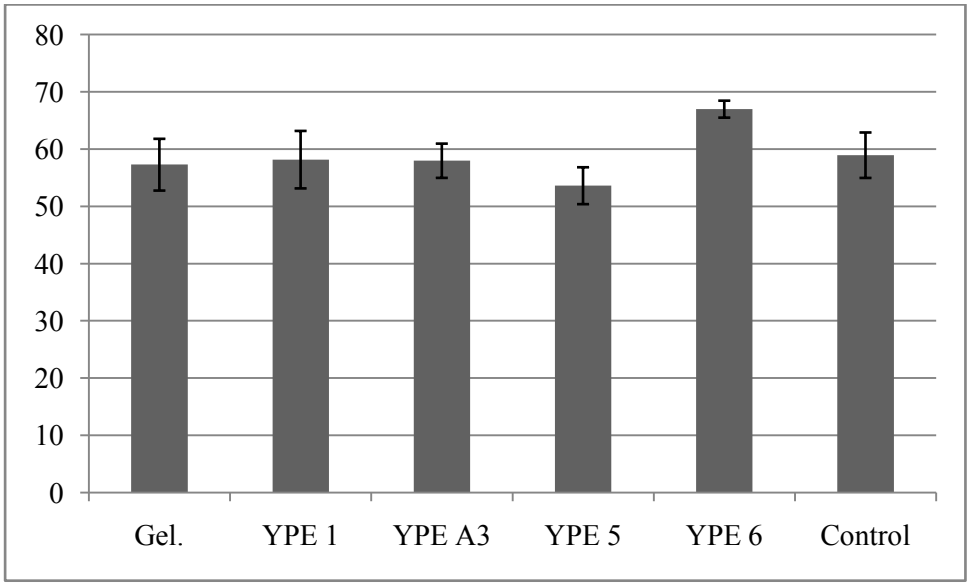

Figure 35: PVPP-index in wines after fining in experiment in carboys of cycle 5 (bars show standard deviation above and below the means)

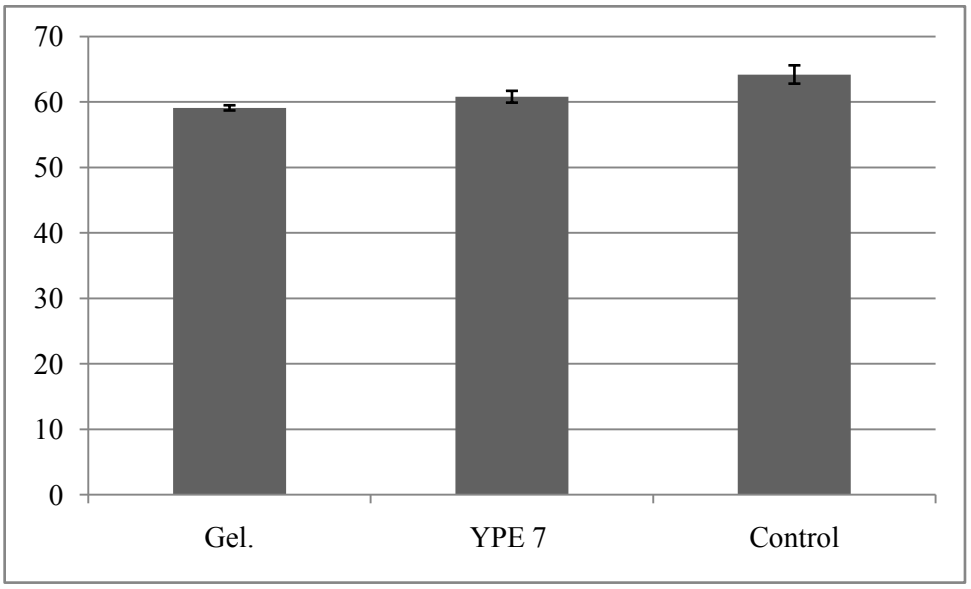

Figure 36: PVPP-index in wine after fining with YPE 7 in experiment in bottles of cycle 5 (bars show standard deviation above and below the means).

The index of total polyphenol, absorbance (O.D.-optical density) at 280 nm of the cuvee of red wines was not influenced by fining with gelatine or YPE1, A3, 5, 6, 7 in the experiments in carboys or bottles when standard deviations were considered (figures 37 and 38).

The BSA-index, regarded as an index of wine astringency, was diminished by 45 % when wine was fined with gelatine in experiment in carboys and not influenced by the other treatments taking into account standard deviations. BSA index was however not changed considering standard deviations when wine was fined with gelatine in the later experiment in bottles and diminished by 20 % after treatment with YPE 7.

The wines of the experiment in carboys were tasted by 15 experienced panelists after fining. The tasters could not distinguish significantly (α of 0.05 evaluated according to DIN 10951) the wine fined with gelatine, YPE 1, A3, 5 or 6 from the wine of the control variant.

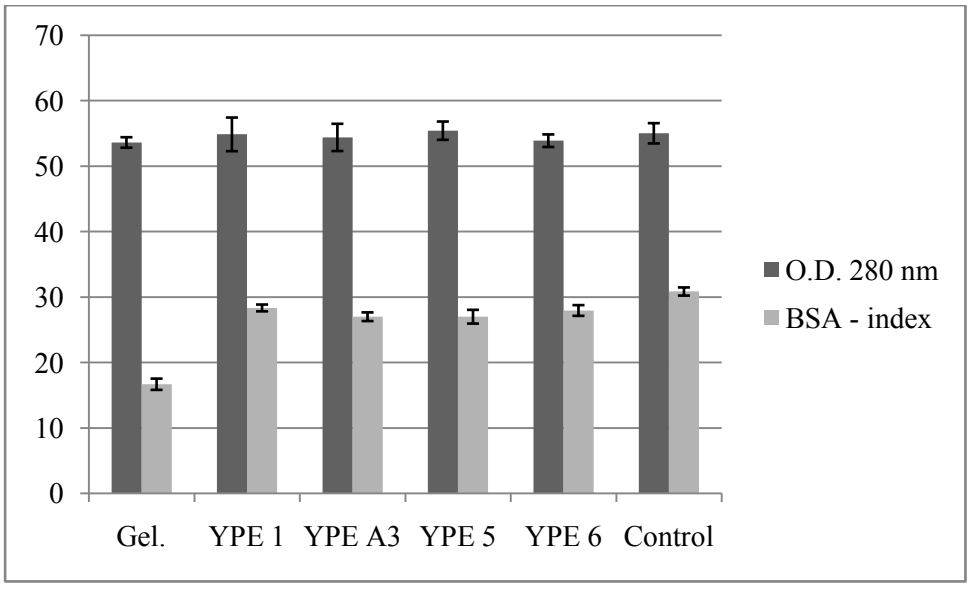

Figure 37: Indices of polyphenols in wines after fining in experiment in carboys of cycle 5 (bars show standard deviation above and below the means)

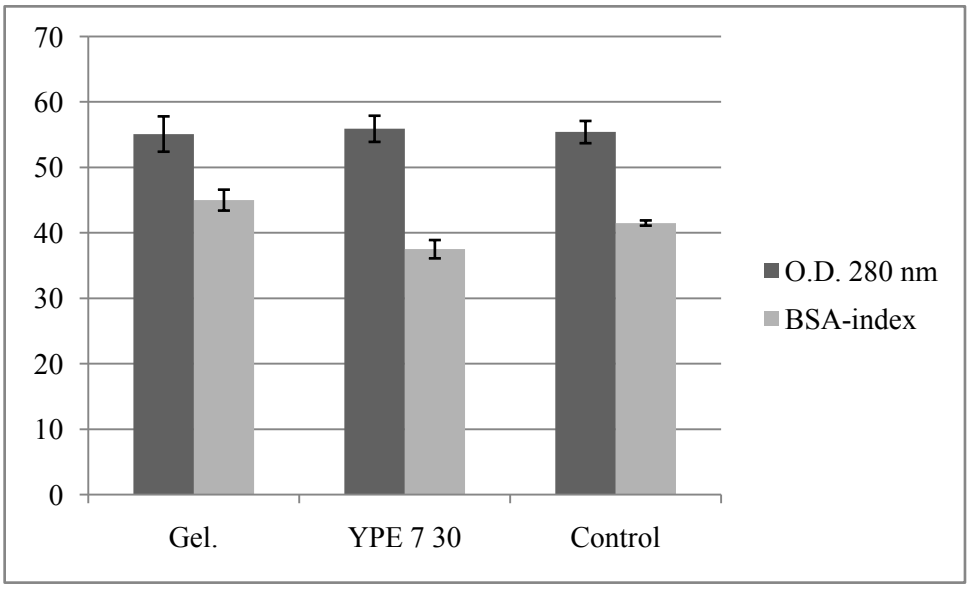

Figure 38: Indices of polyphenols in wines after fining in experiment in bottles of cycle 5 (bars show standard deviation above and below the means)

3.2.6 Fining experiments with the commercial YPE 8 (experiments of cycle 6)

The commercial YPE 8 was tested in three red wines. The first one was a wine of the variety Dornfelder of the viticultural area Palatinate of vintage 2011, which was stored 18 months after maceration and alcoholic fermentation and was racked when malolactic fermentation was completed.

The second wine was a cuvee of red grape varieties of the Department of Grapevine Breeding of Hochschule Geisenheim University of vintage 2012, which was stored 6 months after maceration and alcoholic fermentation and which had finished malolactic fermentation. The third wine was a cuvee of equal parts of two red wines of vintage 2013 of the grape varieties Rondo and Bolero respectively also

provided by the Department of Grapevine Breeding of Hochschule Geisenheim. The wines were stored for two months after maceration and alcoholic fermentation and until malolactic fermentation of the wines was finished.

The chemical parameters of the three wines are detailed in table 23. All three wines were dry and had completed malolactic fermentation. The alcohol concentrations were similar but the pH values were slightly different.

Table 23: Chemical parameters of red wines of cycle 6

	Dornfelder 2011	**Cuvee 2012**	**Cuvee 2013**
Alcohol	13.4 ml/100 ml	13.6 ml/100ml	13.7 ml/100 ml
Reducing sugars	2.2 g/l	0.4 g/l	0.1 g/l
Tartaric acid	1.8 g/l	1.9 g/l	3.1 g/l
Malic acid	< 0.1 g/l	< 0.1 g/l	< 0.1 g/l
Lactic acid	2.7 g/l	1.9 g/l	1.9
pH	3.6	3.7	3.5

Fining experiments of this cycle used YPE 8 in concentrations of 5, 10, 20 or 40 g/hl and gelatine served as a reference treatment used at 100 ml/hl.

The red wines of Dornfelder and the cuvee 2012, which were fined both in May 2013, but after different storage times (cf. above), were both clear with a limpidity measured by nephelometry of below 7 NTU characterizing a limpid wine. The fining did not change limpidity of the wine to levels above 10 NTU (above 10 NTU a slight haze in be visible in some wines).

Cuvee 2013 was fined immediately after malolactic fermentation and had an initial turbidity of 15 NTU. Such a value is typical for a wine with a slight haze and the turbidity of the wine was reduced to a level of limpidity (of below 10 NTU) when it was fined with gelatine or YPE 8 in all concentrations (figure 39). The fining behaviour as far as formation of visible flakes (named here flocculation) and the beginning of sedimentation of lees are concerned is shown in tables 24 and 25.

Gelatine flocculated in all three wines and YPE 8 also formed flakes when used in concentrations of 10 g/hl and above. Beginning of flocculation was much faster in cuvee 2013 compared to the other two wines. YPE 8 used at 5 g/hl did not form visible flakes, but its compounds, presumably proteins, precipitated in all three wines. All fining treatments showed formation of precipitates and settling of fining lees. The wine of the control variant formed only small sediment of lees of below 1 ml/100 ml in cuvee 2013, the wine with the highest initial turbidity and the shortest storage time before fining.

The volume of lees was 1 ml/100ml in all three wines after fining with YPE 8 in all concentrations and 2 ml/100 ml in wine after fining with gelatine.

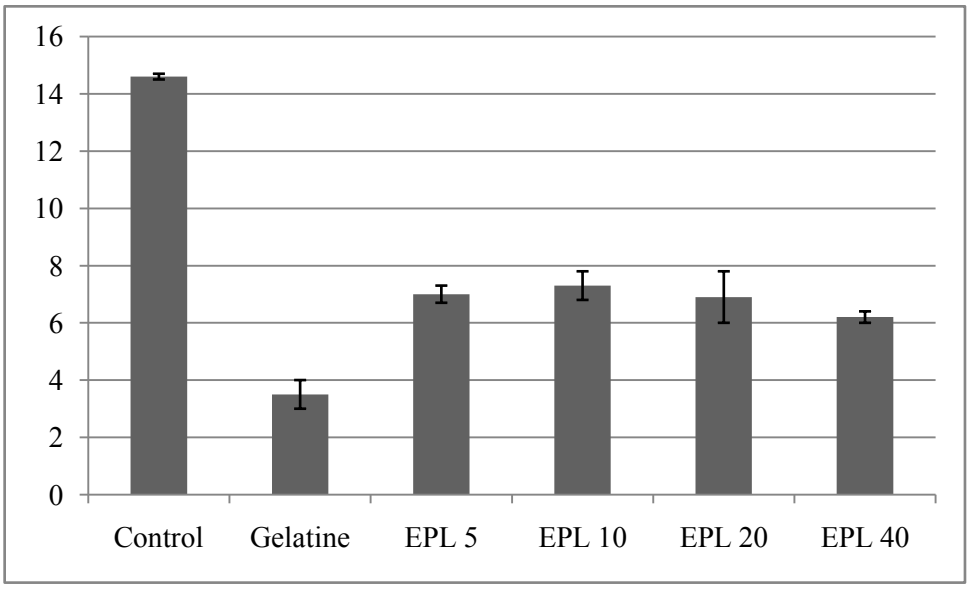

Figure 39: Turbidity [NTU] after fining of cuvee 2013 of cycle 6
(bars show standard deviation, numbers are g/hl)

Table 24: Flocculation during fining of YPE 8 and gelatine in three red wines

	Beginning of flocculation [hours]		
	Dornfelder 2011	**Cuvee 2012**	**Cuvee 2013**
YPE 8 5 g/hl	not in 72 h	not in 72 h	22
YPE 8 10 g/hl	6	6	4
YPE 8 20 g/hl	2	4	0.5
YPE 8 40 g/hl	1	1	0.25
Gelatine	6	1.5	0.25
Control	not in 72 h	not in 72 h	not in 72 h

Table 25: Sedimentation of lees during fining of YPE 8 and gelatine in three red wines

	Beginning of sedimentation [hours]		
	Dornfelder 2011	**Cuvee 2012**	**Cuvee 2013**
YPE 8 5 g/hl	21	21	2
YPE 8 10 g/hl	6	6	1.5
YPE 8 20 g/hl	2	4	0.5
YPE 8 40 g/hl	2	1.5	0.5
Gelatine	6	2	0.5
Control	not in 72 h	not in 72 h	2

The colour intensity of the three red wines tested was decreased when YPE 8 was used at dosages equal or above 20 g/hl by 5 to 10 % in all three wines. Fining with gelatine caused a decrease of 2 to

5 %. The hue and the index of total polyphenols of all three wines were not influenced by the fining considering the standard deviations.

Cuvee 2012 was also treated with 20 and 40 g/hl YPE 8 in volumes of 10 l in carboys integrating a control variant. The wines fined with 20 or 40 g/ hl YPE 8 were judged as being less astringent during a tasting 4 weeks after fining by the majority of 10 experienced panelist.

It has to be remarked that the wine fined with 40 g/hl in the carboy and racked after three days like the other variants showed an increased turbidity compared to the limpid control wine.

Cuvee 2013 was as well used for a fining experiment in carboys with the variants 10 g/hl YPE 8, 100 ml/hl gelatine and a control variant: A sensory evaluation 4 weeks after fining did not show differences between the wine fined with YPE or gelatine and the control variant in triangle tests according to DIN10951.

3.3 Discussion

3.3.1 Experiments of cycle 1

The white must used in this fining experiment showed a good auto clarification resulting in the formation of lees and a turbidity of 70 NTU after 48 hours. Must should present turbidity values between 100 and 250 NTU as reviewed by Ribéreau-Gayon et al. (2004a, pp.561-572) before fermentation. It could be concluded that this must presented a turbidity that was convenient to start fermentation after 2 days of settling without any fining agent. On the other hand using fining agents could reduce the time necessary to reach turbidity in the convenient range, which could constitute an economic and processing advantage. A continuous sampling of the must to monitor the evolution of the turbidity during clarification was not possible in the type of assay performed, but the formation of flakes and the onset of formation of sediment in the must, which is a prerequisite of clarification, were continuously observed. An addition of tannins out of chestnut, mainly composed of ellagitannins according to the supplier, was done in all fining treatments except treatment with isinglass. This addition of oenological tannins is supposed to accelerate or to reinforce the formation of protein-polyphenol-composites resulting in particles that settle down by gravity (cf. general considerations on fining under 1.3.3.1.). The addition of tannins during fining should also allow a more complete precipitation of the proteins of the fining agent. This effect seems to be however more based on empirical observations than on scientific studies. The addition of oenological tannins or silica gel is though a common practice used during the fining of white must or wine, which is poor in phenolic compounds and especially in proanthocyanidins. Most of the studies on protein tannin interactions in wine and wine conditions cited in the sections 1.3.3.3 and 1.3.3.4. of the introduction dealt in deed with interactions between proanthocyanidins, the main class of tannins in must and wine and proteins (cf. e.g. Ricardo da Silva et al. 1991, Yokotsuka and Singleton 1987). Isinglass is said to flocculate in white must or wine with a low level of total polyphenols, which is once again a fact reported in the "oenological literature" (cf. Ribéreau-Gayon 2004 b), but which seems to be based on empirical observations. Isinglass was the only fining agent used without tannin out of chestnut and showed an immediate visible flocculation whereas flocculation and formation of a sediment in the other variants treated with YPE and tannin of chestnut was not observed sooner than in the control variant. No fining treatment decreased the final turbidity of the must stated at the end of the fining. The fining of must with YPE and tannin resulted in an enrichment of the must with polyphenols after fining as indicated by a rise in the absorbance at 280 nm. This enrichment in polyphenols seemed to be related with a slight increase in absorbance at 420 nm characteristic of a yellow colour (Ribéreau-Gayon et al. 2004b, pp. 253-255). It could be summarized that only the treatment of must with isinglass had a potential to accelerate must clarification. The oenological tannin added before fining with YPE did not completely precipitate with proteins of the YPE or with other must proteins or constituents.

The clarification of the white wine was slow taking 5 days and isinglass was again the fining agent showing the fastest visible flocculation. YPE 1 and gelatine combined with oenological tannin formed

also visible flakes, but after 9 instead of 4 hours. YPE 3 and 4 combined with oenological tannin precipitated in the wine and started forming sediment at 9 hours. It is possible that pectins were present in white grape must inhibiting the precipitation of proteinaceous fining agents (Ribéreau-Gayon et al. 2004b, pp. 383-390 and de Freitas et al. 2003). These pectins and polysaccharides could be hydrolyzed or precipitated during the vinification. That could explain why visible flocculation of YPE 1 combined with tannin was observed in the white wine but not in the white must. The control variant presented a turbidity of around 15 NTU, which is barely visible as observed in the studies presented in this thesis. Only fining with YPE 3 and 4 resulted in a white wine having turbidity below 10 NTU meaning limpidity as observed in the presented studies. Treatments with proteinaceous fining agents combined with oenological tannin resulted in an enrichment of the white wine with polyphenols monitored by the rise in absorbance at 280 nm, which was already found when fining of must with YPE combined with oenological tannin was performed. The absorbance at 320 nm, characteristic of derivatives of cinnamic acid, was also increased, when the tannin out of chestnut was added to white wine during fining. Tannins of chestnut were however not reported to contain derivatives of cinnamic acid as main compounds (Ribéreau-Gayon et al. 2004b, pp. 409-410). Fining white wine with isinglass did not diminish the concentration of total polyphenols (absorbance at 280 nm) whereas Cosme et al. (2008) found a decrease of procyanidins by such a treatment. It has to be mentioned that in case of white wines a part of the absorbance at 280 nm can also be caused by wine proteins (Somers and Ziemelis 1985), which could mask small decreases in the concentrations of procyanidins absorbing UV-light at 280 nm. Isinglass showed the fastest flocculation of all fining treatments in this experiment, but did not result in a wine that was more limpid after 5 days of observation contrary to results shown by Cosme et al. (2008) and Marchal et al. (2002b).

Red wine of Pinot noir could be clarified by fining with gelatine and YPE 1, 3 and 4 in both experiments, in cylinders and at bigger scale, in bottles. Gelatine was the only product however that reduced turbidity below 20 NTU, which is a value characterizing a wine of low turbidity but not presenting complete brilliant limpidity according to the experience of the studies presented herein. Gelatine and YPE 1 showing flocculation visible by naked eye in the trial in cylinders were also the fining agents showing the fastest onset of sedimentation in the bigger scale experiment. The clarifying effect of YPE in case of red wines made from Pinot noir stated by Charpentier et al. (2006) was confirmed by these studies. A red wine of Cabernet Sauvignon, a typical grape variety of the Bordeaux-region, could also be successfully clarified with YPE 1, 3 or gelatine. That confirmed the results of fining of red wines from Bordeaux with other YPE as presented by Charpentier et al. (2006) and Iturmendi et al. (2012). The colour intensity of the Pinot noir wine variants fined with YPE or gelatine was diminished by 5 % to 10 %, which was also observed by Charpentier et al. (2006). On the other hand PVPP-index of the red wine fined with YPE 1, 3, 4 or gelatine was increased reflecting a higher proportion of tannin-anthocyanin-combinations among the anthocyanins measurable by decolorization with sulphur dioxide. These tannin-anthocyanin combinations were described as being

more stable against chemical degradation during wine storage (Glories 1984). The colour intensity of Cabernet Sauvignon wine was diminished by 10 % by fining with YPE 1 and 3, which was also observed in the study of Charpentier et al. (2006), but the potential colour stability monitored by PVPP-index (cf. above) was not changed by the fining treatments. The concentration of total polyphenols of Pinot noir red wine fined with YPE 1 or 3 was diminished by 5% to 10 %, but the concentration of total polyphenols and the BSA- index reflecting tannins' astringency of Cabernet Sauvignon wine was not reduced by fining with gelatine or YPE. The panel of tasters did only distinguish the Pinot noir fined with YPE 3 from the control wine in triangle-tests, whereas the variants fined with gelatine, YPE1 and 4 did not show sensorial differences compared to the control variant. Tasters were asked to rate the red wine variants also on the descriptors "red berries", "bakers yeast" and "reductive notes", which were chosen by some experienced wine tasters beforehand, but no differences between the control wine and wine fined with YPE 3 was found. Red wine of Cabernet Sauvignon fined with gelatine or YPE 1 and 3 could not de differentiated from control samples. It could be concluded that wine's sensory quality was preserved, when red wines of Pinot noir or Cabernet Sauvignon were fined with YPE 1 or 3 and that according to sensory analysis no aroma compounds typical of yeast extracts (Mahadevan and Farmer 2006; Münch and Schieberle 1998a) or inactive yeast derivatives designed for oenological use (Comuzzo et al. 2006) were transmitted to both red wines used in cycle 1 of the studies.

One question that should be answered in the fining experiments of cycle 1 was if YPE 1, that proved to be a potential fining agent according to the supplier Oenofrance, could be replaced by another YP produced by a less complex and ideally less expensive production process. YPE 3 showed a potential to replace YPE 1, but it was not retained as its protein profile did not match the objectives set for an ideal YPE useable for fining (cf. part III of the thesis).

3.3.2 Experiments of cycle 2

The first batch of YPE of the three yeast strains selected in part I of the thesis was available for this cycle. All these three YPE were produced by an identical manufacturing process.

It was decided to focus on the treatment of white grape must and white wine with a high concentration of polyphenols, that should be extracted by a rougher grape treatment and pressing of the must. Proanthocyanidins are the type of polyphenols of grape that readily precipitate with proteins as shown in the introduction (cf. e.g. Poncet-Legrand et al. 2007b; Ricardo da Silva et al. 1991 and Yokotsuka and Singleton 1987). Proanthocyanidins are indeed mainly located in and extracted from grape skins, stems and seeds but not from the pulp (as reviewed in Ribéreau-Gayon et al. 2004b, pp.179-259). Most of the compounds of white grape must obtained by conventional grape processing and pressing derive from the pulp. However, the final concentration of total polyphenols in the Riesling must and Riesling wine, fermented from the same must used in cycle 2, was in the same range as in the must and wine used in cycle 1 of the fining experiments.

The initial turbidity of the Riesling must was 150 NTU, a level, that would be convenient to start alcoholic fermentation as outlined in discussion of results of fining cycle 1. Casein and isinglass used as reference fining treatments were the only products that showed a faster onset of formation of lees in the must than was observed in the control variant. The yellow colour of must estimated by absorbance at 420 nm was increased after fining with YPE B1, but that could also be caused by a slight rise in turbidity to 20 NTU compared to the control (8 NTU), as particles in suspension could reflect parts of the light at 420 nm. Casein reduced the concentration of total polyphenols, as observed by Cosme et al. (2008) in case of proanthocyanidins in white wine. The absorbance at 320 nm characteristic of derivatives of cinnamic acid (Somers and Ziemelis 1985) was also decreased by treatment with casein.
Isinglass and casein were in case of the Riesling wine the only fining agents that showed visible flocculation, but only isinglass clarified the wine. YPE B1 increased the turbidity during three days of fining. A microscopic analysis of this YPE showed that it seemed to contain parts of yeast cell walls, which could cause turbidity. The absorbance at 420 nm was also increased in Riesling wine treated with YPE B1 as in the grape must. Casein was as already observed in the Riesling must the only fining agent diminishing total polyphenols and cinnamic acid compounds. It has to be kept in mind that the enrichment of the must in polyphenols, mainly procyanidins, was not successful, what could partly explain the poor performance of flocculation, sedimentation and clarification of YPE 1, A 1, B1 and C 1 (only used in wine fining). Another factor that could reduce the precipitation of proteins of fining agents with polyphenols of the must or wine could be the low pH, as outlined in the studies with gelatine derivatives with polyphenol fractions of Yokotsuka and Singleton (1987, 1995). A low pH was characteristic of Riesling must and wines of 2010 in the Rheingau region.
Cycle 2 of the fining experiments also showed that red wines having a very high initial turbidity and a lot of pectins or glucans, detected by an “ethanol-test”, could not be successfully clarified by gelatine. The ethanol test, consisting in adding one part of pure ethanol to two parts of wine, detected glucans, if white filaments appeared, or a high level of pectins by an increase in turbidity or formation of a heavy precipitate. Pectins and polysaccharides probably inhibited the precipitation of proteins of gelatine with tannins of the red wine (results not shown). This inhibition of precipitation of proteins in wine conditions by high concentrations of pectins and polysaccharides has been already described (Ribéreau-Gayon et al. 2004b, pp. 383-390 and de Freitas et al. 2003).
It was decided that red wines should be used for fining experiments with YPE in which the chosen gelatine could flocculate and settle down. YPE showing a good precipitation and clarification capacity could then be used for fining to examine if YPE would be less sensible to pectins than gelatine preparations in further experiments (beyond the work of this thesis).
The cuvee of German red wines used for the fining experiment of cycle 2 could be clarified by YPE 1 and gelatine and these two fining agents were the only ones that precipitated in the must and started sedimentation before “natural sedimentation” of the lees was observed in the control wine variant.

YPE A1, B1 and C1 did not present visual flocculation in the red wine used for the experiment, which was successfully clarified by gelatine.

Red wines are naturally rich in proanthocyanidins (cf. review of Ribéreau-Gayon et al. 2004b, pp.179-259), the tannins of wine that precipitate readily with proteins (cf. above of this section). A YPE convenient for fining of wine could thus show a fast precipitation, flocculation and sedimentation ,linked with clarification, in red wine, but not in white wine or grape must (cf. above). That was however not observed in case of the three YPE A1, B1 and C1.

Furthermore the pH of the cuvee of red wines was 3.4 and thus in the normal range of German red wines and 0.4 to 0.5 unities higher than in the white Riesling must or wine, which should also favour a precipitation of wine tannins with proteins of YPE according to Yokotsuka and Singleton (1987, 1995). It could be concluded that these first batch of YPE from the three selected strains was not convenient for fining.

It was decided that the future research was focused on fining of red wines and on red wines that could be fined with gelatine cf. above.

3.3.3 Experiments of cycle 3

A new batch of YPE of the three selected strains produced with a different technology than used for manufacture of YPE A1, B1 and C1 was employed for this cycle of experiments.

Two batches of YPE were delivered in case of strain A, but which were produced with the same method according to the information of the supplier. YPE 1 and A1 were used to compare their performance in the new red wine cuvee, which could be also fined with gelatine (result not shown).

YPE 1 and A 2.2. clarified the red wine in both concentrations tested, but YPE A2.1., that was reported to be produced identically than YPE A2.2. showed a non-reproducible and inferior fining and clarification capacity as well as YPE A1. YPE B2 and C2 clarified the red wine when used alone, but not when combined with oenological tannin, that should facilitate protein precipitation during fining (cf. discussion of experiments of cycle 1). It seemed that particles of protein-tannin-precipitate were formed in case of a combined treatment of YPE B2 and C2 with oenological tannin, which did not settle down during the three days of fining and observation. Precipitation and sedimentation of lees was however observed in these variants but not in the control variants (result not shown). It was later tried to analyze the proteins precipitated during fining and in the supernatant of YPE B2 and C2 by SDS-PAGE to gain an insight into that feature. The stable turbidity formed by these YPE when used in fining combined with oenological tannin could however not be reproduced.

Colour intensity and index of total polyphenols of the red wine cuvee was not diminished by fining with 30 g/hl of YPE 1, A2.2., B2 and C2 or at maximum by 5 % and comparable to fining with the reference product gelatine, which confirmed the findings of Charpentier et al. (2006).

It could be concluded that YPE used in fining cycle 3, obtained from the three selected yeast strains and produced with another manufacture method than YPE A1, B1 and C1 of cycle 2, had a potential to constitute an alternative fining agent for red wine.

The general ability of YPE as fining agents stated by Charpentier et al. (2006) and Iturmendi et al. (2012) could be confirmed. YPE A2.2. had a performance comparable to YPE 1, but was produced with a less complex production method. It seemed that the yeast strain could have an influence on the fining performance of the YPE of the three strains, but on the other hand the two batches of same manufacture of strain A, i.e. A2.1. and 2.2. behaved differently in fining. A conclusion on the influence of the yeast strain on fining ability of the YPE was not yet possible.

3.3.4 Experiments of cycle 4

A new batch of YPE of the three strains was produced, i.e. A3, B3 and C3, with an alternative technique by the yeast supplier. The protein profiles of the three strains obtained during autolysate production at the laboratory (cf. part I of this thesis) were indeed different from the profiles of YPE A2.2., B2 and C2 (cf. part III of this thesis). Extracts with proteins of high molecular masses were searched for as former studies (Tschiersch et al. 2010 and Yokotsuka and Singleton. 1987 and 1995) showed that fining agents of a low degree of hydrolysis precipitated more completely in wine or had a higher influence on its polyphenol profile.

A red wine of Syrah that could be clarified by fining with gelatine was used for the first series of experiments of cycle 4. YPE 1 used since cycle 1 of the experiments, as well as YPE A2.2., that was the most interesting YPE for fining of cycle 3 were integrated in this research cycle. Gelatine showed a superior fining performance than all the YPE in Syrah. YPE 1 and A3 could slightly improve or "maintain" the limpidity of Syrah wine, as well as YPE A2.2., B3 and C3 used at 50 g/hl without adding oenological tannin. These YPE also showed precipitation of probably protein-tannin-composites and sedimentation of the precipitates, which was not observed in the control variants except a formation of lees in 3 of 18 cases, but with a smaller volume of lees than in the fined variants. YPE1 showed the fastest onset of precipitation of the YPE followed by YPE A3, but YPE A3 used at 30 g/hl did not precipitate in the Syrah wine. YPE A3 did not form protein-tannin-particles that were not settled down within three days and increased the turbidity of the wine. Formation of particles not settling down within three days of fining was however observed in case of YPE 1, A2.2., B3 and C3 when used in combination with tannin and was already observed in case of fining with YPE B2 and C2 in cycle 3. The colour intensity of Syrah wine was at maximum diminished by 5 % by fining with YPE or gelatine, which was already reported by Charpentier et al. (2006). The concentration of total polyphenols (absorbance at 280 nm) was not diminished by any fining procedure in the Syrah wine. The YPE that seemed to be interesting for fining, i.e. YPE 1, A 2.2. and A3, were tested in a second red wine of the variety Rondo. That second red wine had a colour intensity and a concentration of total polyphenols that was 50 to 60 % higher than in the Syrah wine and presented a richness in tannins that caused a high astringency in this red wine as observed by some experienced tasters. This red wine had

initially a high concentration of pectins and was thus treated with enzymes with pectinase and glucanase activity (cf. material and methods) to overcome an inhibition of precipitation of proteins of the fining agent observed before enzyme treatment and also described by Ribéreau-Gayon et al. (2004b, pp. 383-390) and de Freitas et al. (2003). Gelatine was also in the Rondo wine the fining agent showing the fastest onset of precipitation of proteins and settlement of lees, followed by YPE1. Gelatine was as well the fining agent having the highest clarification performance of all treatments, but was followed by YPE A3. The combined treatment of Rondo wine with oenological tannin and YPE A2.2. did not result in an increase of wine turbidity, which was observed in case of Syrah wine. A reason for that may be that the Rondo wine had a much higher concentration of polyphenols and probably proanthocyanidins, which would facilitate the precipitation of protein fractions of the YPE2 causing turbidity. None of the fining treatments did change the composition of phenolic compounds in that Rondo wine being very rich in such compounds and that was also observed by Ricardo da Silva et al. (1991) and Fischerleitner et al. (2003) in red wines fined with gelatine.

It could be concluded that gelatine was still the most performative fining agent of cycle 5, as far as speed and extent of clarification was concerned, and that YPE 1 could be used as fining alternative in some treatment variants. On the other hand YPE A3 manufactured by a less complex production method than YPE 1 showed a better final clarification performance when combined with tannin in Syrah wine and in all treatments in Rondo wine compared to YPE 1 but had a slower clarification kinetic. Furthermore the protein profile of YPE A3 showed proteins of higher molecular mass and practically identical to the autolysates of the three yeast strains produced in the laboratory whereas YPE 1 was only composed of polypeptides.

The most successful YPE besides YPE1 were produced from strain A and thus it could be concluded that the factor “strain” could have an influence on fining performance of an YPE

3.3.5 Experiments of cycle 5

YPE A3 of fining cycle 4 already presented a protein profile identical to the autolysates of laboratory (cf. part I and III of the thesis) and showed a clarification performance comparable to YPE 1 in most cases although the fining and clarification process still took longer than in case of YPE1.

Furthermore YPE 1 known as potential fining agent but having disadvantages already described (complex production process and high degree of protein hydrolysis during manufacturing) and YPE A3 still did not reach the clarification performance of the gelatine preparation employed as reference product.

New YPE tested in fining cycle 5 were produced from strain A (YPE A4) and from other yeast strains mainly from the genus *Saccharomyces* by different confidential production processes. Their protein profile corresponded globally to those of the autolysates produced in laboratory of the three selected yeast strains (cf. part I and III of the thesis).

A cuvee of four different red wines was used for this cycle of experiments that had turbidity values comparable to the red wines used in fining cycle 4. Such turbidity characterizes a red wine not heavily

charged with particles causing turbidity, but not yet being limpid. The colour intensity was inferior to Syrah wine of cycle 4 (8 instead of 12) and much lower than in Rondo wine of cycle 4 (18).
The index of total polyphenols of the cuvee was comparable to Syrah wine and half of that of Rondo wine of cycle 4.
YPE 1, 5 and 6 presented the highest clarification capacity of the YPE and fining with them resulted in a limpid wine as did fining of the red wine cuvee with gelatine. YPE 1 and 6 were furthermore the YPE which had the fastest clarification kinetic, that was still a bit slower than in case of gelatine (start of precipitation and sedimentation at 1 hour). Clarification started however at least in the first 6 hours when used at 30 g/hl and YPE 6 was thus the YPE produced with another less complicated and complex production technology that showed a fining performance comparable to YPE 1.
The fining experiment in carboys confirmed the good clarification capacity of YPE 1 and 6 at 30 g/hl, but also of YPE A3 at 50 g/hl as well as gelatine at 100 ml/hl (corresponding to 10 to 12 g/hl dry mass according to information of the supplier).
The colour intensity of the wine was at maximum diminished by 10 % by YPE, which was comparable to gelatine fining and was again in line with the results of (Charpentier et al. 2006). YPE 6 seemed to increase slightly the colour stability of the red wine as monitored by an increase of PVPP-index (cf. discussion cycle 1).
The index of total polyphenols was not influenced by any of the fining treatments as was found in case of gelatine by Fischerleitner et al. (2003) and Ricardo da Silva et al. (1991).
The BSA-index that is reported to present the tanning power of a red wine according to the work of de Freitas. (1995) and should reflect red wines' astringency was diminished when wine was fined with gelatine. That was striking as the total concentration of polyphenols (index of absorbance at 280 nm) was not diminished, but Sarni-Manchado et al. (1999) pointed out that after fining red wine with gelatine soluble tannin-protein combinations remained in the wine. Tannins involved in such combinations could still absorb UV-light at 280 nm but react no more with BSA.
15 experienced tasters evaluated the wines fined with gelatine, YPE 1, A3, 5 and 6 in triangle tests and could not distinguish them from non-treated control wine. The potential decrease of astringency that could be expected in case of wine fined with gelatine as BSA-index decreased was not confirmed by sensory analyses. Aroma compounds typical of yeast extracts (Mahadevan and Farmer 2006; Münch and Schieberle 1998a) or of inactice yeast derivatives marketed for wine treatments (Comuzzo et al. 2006) characterized with "cheesy", "yeasty" or "meaty" aroma notes were not transferred to the cuvee of red wines by fining with YPE 1, A3, 5 and 6 in concentrations that were detectable by the panel of tasters during sensory analysis.
The clarification performance of YPE 7 was difficult to evaluate as the fining experiment could only be done three month after the other trials of this cycle and at that time the red wine cuvee was already limpid. The clarification kinetic was comparable to gelatine, but it has to be kept in mind that the control wine was at that time completely limpid, which was not the case in the other experiments of

cycle 5. It is possible that colloids constituting or stabilizing particles causing turbidity, like pectins or polysaccharides, were precipitated or hydrolysed at the time of fining with YPE 7. Pectins or polysaccharides could inhibit and slow down the flocculation of YPE of the other assays of cycle 5 according to de Freitas et al. (2003) and Ribéreau-Gayon et al. (2004bpp. 383-390).

The colour intensity of the red wine was decreased by YPE 7 to the same extent than did the other YPE used in that fining cycle 5. The PVPP index was not influenced by fining with YPE7, as was neither the index of total polyphenols and the BSA-index. A sensory evaluation of red wine fined with YPE 7 was not possible due to the small volume of red wine cuvee still available when the fining took place.

3.3.6 Experiments of cycle 6

Oenofrance a company of the Sofralab group, the supplier of all YPE used in these fining studies succeeded due to the experiences made in fining cycles 3 to 5 and to the product characterization (cf. part III) in establishing the commercial YPE 8 named for fining of must and wine.

This commercial YPE 8 was tested in three red wines at two times. The red wines had at that time different periods of storage since vinification. The first two wines, a Dornfelder of vintage 2011 and a cuvee of German red wines of vintage 2012 were fined 18 and 6 month after end of maceration and alcoholic fermentation. The wines were already practically limpid (NTU around 10) at that time.

YPE 8 showed a behaviour of precipitation/flocculation and sedimentation comparable to gelatine (100 ml/hl) when used at or above 20 g/hl. The influence on wine colour and index of total polyphenols was also comparable to that of gelatine. The same behaviour was observed in a red wine cuvee of 2013, which was fined immediately after malolactic fermentation, which occurred 2 month after the end of maceration and alcoholic fermentation. This third wine had still a small turbidity of 15 NTU at that time and fining it with gelatine or YPE 8 at or above 10 g/hl yielded a completely limpid wine of a value below 10 NTU. The volume of fining lees was smaller in case of fining with YPE 8 (1l/100l) than in case of gelatine (2l/100l) and this tendency was already observed in cycle 1 and 5 of the fining experiments.

Fining lees are normally discarded in the production of quality wine or the wine contained in the lees is recycled by filtration which consumes time and material. In that way a smaller volume of fining lees constitutes an economical advantage in wine production.

The studies of Charpentier et al. (2006), Iturmendi et al. (2010) and Iturmendi et al. (2012) stating an ability of YPE to replace traditional fining agents in red wines could be confirmed at the end of 6 cycles of fining experiments.

The red wine cuvees of vintage 2012 and 2013 were also submitted to sensory evaluation.

The cuvee 2012 was judged by the majority of 10 experienced tasters as being less astringent than the control wine after fining with 20 g/hl or 40 g/hl YPE 8. It has to be pointed out that the small scale fining trial in cylinders showed still a presumably complete precipitation of YPE 8 at 40 g/hl as the final turbidity of the wine was not increased. The final turbidity of the wine was however slightly

increased to 20 NTU in the experiment in carboys, which could be due to overfining. Overfining would occur if proteins of the YPE stayed in the wine, which could not be precipitated by the concentration of polyphenols present. It could be possible that three days of storage after fining were too short to allow the complete sedimentation of the protein-tannin precipitates.

Cuvee 2013 fined with 10 g/hl YPE 8 was not distinguished in a triangle test from the control variant, which could be due to the lower concentration of YPE that was necessary to clarify cuvee 2013 successfully, but which did not reduce wine's astringency.

A decrease of astringency after fining with YPE 8 could be due to precipitation of polyphenolic compounds that cause astringency (Hufnagel and Hofmann 2008; Sun et al. 2013; Vidal et al. 2004; Weber et al. 2013) by the protein fining agents as observed by Maury et al. (2001).Yet, the concentration of total polyphenols was not changed by the fining treatments. Another cause of a decrease of astringency could be the potential presence of mannoproteins in YPE 8 which interact with wine's polyphenols (Charpentier et al. 2006; Poncet-Legrand et al. 2007a; Riou et al. 2002) probably by weak chemical bonds. Mannoproteins were indeed found in preparations of inactive yeast by Comuzzo et al. (2012) and their binding to polyphenols could reduce their reactivity with proteins of human saliva or mucous membranes in the mouth and thus their sensory perception as found by Vidal et al. (2004) and Escot et al. (2001).

4 Thesis Part III: Partial characterization of yeast protein extracts and protein fining agents

4.1 Material and methods

4.1.1 Chemical substances

All substances and compounds used were of analytical grade unless otherwise specified.

Water purified in a Genpure-CAD-apparatus of TKA (part of Thermo Electron LED, Niederelbert, Germany) was used for all analyses. Ethanol, methanol, sodium hydroxide, sodium chloride, phosphoric acid 85 %, acetic acid, Tris (Tris (hydroximethyl) aminomethane), glycine and hydrogen peroxide 30 % were from Carl Roth (Karlsruhe, Germany).

Sulphuric acid, hydrochloric acid 37 % fuming, sodium dodecyl sulphate (SDS > 85%), manganese chloride tetra hydrate, copper sulphate pentahydrate, sodium potassium tartrate tetrahydrate, sodium carbonate, Folin Ciocalteu's reagent and glycerol anhydrous were delivered by Merck (Darmstadt, Germany). Calcium chloride dihydrate, Coomassie Brilliant Blue G-250 (Sigma reference number 27815), bromophenol blue (Sigma reference number B 0126), bovine serum albumin (BSA, Sigma reference number A 7906), concanavaline A (Sigma reference number C2010), peroxidase from horseradish (Sigma reference number P 6782), DL- dithiothreitol, polysorbate-20, 4-chloro-1-naphthol (Fluka reference number 25328) were obtained from Sigma-Aldrich Chemie (Munich, Germany).

4.1.2 Quantification of proteins in fining products

Samples were dissolved in water, except for YPE 6 and proteins were quantified in convenient dilution according to Bradford (1976). Proteins in YPE were also determined quantitatively as described by Lowry et al. (1951), as this method is mentioned in the resolution OIV-OENO-452-2012 of the International Organisation of Vine and Wine. BSA served as reference protein for establishing the calibration of both methods and measurements were done in triplicate.

All spectrophotometric measurements were performed in a Unicam UV 300 apparatus of Thermo Fisher Scientific (Dreieich, Germany).

4.1.3 SDS-PAGE of yeast proteins and fining products

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Mini PROTEAN- 3-Cell of Bio-Rad Laboratories (Munich, Germany). The procedure was based on Laemmli (1970), but precast Mini-PROTEAN TGX-gels of Bio-Rad with a polyacrylamide gradient of 4 to 20 % were used and the sample treatment and protein detection were modified (cf. below). Fining products were dissolved in water to have a concentration of 60 g/l and in case of YPE 4 the liquid extract was lyophilised in an Alpha 1-2 apparatus of Martin Christ (Osterode, Germany) before analysis. The solutions of fining products were mixed with 2 volumes of sample buffer (pH 6.8, 62.5 mM Tris-HCl, 250 ml/l glycerol, 20 g/l SDS, 40 mM DTT,125 mg/l bromophenol blue) to a final concentration of 20 mg/l and heated 5 min at 95°C. Isinglass and casein were dissolved in

water at a concentration of 6 mg/ml and then treated as the other samples yielding a final concentration of 2 mg/ml. 15 µl of final sample were applied per gel well. SDS-PAGE molecular weight standards of Bio-Rad (reference number 161-0317) were treated as the samples and 1 µg of the individual proteins were applied per well. Electrophoresis was done at a voltage of 150 V until bromophenol blue left gel at ambient temperature.

4.1.4 Detection of proteins and glycoproteins on the SDS-PAGE gels

Total proteins on the gels were detected by staining with Coomassie Brilliant Blue according to Schägger and von Jagow (1987).

Glycoproteins on the gels were stained with a Pierce ® Glycoprotein Staining Kit (Pierce Biotechnology, Rockford, USA). The detection is based on PAS-method, coupling oxidation of sugars to aldehydes and detection of aldehydes by Schiff's reaction.

Gels were documented in an Alpha Imager ® EC of Alpha Innotech (now part of ProteinSimple, Santa Clara, USA). SDS-PAGE analyses were performed with two repetitions.

4.1.5 Detection of glycoproteins on SDS-PAGE gels by Western Blotting

The detection of glycoproteins was done according to Hawkes (1982) with some modifications.

This method is based on Western Blotting and the coupling of the blotted proteins containing α-mannose or α-glucose to a peroxidase from horse radish using concanavalin A. Glycoproteins are then detected by peroxidase activity. SDS-PAGE was performed as outlined above and proteins were transferred on a Bio-Rad Immun Blot PVDF (polyvinylidene fluoride) membrane in a Bio-Rad Trans Blot Cell at 100 V, 350 mA for 90 min using a buffer of 25 mM Tris and 192 mM glycine.

After protein transfer free protein binding sites on the membrane were blocked for 1 h in a solution of 10 g/l BSA in Tris buffered saline (TBS) of 50 mM Tris, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 0.5 g/l polysorbate-20, at pH 7.4. After two wash steps of 5 min in TBS, membrane was incubated in TBS with 10 g/l BSA and 50 mg/l concanavalin A for 1 h. Membrane was then washed three times 5 min in TBS and subsequently incubated in TBS with 10 g/l BSA and 50 mg/l peroxidase for 1 h. This was followed by three washing cycles of 5 min in TBS. The detection of glycoproteins combined with peroxidase was done as outlined by Hawkes (1982) using the peroxidase substrate 4-chloro-1-naphthol. Membranes were subsequently rinsed in water until purple bands were clear. Blots were run in triplicate for all samples and pictures were taken with Alpha Imager (cf. above).

4.1.6 Detection of mannose and glucose in yeast products

Yeast products were hydrolysed with two repetitions modified according to Dupin et al. (2000 b) in order to quantify also both sugars in polymeric forms derived from yeast cell wall. Hydrolysis was done for only 60 min instead of 90 min and hydrolysates were neutralised with 5M NaOH. Sugars were then quantified with a Megazyme D-mannose, D-fructose and D-glucose enzymatic kit (Megazyme International, Ireland).

4.2 Results

The protein profile of YPE as well as the protein fining agents gelatine, isinglass and casein was characterized by SDS-PAGE. The protein concentration was determined in protein fining products and YPE used since fining cycle 2.

Glycoproteins and sugars were mainly determined in YPE from fining cycle 2 to 6 except YPE A4, which was judged to have an inferior fining performance than A3 and was not further retained.

4.2.1 Protein quantification in YPE and fining products

Figure 40 shows the protein concentration determined according to Bradford (1976) of all charges of gelatine used in cycles 2 to 6, as well as of casein and isinglass used in cycle 2 of the fining experiments. Bovine serum albumin was used as standard for calibration. The three lots of gelatine differ in their protein concentration expressed in g/100 g of dry gelatine, although the product was a liquid having 10 to 12 g/100 ml gelatine concentration. Isinglass and especially casein had a much higher protein concentration than gelatine of 10 or 32 g/100g, but in case of casein the standard deviation was also high (17 g/100 g).

Most of the YPE had protein concentrations between 1 and 2 g/100 g (figure 41). YPE A3, 5, 7 and 8 showed protein concentrations between 2 and 5 g/100 g and YPE 6 had by far the highest protein concentration of all YPE of 14 g/100 g

The protein concentration of a selection of YPE of cycles 5 and 6 was also analysed by the method of Lowry et al. (1951) and table 26 shows the values compared to values of the method of Bradford (1976). All YPE showed a higher protein concentration determined by the method of Lowry et al. (1951), although the same lot of BSA was used for calibration of both methods. Furthermore YPE1 had the lowest protein concentration measured by Bradford's method and the highest concentration determined by Lowry's method. YPE 6 had the highest protein concentration measured by Bradford's method and a medium concentration determined by Lowry's method.

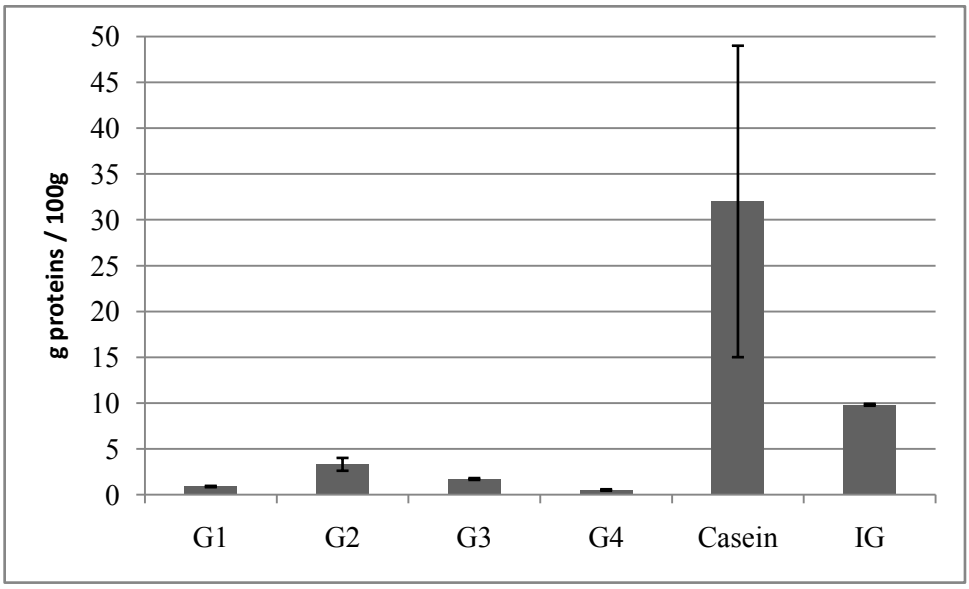

Figure 40: Protein concentration in gelatine, casein and isinglass measured according to Bradford (1976) (G1 to G4 are 4 lots of gelatine; IG means isinglass; bars show the standard deviation)

Table 26: Protein concentration in YPE determined according to Bradford (1976) and Lowry et al. (1951)

Product	Method of Bradford (1976)		Method of Lowry et al. (1951)	
	Concentration [g/100g]		Concentration [g/100g]	
	Mean	Standard deviation	Mean	Standard deviation
YPE 1	0.2	0.03	35.6	4.5
YPE A3	2.5	0.03	26.4	1.19
YPE 5	2.5	0.13	16.3	3.52
YPE 6	13.8	1.03	25.6	2.48
YPE 7	2.3	0.24	19.1	2.26
YPE 8	4.6	0.2	21.8	1.4

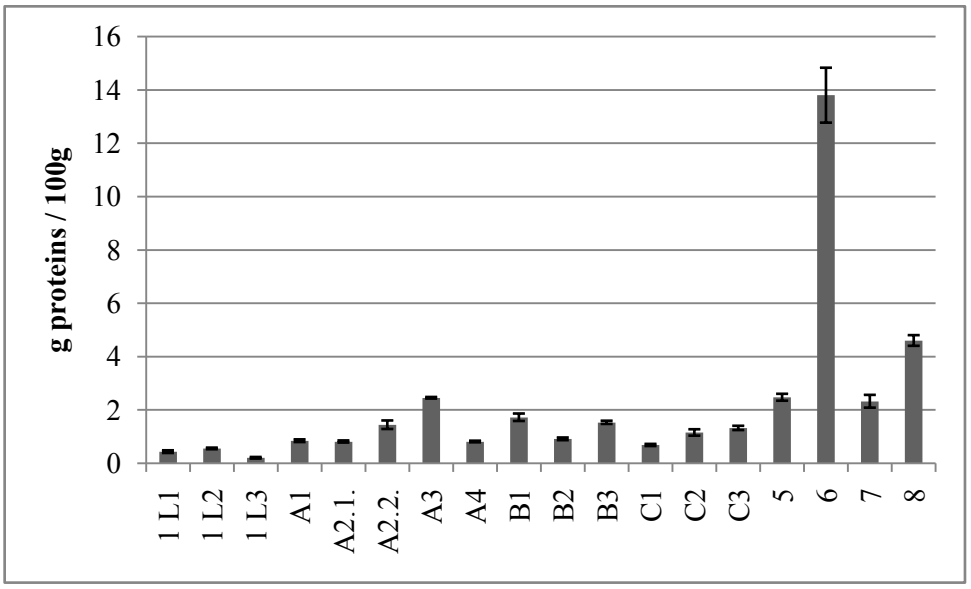

Figure 41: Protein concentration in YPE of fining cycle 2 to 6 measured according to Bradford (1976) (L1 to L3 denote three lots of YPE1; bars show the standard deviation)

4.2.2 Distribution of molecular masses of proteins

Table 27 shows the molecular mass distribution of proteins in the reference fining products and the YPE. Gelatine and all YPE were analyzed at a concentration of 20 mg/ml, resulting in 300 µg in the gel well. In case of gelatine a dry mass concentration of 100 g/l was used for calculation and casein and isinglass that were richer in proteins as measured according to Bradford (1976) were applied at 2 mg/ml resulting in 30 µg product per gel well. Molecular mass markers had an individual concentration of 1µg. YPE A1, B1 and C1, as well as A2.1., A2.2., B2, C2 as well as A3, B3 and C3 are from production cycles 1, 2 and 3 respectively. Total proteins were stained with Coomassie Blue.

Table 27: Molecular mass distribution of fining products and YPE determined by SDS-PAGE

Product	**Zones of molecular masses in kDa**	**Product**	**Zones of molecular masses in kDa**
Gelatine lot 1 to 4	Smear in range from 60 to 20	YPE B2	Faint band at 27 kDa Bands between 5 and 15
Isinglass	Bands at 200, 110 to 120, 45 to 50	YPE B3	Bands at 40 25 to 30 20
Casein	Bands at 30 to 35		

Table 27: continued

Product	Zones of molecular masses in kDa	Product	Zones of molecular masses in kDa
YPE 1	Polypeptides below 7 Center of the band around 4	YPE C1	2 bands between 10 and 15
YPE 2		YPE C2	Faint band at 27 Bands between 5 and 15
YPE 3		YPE C3	Bands at 40 25 to 30 20
YPE 4		YPE 5	Bands at 80 to 90 40 to 50 30 10 to 20
YPE A1	2 bands between 10 and 15	YPE 6	Bands at 40 to 50 27 to 33 5 to 20
YPE A 2.1. A2.2.	Faint band at 27 Bands between 5 and 15	YPE 7	Faint zone between 30 and 40 Main zone 5 to 20
YPE A3	Bands at 50 to 55 40 to 45 30 to 35 15 to 20	YPE 8	50 to 60 40 30 Main zone between 8 and 23
YPE A4	Bands at 50 to 60 25 to 35 15 to 20		
YPE B1	Bands at 50 to 55 45 37 to 40		

Gels of SDS-PAGE showing YPE of strain A, B, C of the three production methods and used in fining cycles 2 to 5, as well as gelatine, YPE1, YPE 5 to 7 and YPE 8 are shown in figures 42, 43, 44 and 45. YPE used in fining cycle 2 and 3 of strain A, B, and C and produced by the first and second production method, i.e. A1, A2.1., A 2.2., B2, C1 and C2, were mainly composed of proteins of molecular masses below 20 kDA. YPE B1 was the exception having proteins of higher masses than 20 kDa, but YPE B1 was not successful in fining as far as flocculation, sedimentation of fining precipitate and clarification was concerned. YPE of the third production method, i.e. A3, B3 and C3 as well as YPE 4, 5, 6 and YPE 8 had protein fractions of masses higher than 20 kDa and the protein profiles were similar to those of the autolysates of strain A, B and C produced at laboratory scale during yeast selection and pilot production.

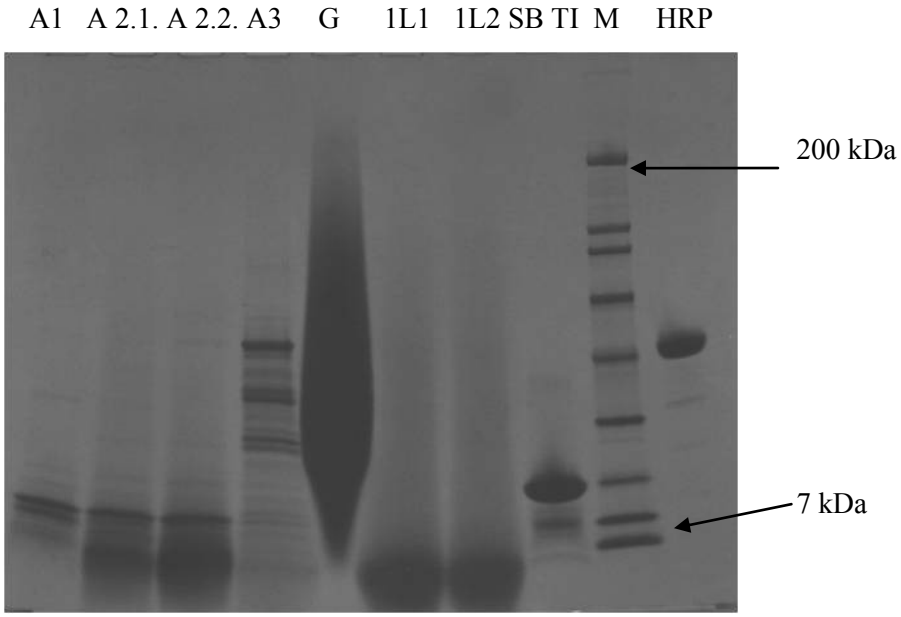

Figure 42: SDS-PAGE gel showing YPE of strain A, gelatine and YPE 1
(cf. YPE determination above figure; G and L denotes gelatine and lot;
M is the mixture of molecular mass standards going from 200 to 7 kDa;
SB TI is Soybean Trypsin Inhibitor; HRP is peroxidase from horseradish)

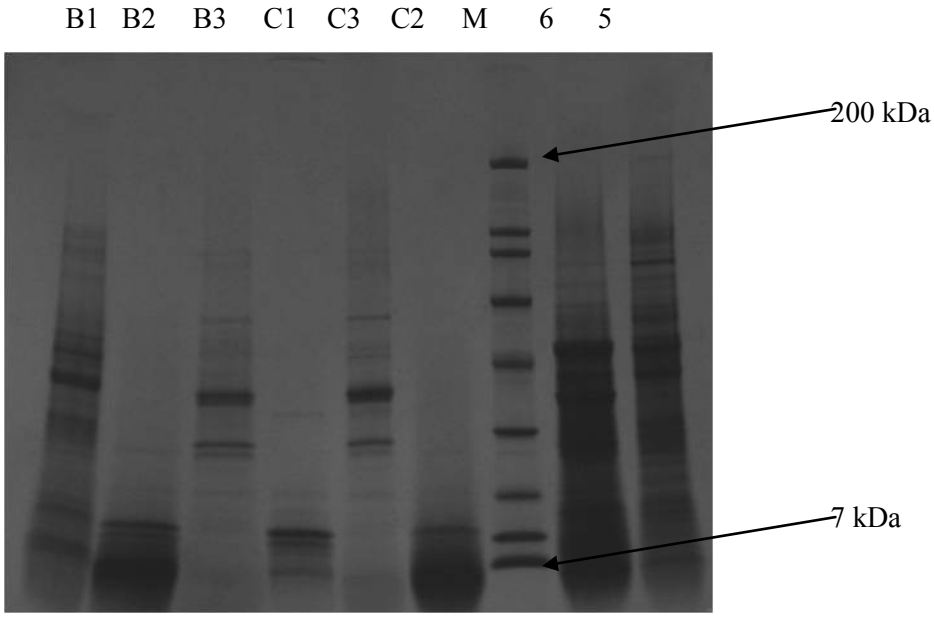

Figure 43: SDS-PAGE gel showing YPE of strain B, C, YPE 5 and 6
(cf. YPE determination above figure; M is the mixture of molecular mass standards going from 200 to 7 kDa;)

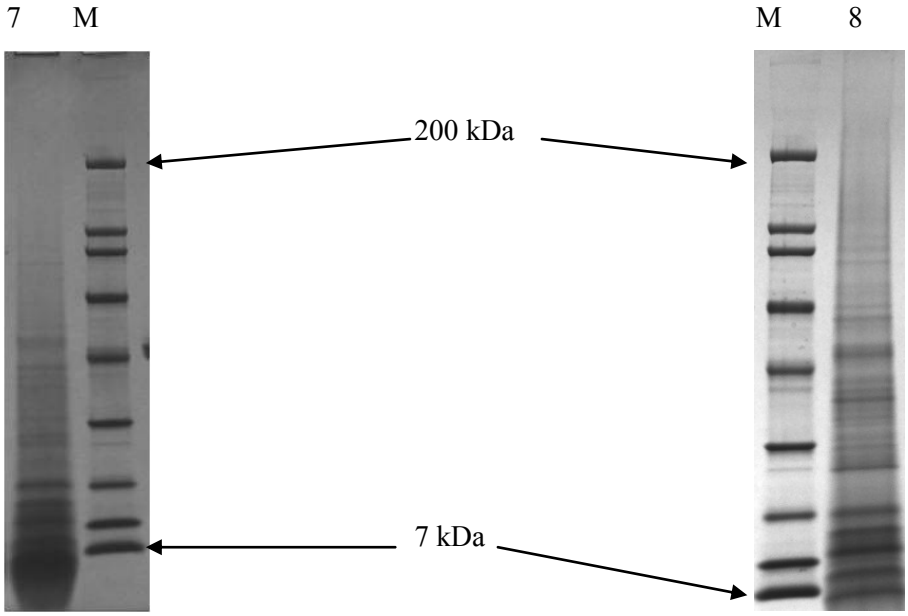

Figure 44: SDS-Page gel showing YPE 7
(YPE determination above figure
M Mass standards cf. fig. 42 and 42)

Figure 45: SDS-Page gel showing YPE 8
(YPE determination above figure; M Mass standards cf. fig. 42 and 43)

4.2.3 Glycoproteins detected in YPE and gelatine

4.2.3.1 Detection of total glycosylated proteins

The Pierce ® Glycoprotein Staining Kit using the PAS method detected all glycosylated proteins that could be present in YPE and gelatine. Horseradish Peroxidase, a glycoprotein, was used as positive control and Soybean Trypsin Inhibitor as negative control.YPE and gelatine were used at a concentration of 20 g/l unless otherwise stated. The detection limit of the method was in a g/100 g range.

The molecular mass of horseradish peroxidase was estimated to be 45 kDa, which was confirmed by indication of the supplier Sigma-Aldrich Chemie (Munich, Germany). All YPE showed a smear of glycoproteins having molecular masses of possibly above 100 kDa (figures 46 to 49). It has to be kept in mind that sometimes glycosylated proteins are difficult to separate by molecular mass in SDS-PAGE analysis (Strayer Leach et al. 1980). Lots 2 and 3 of YPE 1 were analyzed and showed equal results. No glycoproteins were detected in case of gelatine (result not shown).

Proteins of masses above 100 kDa were not detected on SDS-PAGE gels by Coomassie blue (cf. table 27 and figures 42 to 45).

A1 A 2.1. A 2.2. A3 B1 B2 B3 C1 HRP SB TI

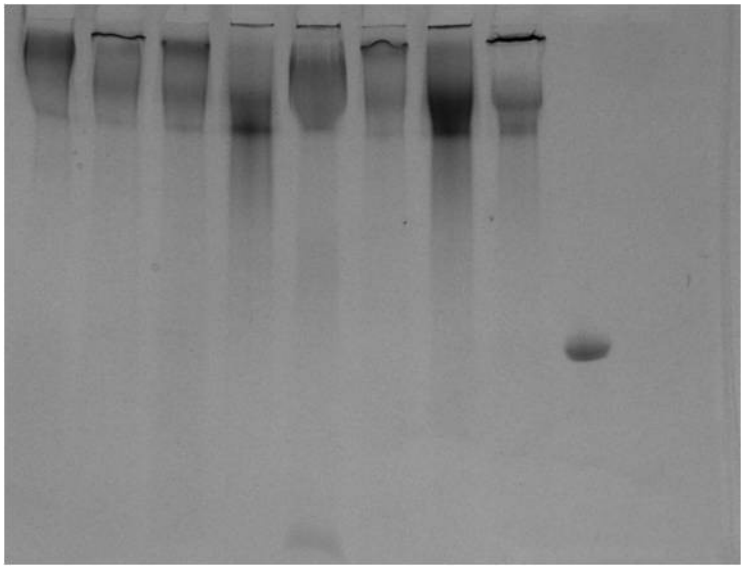

Figure 46: Total glycoproteins on SDS-PAGE gels (cf. YPE determination above figure; HRP is horseradish peroxidase at 6.7 µg; SB TI is Soybean Trypsin Inhibitor at 13.3 µg)

C2 C3 1 SB TI HRP

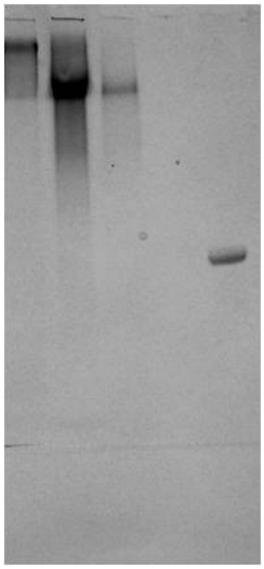

Figure 47:
Total glycoproteins on SDS-PAGE gels (explanations cf. figure 46)

5 6 6 7

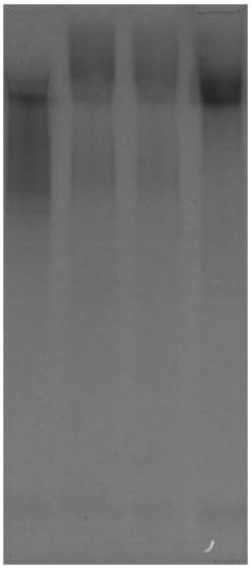

Figure 48:
Total glycoproteins on SDS-PAGE gels (explanations cf. figure 46; HRP on same position as in fig 47)

8 HRP

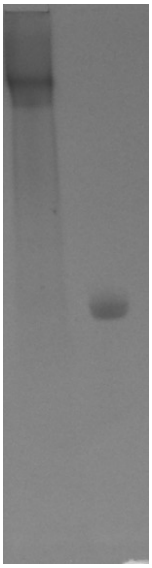

Figure 49:
Total glycoproteins on SDS-PAGE gels (explanations cf. figure 46)

4.2.3.2 Detection of glucosylated and mannosylated proteins after Western Blotting

The modified method of Hawkes (1982) was used to quantify proteins that were α-mannosylated or α-glucosylated. Mannoproteins of yeast cell wall could be detected by this method, but glucose seems to be present only in ß-form in polymers in cell wall of Saccharomyces cerevisiae (Klis et al. 2002). Horseradish peroxidase, a glycoprotein, was used as positive control and Soybean Trypsin Inhibitor as negative control.YPE and gelatine were used at a concentration of 20 g/l unless otherwise stated. Figures 50, 51, 52 and 53 show proteins separated by SDS-PAGE, transferred on Blotting membrane and detected on membrane with modified method of Hawkes (1982).

Horseradish peroxidase was used for Blotting in the same concentration as on gels where glycoproteins were directly detected by PAS method with kit, but the "spot" on the gel is much bigger than on PAS gels. Hawkes (1982) reported indeed that glycoproteins in a range of mg/100 g could be detected with his Western Blot method. Ovalbumine, which is the 45 kDa standard used in protein mass standard mixture on SDS-PAGE gels (denoted M), is detected by Hawkes'method on the blots. Ovalbumine is a glycoprotein containing α-mannose and β-glucosamine (Conchie et al. 1969), but it was not detected by the PAS method (result not shown). That is another indication that the Western blot method used modified from Hawkes (1982) is more sensitive than the PAS method used in the

Pierce ® Glycoprotein staining Kit, which was also found for concanavalin-A- horseradish peroxidase bridge staining compared to PAS staining by Allen et al. (1976).

All YPE showed glycosylated proteins near the gel wells, of possibly high molecular mass, which were also detected with the Pierce Staining Kit using the PAS method. Furthermore the modified method of Hawkes (1982) detected α-mannosylated and perhaps α-glucosylated proteins of a possible mass range of over 100 to below 45 kDa (mass of HRP), possibly to around 30 kDa, in all YPE of strain A and B and in YPE C1, C3, 5, 6, 7 and 8. It has to be remarked that in case of YPE1, A1, A2.1., A2.2., B2, C1 and C2 no proteins were detected with Coomassie Blue in a mass range over 30 kDa.

A1 A2.1. A 2.2. A3 B1 B2 B3 M HRP HRP/10

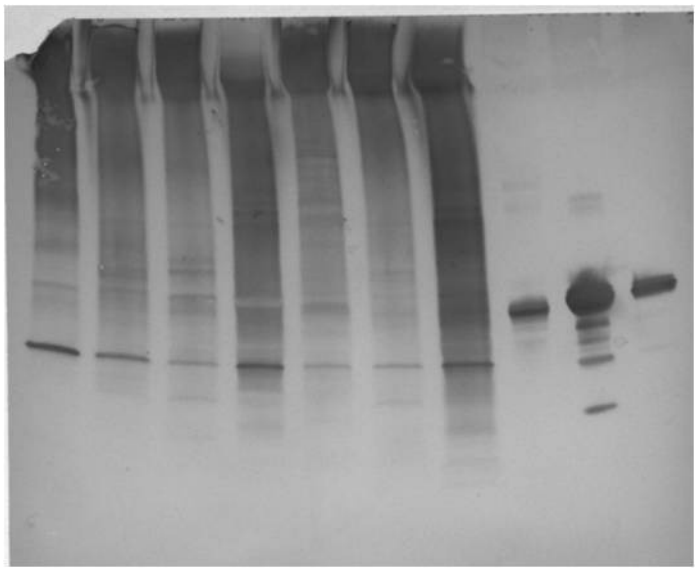

Figure 50: Glycoproteins of YPE of strains A and B on Western Blot membrane
(find YPE terms above the figure; M denotes standard protein mixture for molecular masses; HRP horseradish peroxidase at 6.7µg; HRP/10 horseradish peroxidase at 0.67µg)

C1 C2 C3 G G/10 1 L2 1L3 SB TI M HRP

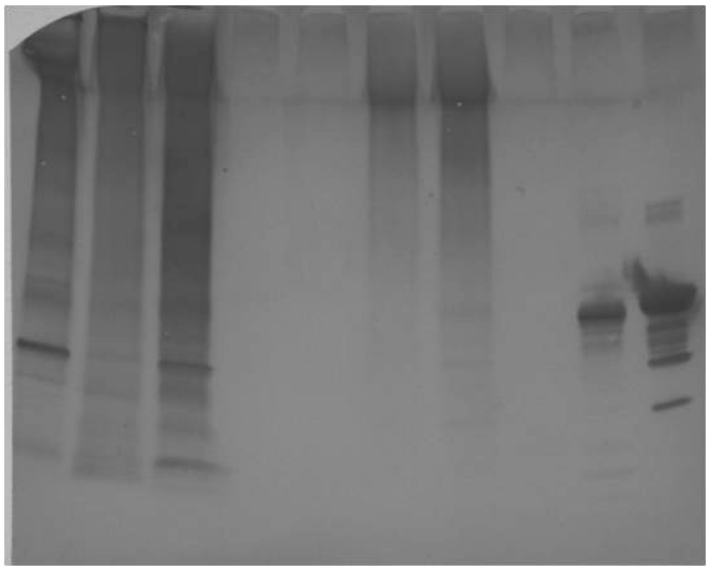

Figure 51: Glycoproteins of YPE of strain C, gelatine and YPE 1 on Western Blot membrane (find YPE terms above the figure; G denotes gelatine at 20 g/l and G/10 gelatine at 2 g/l, L is lot; M is standard protein mixture for molecular masses;
SB TI SoybeanTrypsin Inhibitor at 13.3 µg; HRP horseradish peroxidase at 6.7µg)

5 6 6 7 M HRP

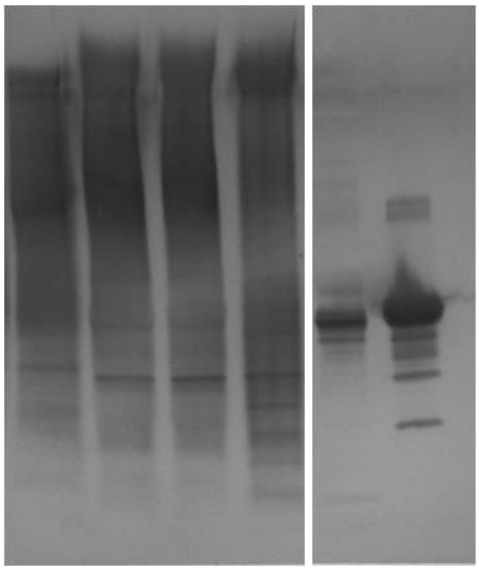

Figure 52: Glycoproteins of YPE 5, 6 and 7 on Western Blot membrane (find YPE terms above the figure; M is standard protein mixture for molecular masses
HRP horseradish peroxidase at 6.7 µg)

8 HRP

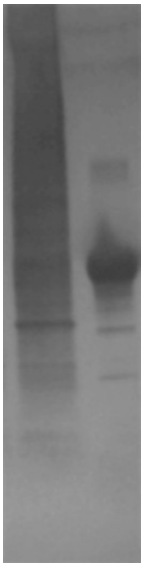

Figure 53: Glycoprotein in YPE 8 (terms cf. figure 52)

4.2.4 Sugar concentration in selected YPE

Figure 42 shows the sugar concentration determined after acid hydrolysis of most of the YPE used in cycles 2 to 6. Mannose and glucose determined by this method could derive from cytoplasm or the cell wall (refer to discussion).YPE 1 had a low concentration of glucose and mannose in both lots measured. YPE A1 had a much higher glucose concentration than YPE A2.1., A2.2. and A3. YPE B1, B2, C1 and C2 had all a higher glucose concentration than YPE 1 and A3. Commercial YPE 8 shows a low glucose concentration and 5 g / 100 g mannose. Mannose was clearly detectable after hydrolysis in YPE A2.1., A 2.2., A3, B2, C1, C2, 5, 6, 7 and 8, which is another indication that these YPE contain mannoproteins which could be also detected by the two methods of glycoprotein detection used within this work.

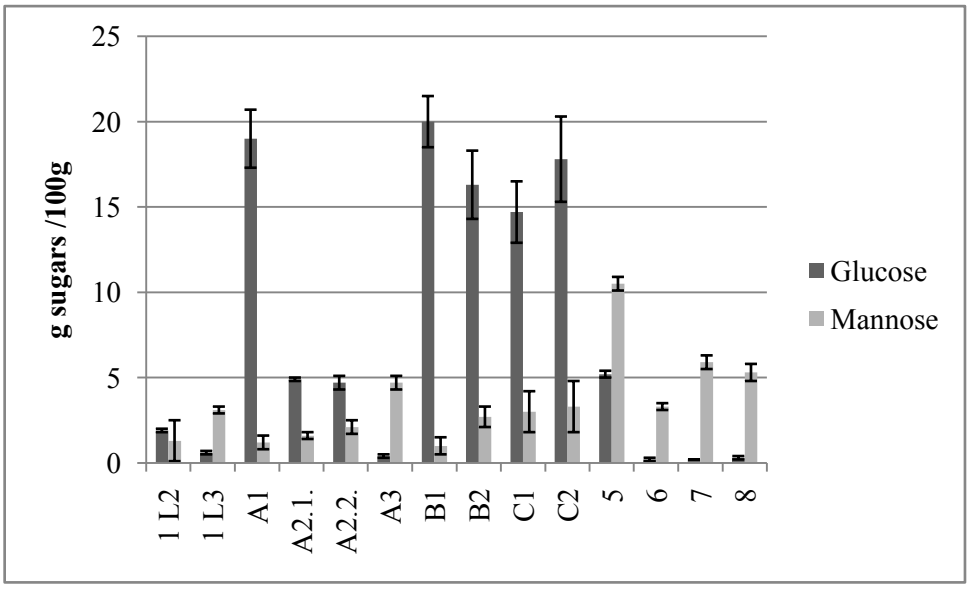

Figure 42: Sugar concentration in YPE (L denotes lot; bars give the standard deviation)

4.3 Discussion

4.3.1 Quantitative analysis of proteins in YPE and reference protein fining products

The protein concentration in gelatine, isinglass, casein and the YPE were determined by the method of Bradford (1976). This method uses the stain Coomassie Brilliant Blue G which was also used in the staining of the SDS-PAGE gels to detect total proteins. Furthermore the quantitative protein analysis in autolysates in part I of the thesis by the method of Lowry et al. (1951) and the tentative quantitative analysis by densitometry on SDS-PAGE gels stained with Coomassie Blue, a stain widely used for protein detection on PAGE gels, showed a poor correlation. This may be explained by different detection principles of the method of Lowry et al. (1951) and of Coomassie blue (cf. discussion in part I of the thesis).

Gelatine showed varying protein concentrations in the different charges used and it was reported that a gelatine preparation of bovine skin had a very different response factor in the assay of Bradford (1976) than the standard protein BSA (bovine serum albumin) (Stoscheck 1990). That may be due to a different composition in amino acids in the gelatine used and BSA.

It can be seen from the arguments mentioned above that the quantification according to Bradford (1976) chosen for protein quantification in YPE and the other fining product is also a compromise, but in random samples this quantification gave results in the same order of magnitude than a quantification on SDS-PAGE gels by densitometry (results not shown).

One charge of BSA was used for all quantitative protein analyses by the methods of Bradford (1976) and Lowry et al. (1951) shown in this thesis.

YPE 6 was by far the yeast extract with the highest protein concentration of 14 g/100 g followed by YPE A3, 5 and 7 having between 2 and 5 g/100 g in the Bradford assay. YPE 1 however that also proved to be a reliable fining agent in red wines had in all charges analyzed a much lower protein concentration ranging between 0.2 and 0.5 g/100 g measured with the same method.

The protein quantification by the Bradford assay can thus not be used as single criterion for the selection of a performing YPE for fining (cf. also part II of the thesis).

The protein concentration in YPE used in cycle 5 and 6 of the fining experiments was also analyzed with the assay of Lowry et al.(1951) as this method of quantification is proposed in the monograph of OIV on YPE (OIV-OENO 452-2012). The Lowry method gave completely different results than the assay of Bradford (1976) in the YPE analyzed. YPE 1 had the highest protein concentration of 36 g/100 g proteins, followed by YPE A3 and 6 having around 25 g/100g of proteins.

Such striking differences may be once again explained by different detection principles of both methods (cf. above).

A protein concentration of 50 % as demanded in the OIV-monograph was not attained in the YPE used within the studies presented here, but as the quantification method is far as the author of this thesis understood not definitely specified such a value seems to be still discussable.

4.3.2 Qualitative protein characterization by SDS-PAGE

The profiles of molecular masses of YPE used in the different cycles of fining experiments varied widely.

All commercial YPE used in fining cycle 1, i.e. YPE 1 to 4, contained only polypeptides in mass ranges below 7 kDa. All autolysates produced under laboratory conditions in experiments of this thesis (cf. part I) and analyzed for their protein profile contained however proteins in ranges from 50 kDa to 10 kDa. It is probable that in case of production of YPE 1, 2, 3 and 4 some of the activity of the wide array of proteases found in yeast and especially studied in case of *Saccharomayces cerevisiae* was still functional during extraction of yeast cells in manufacture of these products

YPE used in cycle 2 and 3 of the fining cycles had strong and defined band at 15 and 5 kDa and YPE A2, B2 and C2 also a fine but defined band at around 27 kDa. YPE B1 was an exception having proteins of bigger masses between 37 to 55 kDA. The fining performance of these YPE was however different with YPE A2.2. showing the best results in red wine.

YPE of fining cycle 4, 5 and 6 had all proteins of bigger masses than observed in the former cycles with exception of YPE B1 and the profiles were as a whole comparable to those observed in autolysates made in the autolysis experiments of part I of the thesis.

The mass distribution demanded by the OIV monograph OIV-OENO 452-2012, stating that 50 % of the proteins should have a mass above 15 kDa , could be fulfilled in case of YPE used in cycle 5 and 6 of the fining experiments except YPE1. It has to be mentioned however that the approach done in this thesis is only semi-quantitative as no densitometric data of SDS-PAGE profiles are shown. Densitometric measurements which were performed with repetitions of the same YPE were not considered enough accurate to allow a completely quantitative judgment (results not shown).

Gelatine charges used in the fining experiments showed no defined protein fraction, but only smear on SDS-PAGE gels and the masses ranged probably between 20 and 60 kDa. Gelatines used for fining of red wine did neither contain bands of distinct molecular masses in the studies of Cosme et al. (2009) and Marchal et al. (2002a).

It can be concluded that the production technique had an influence on the molecular masses of proteins in the YPE, probably by favouring or limiting endogenous protease activity.

The fining performance of YPE with a very different distribution of protein masses such as YPE 1 compared to YPE 5 and 6 was comparable. YPE used for successful fining in the study of Iturmendi et al. (2012) were also only composed of proteins and polypeptides of masses of 10 kDa and below 10 kDa. The distribution of protein masses of a YPE did not alone determine its fining performance.

The factor molecular mass distribution had on the other hand a clear influence on tannin-protein interactions in the studies of Ricardo da Silva et al. (1991) with synthetic poly-proline and a higher binding capacity in case of poly-proline-molecules above 19 kDa was found.

On the other hand Yokotsuka and Singleton (1987, 1995) reported that a wide array of gelatine fragments of masses between 2 and 10 kDa readily precipitated with proanthocyanidins, but only

gelatine of a mass of 70 kDa was completely precipitated under such conditions. Gelatine preparations of the same origin with different molecular masses showed the highest efficiency in adsorption of tannins in case of a preparation of a mean degree of hydrolysis having an average molecular mass of 25 kDa (Sarni-Manchado et al. 1999). Tschiersch et al. (2010) pointed out that plant proteins of a higher degree of hydrolysis changed to a minor extent the composition of polyphenols of red wines, but the plant origin had also a clear influence on fining performance of the preparations.

Studies done in part II and III of this thesis cannot state a clear influence of the distribution of molecular masses on the fining performance as far as precipitation and settlement of the tannin-YPE combinations and influence of fining on colour and other polyphenol indices measured is concerned.

The profile of molecular masses alone can thus not predict the fining performance of an YPE, but SDS-PAGE profiles are very useful to control production process and identity of YPE.

4.3.3 Presence of glycoproteins in YPE

Yeast cell wall can be partially degraded during yeast autolysis and thus polysaccharides and glycoproteins may be set free (Hernawan and Fleet 1995; Kollar et al. 1993) which was also shown in yeast autolysates by Comuzzo et al. (2012). Polysaccharides or glycoproteins could thus be part of the YPE and they were reported to inhibit protein precipitation with tannins in wine (de Freitas et al. 2003). Mannoproteins also prevented the precipitation of heat-unstable proteins sometimes causing turbidity in wine (Dupin et al. 2000 b; Dupin et al. 2000 a; Moine-Ledoux, Dubourdieu 1999; Waters et al. 1994). Futhermore mannoproteins were shown to diminish tannins' (i.e. procyanidins') reactivity as they hinder tannin aggregation (Charpentier et al. 2004; Poncet-Legrand et al. 2007b; Riou et al. 2002). The presence of glycosylated protein in YPE could thus diminish their fining performance by inhibiting the precipitation of their proteins.

On the other hand it was reported that mannoproteins can bind wine tannins, but with a lower affinity than pure proteins (Rowe et al. 2010) and glycosylated proline-rich proteins of human saliva were more resistant to precipitation than non-glycosylated ones (Pascal et al. 2008; Sarni-Manchado et al. 2008). Studies presumed (Guadalupe, Ayestaran 2008) that yeast mannoproteins can precipitate in red wine and it was proven for patatin (Gambuti et al. 2012), a glycoprotein out of potato containing mannose (Shewry 2003).

It can be seen from the studies cited above that studying the presence of glycosylated proteins and especially mannoproteins in YPE seems to be useful.

The first attempt to detect glycoproteins in YPE of the studies presented herein was made by PAS staining of SDS-PAGE gels using the Pierce ® Glycoprotein Staining Kit. This staining is based on oxidation of the sugar residues of glycoproteins by periodic acid and detection of the resulting aldehydes with the Schiff reaction using basic fuchsin sulphate. All YPE produced of strain A, B, and C and used in fining cycles 2, 3, 4 and 5 showed a fraction of glycosylated mannoproteins of a molecular mass probably above 100 kDa. No proteins were detected by Coomassie Blue staining in that range and the same feature was observed by Comuzzo et al. (2012) in case of yeast autolysates

and by Frevert and Ballou (1985) in case of yeast mannoproteins. It may be possible that these glycosylated proteins have a big sugar moiety and a small protein part poor in amino acids to which Coomassie Blue binds (Compton, Jones 1985). They could thus not be detected by Coomassie Blue staining. The sensitivity of the PAS staining method for proteins can be estimated to be in a g/100g range as the glycoprotein horse radish peroxidase serving as positive control on the gel had a concentration of 0.4 g/l and 20 g/l YPE were used for SDS-PAGE analysis.

Glycoproteins were furthermore detected in YPE 1, 5, 6, 7 and 8 and only YPE 5 may contain proteins stained by Coomassie Blue that could be glycoproteins. It is to be mentioned that the sugar moiety of glycoproteins can hinder the separation of glycoproteins according to molecular mass by SDS-PAGE (Strayer Leach et al. 1980).

Glycoproteins were then also detected by specific Western Blot Assay modified from the study of Hawkes (1982) using concanavalin A to couple glycoproteins having residues of α-glucose and α-mannose to horse radish peroxidase, which is also a glycoprotein. The enzyme activity of the peroxidase was then detected by the substrate 4-chloro-1-naphthol staining proteins containing α-mannose residues. Yeast cell wall is composed of ß-glucans and mannoproteins (Klis et al. 2002) and thus this specific staining should only detect mannoproteins in contrast to the PAS-method.

The Western Blot method was reported to be more sensitive for mannoproteins detecting concentrations as low as in mg/100 g range (Hawkes 1982). A higher sensitivity of the Western Blot method was confirmed in this study as the spot of the same concentration of horseradish peroxidase was much bigger and intense on the Western Blot membrane than on the gels stained with the PAS method. YPE of strain A, B, and C all contained a fraction of mannoproteins of high molecular masses probably above 100 kDa that were not well resolved and already detected by PAS method. Furthermore YPE of the three strains showed diffuse fractions of mannoproteins in a range of possibly below 100 kDa until around 40 to 30 kDa. A defined band estimated to be around 30 kDa was also detected in all YPE of the three strains. The same pattern of mannoproteins as in the strains A, B, and C was also found in YPE 5, 6, 7 and YPE 8. YPE1 however showed only mannoproteins of a mass of possibly above 100 kDa.

Mannoproteins in a size range between 50 kDa and 30 kDa were also detected in white wines after sur-lies ageing (Moine-Ledoux and Dubourdieu 1999) and in extracts of yeast cell walls (Nunez et al. 2005). A mannoprotein being a fragment of invertase of *Saccharomyces cerevisiae* of a mass of 32 kDa was described in the work of Moine-Ledoux and Dubourdieu (1999).

It could be concluded from the results of SDS-PAGE gels stained with Coomassie Brillliant Blue, the PAS-method and from the Western Blot results that there was probably a separation between mannoproteins and proteins detected by Coomassie Blue in case of YPE 1, A1, C1, A2.1., A2.2., B2, C2. On the other hand YPE A3, B1, B3, C3 as well as 5, 6, 7 and 8 may contain mannoproteins that are also detected by Coomassie blue or mixtures of proteins and mannoproteins.

It can finally just be stated that YPE successful in fining contained mannoproteins that did not completely inhibit tannin-protein-precipitation which is a prerequisite of successful fining.
It could further be speculated that mannoproteins contained in the commercial YPE 8 could diminish the astringency (Escot et al. 2001) and bitterness (Vidal et al. 2004) of red wines treated with it as already pointed out in discussion of part II of the thesis.

4.3.4 Concentration of sugars in YPE after acid hydrolysis

Glucose and mannose, the sugars being part of the yeast cell wall and especially of the wall of *Saccharomyces cerevisiae,* were determined in the YPE after acid hydrolysis setting free glucose and mannose contained in β-glucans or mannoproteins.
Mannose was detected without doubt in YPE A3, 5, 6, 7 and commercial YPE 8 in which glycosylated proteins were also detected by PAS method and Western Blot method according to Hawkes (1982). That confirmed that the glycosylated proteins should be mannoproteins as pointed out in the previous section. YPE A1, B1, C1 and C2 all contained higher concentrations of glucose after hydrolysis than the other YPE. It could not be discriminated whether this glucose was derived from β-glucans extracted from yeast cell wall or if it was set free from trehalose, a disaccharide of two molecules of glucose which is synthesized by yeasts in case of stress conditions (D'Amore et al. 1991; Li et al. 2010).

4.4 Final conclusions

The studies of this thesis demonstrated the suitability of YPE as alternative fining agents for red wines, which was also stated by Charpentier et al. (2006), Iturmendi et al. (2010) and Iturmendi et al. (2012).

The YPE developed during the course of the fining studies by an external yeast producer from yeast strains selected during the initial part of the studies of this thesis and from other strains had a well defined profile of proteins with masses above 10 kDa. That was not shown or was not the case in the other work on YPE for fining mentioned above. The commercial YPE 8 was produced by a less complex production process than YPE 1, had a much lower degree of protein hydrolysis and corresponded as a whole to the autolysates made in laboratory in part I of the studies of this thesis.

YPE 8 should fulfill the requirements of the OIV on YPE (OIV-OENO 452-2012) concerning its protein profile and the question of the final definition of a minimum concentration of proteins as made in this OIV-monograph remains to be solved.

Furthermore YPE are not regarded as food constituents causing allergies in sensitive people by European laws (regulation EC No 1169/2011) and are since 2013 allowed for fining of must and wine within the European Union (regulation EC No 144/2013).

The characterization of proteins, mannoproteins and sugars in the YPE of this study did not allow a conclusion whether the YPE were composed of proteins and mannoproteins or only of mannoproteins and which proteins were involved in the precipitation in red wine.

Fining red wines with YPE 8 maintained the flavour quality of the red wines. No aroma impressions typical of other commercial yeast extracts (Mahadevan and Farmer 2006; Münch and Schieberle 1998b) and yeast derivatives used in vinification {Comuzzo et al. 2006) such as “meaty” and “cheesy” notes were detected in the red wines after fining by sensory analysis. A quantitative analysis of aroma compounds found in commercial yeast extracts and contributing to “meaty” and “yeasty” aroma , such as furan derivatives, which could be performed in YPE and in wines after fining with YPE, could complement the sensory approach as done in this thesis.

The fining of white grape must and wine with YPE was only studied in the first steps of the work of this thesis and then fining was focused on red wines. Red wines contain in most cases more procyanidins, which are the compounds precipitating in case of must and wine preferentially with proteins. However further studies examining the potential of YPE in fining of white must and wine containing high amounts of flavonoids (procyanidins) seem to be a useful subject for further work

Flavonoids can indeed cause excessive bitterness and astringency, features affecting negatively flavour of white wines.

5 Summary

Fining must or wine with proteins of animal origin such as out of egg or milk is a traditional treatment. On the other hand proteins of milk or egg can cause intolerances and allergies in some people. Traces of these proteins out of egg or milk may remain in the wine after fining and can cause such health problems. Treatment of must or wine with egg or milk products has thus to be declared towards the consumer in the European Union (regulation (EC) No 579/2012) since 2012. Therefore alternative sources of proteins were looked for.

The objective of the studies of this doctoral thesis was to obtain protein extracts by yeast autolysis that could be used for fining treatments of musts and wines.

Mutants of a yeast strain of the species *Saccharomyces cerevisiae* were created by mutagenesis induced by UV-radiation. These mutants showed reduced viability and autolysis when exposed to stressors such as elevated temperature or change in osmotic pressure. A methodology was established at laboratory scale to produce protein extracts out of selected mutants which were forced to autolyse by stress conditions. Upscaling of the protein extraction process to industrial conditions was possible.

The yeast protein extracts could successfully clarify red wines and had a clarification capacity and kinetic as well as an influence on wine colour comparable or similar to the traditional fining agent gelatine. The sensory quality of the red wines was maintained after fining with yeast protein extracts and no aroma impressions typical of other yeast extracts used in the food industry such as “cheesy” or “meaty” notes were detected in the wines. A partial characterization of proteins, glycoproteins and sugars (after acid hydrolysis) of the yeast protein extract was also performed. The quantitative analysis of proteins by two methods gave differing results. Therefore, no simple conclusion about the relation between protein concentration and clarification behaviour (onset of visible flocculation and speed of sedimentation) could be drawn. A qualitative analysis of molecular masses by SDS-PAGE (electrophoresis on polyacrylamide gels after unfolding of proteins with SDS (sodium dodecyl sulphate)) showed that proteins were in a mass range at the end of the developing process of the industrial extract comparable to extract produced at the laboratory. However, no direct relation between the profile of molecular masses of the protein extracts and their clarification performance and influence on wine colour could be stated. All yeast protein extracts contained mannoproteins and mannose was detected in them after acid hydrolysis

Yeast protein extracts are now permitted for fining of must and wine in the European Union (regulation (EC) No 144/2013) and offer an alternative for wine professionals.

6 Zusammenfassung

Die Schönung von Mosten und Weinen mit tierischem Eiweiß, z.B. aus Eiern oder Milch ist ein traditionelles Behandlungsverfahren. Andererseits kann Eiweiß aus Milch oder Eiern Unverträglichkeiten und Allergien bei bestimmten Personen auslösen. Spuren von diesem Eiweiß können in den Weinen nach der Schönung verbleiben und solche gesundheitlichen Probleme auslösen.
Die Behandlung von Most oder Wein mit Produkten aus Eiern oder Milch muss deswegen für den Verbraucher in der Europäischen Union seit 2012 gekennzeichnet werden (Verordnung (EG) Nr. 579/2012). Daher wurde nach alternativen Eiweißquellen gesucht.
Das Ziel der Studien dieser Doktorarbeit war es, Eiweißextrakte durch Autolyse von Hefe zu gewinnen, die zur Schönung von Mosten und Weinen genutzt werden könnten.
Mutanten eines Hefestammes der Art *Saccharomyces cerevisiae* wurden durch Mutagenese, die durch UV-Bestrahlung ausgelöst wurde, erzeugt. Diese Mutanten zeigten eine reduzierte Vitalität und Autolyse, wenn sie Stressbedingungen, wie hohen Temperaturen oder einem Wechsel des osmotischen Druckes ausgesetzt wurden. Eine Methodik wurde im Labormaßstab entwickelt, um Eiweißextrakte aus den ausgewählten Mutanten, die durch Stressbedingungen zur Autolyse gezwungen wurden, zu erzeugen. Eine Übertragung des Extraktionsprozesses auf industrielle Bedingungen war möglich.
Die Eiweißextrakte aus Hefe konnten Rotweine erfolgreich klären und hatten ein Klärungsverhalten, sowie einen Einfluss auf die Weinfarbe, der dem des traditionellen Schönungsmittels Gelatine vergleichbar oder ähnlich war. Die sensorische Qualität der Rotweine blieb nach der Schönung mit Eiweißextrakten aus Hefe erhalten und Aromanoten, die für andere Hefeextrakte, die in der Lebensmittelindustrie eingesetzt werden, typisch sind, wie Noten von Käse oder Fleisch, wurden in den Weinen nicht gefunden. Eine teilweise Charakterisierung der Proteine, Glykoproteine und Zucker (nach saurer Hydrolyse) wurde in den Eiweißextrakten aus Hefe durchgeführt. Die quantitative Proteinmessung mit zwei Methoden gab unterschiedliche Ergebnisse. Daher konnte keine einfache Schlussfolgerung über den Zusammenhang zwischen der Proteinkonzentration und dem Klärungsverhalten (Beginn einer sichtbaren Flockung und Sedimentationsgeschwindigkeit) gezogen werden. Eine qualitative Bestimmung der Molekülmassen durch SDS-PAGE (Elektrophorese auf Polyacrylamidgelen nach Denaturierung der Proteine mit SDS (Sodium-Natriumdodecylsulfat)) zeigte, dass die Proteine am Ende des Entwicklungsprozesses des industriellen Extraktes in einer Größenordung waren, die der eines im Labor hergestellten Extraktes vergleichbar war. Es konnte jedoch keine direkte Beziehung zwischen dem Profil der Molekülmassen der Eiweißextrakte und ihrer Klärungsleistung und ihrem Einfluss auf die Weinfarbe festgestellt werden. Alle Eiweißextrakte enthielten Mannoproteine und Mannose wurde in ihnen nach saurer Hydrolyse nachgewiesen.
Eiweißextrakte aus Hefe sind jetzt zur Schönung von Most und Wein in der Europäischen Union zugelassen (Verordnung (EG) Nr. 144/2013) und stellen eine Alternative für Weinerzeuger dar.

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9 Annex

9.1 Table 1: Morphology of strains after autolysis and protein concentrations in autolysate

Strain	**Morphology at end of autolysis**	**mg/l proteins in autolysate (Lowry)**		**mg/l proteins in autolysate (densitometry)**	
		Mean/values	**SD**	**Mean/values**	**SD**
1 (mother strain)	**Cells with several vesicles**	**402**	**192**	**7.6**	**5.2**
2	Cells with several vesicles (2/3 cases)	608.4	51	20 4.8	
3	Normal	396.9	166.4		
4	Cells with deformed wall, empty cells	724.9	167.5	15.6 8.6	
5	Deformed cells	490 440		10 14.9	
6	Cells partly deformed and with vesicles	670.5	425	8.7	3.8
7	Few cells deformed and/or with vesicles (2/3 cases)	337.5	161.3		

Remarks: Morphology: abnormalities only mentioned if in majority of autolysate (x/y cases) or in all cases observed

Protein concentrations were calculated based on BSA (bovine serum albumin) and for a cell concentration of 10^8 cells/ml; values are given, when n = 2

Strains 45: densitometry was done, because of interesting phenotype on plate (cf. below)

SD. : abbreviation of standard deviation

Table 1: continued

Strain	Morphology at end of autolysis	mg/l proteins in autolysate (Lowry)		mg/l proteins in autolysate (densitometry)	
		Mean/values	**SD**	**Mean/values**	**SD**
8	Cells with vesicles and deformed cells (2/3 cases)	335	102	3.3	0.9
9	Normal	357.1	21.6		
10	Normal	325	61		
11	Deformed cells	272 317		4.6 14.5	
12	Deformed cells	317.7	60.0	13.2	9.2
13	Normal	487 392			
14	Cells with vesicles and deformed cells	766 697		21 13.3	
15	Cell residues, deformed cells	675.1	333.5	15.4	14.3
16	Some cells with vesicles (2/3 cases)	512	276.6	7.1	1.9
17	Deformed cells	411.7	47.3	3.6	1.6
18	Cells with vesicles and deformed cells (2/3 cases)	375	25	11.5	4.8
19	Deformed cells and with vesicles	817.3	61.8	24.6	4.8
20	Cells with deformed vavuoles or with vesicles (2/3 cases)	602	62	20.2	7.5

Table 1: continued

Strain	Morphology at end of autolysis	mg/l proteins in autolysate (Lowry)		mg/l proteins in autolysate (densitometry)	
		Mean/values	**SD**	**Mean/values**	**SD**
21	Burst cells, cells with vesicles	659.5	200.3	24.9	13.1
22	Deformed cells	503 492		8.6 38.7	
23	Deformed cells	683.1	232.7	6.4	1.9
24	Deformed cells	412		1.2 2.1	
25	Cells with vesicles (2/3 cases)	436.4	94.5	13.8	11.3
26	Deformed cells (2/3 cases)	796.1	183.1	10.8 15.8	
27	Deformed cells or with vesicles	387.8	74.3	3.2	1.5
28	Normal	400 277			
29	Cells with vesicles	118 132		9.4 14.3	
30	Cells deformed and/or with vesicles	1034 843		2.8	
31	Empty cells, cells with vesicles (2/3 cases)	423.1	134.2	12.6	12.2
32	Normal	315	34		

Table 1: continued

Strain	Morphology at end of autolysis	mg/l proteins in autolysate (Lowry)		mg/l proteins in autolysate (densitometry)	
		Mean/values	**SD**	**Mean/values**	**SD**
33	Normal	201	77		
34	Deformed cells and/or with vesicles (2/3 cases)	536	101.9	9.1	5.5
35	Normal	175	17		
36	Cells with vesicles	330.1	74.1	1 1.7	
37	Normal	140	17		
38	Cells with vesicles (1/2 cases)	245 269			
39	Normal	465	87		
40	Normal	589 582			
41	Cells with vesicles	314 322		2.7 2	
42	Cells with vesicles	159 206			
43	Normal	340 335			
44	Normal	211 188			
45	Normal	352.9	97.3	10.2 10.9	
46	Cells with vesicles	267.5	36.4	2.9	1.1
47	Normal	120 164			

Table 1: continued

Strain	Morphology at end of autolysis	mg/l proteins in autolysate (Lowry)		mg/l proteins in autolysate (densitometry)	
		Mean/values	**SD**	**Mean/values**	**SD**
48	Some deformed cells	522 488		1.7 1.7	
49	Normal	375 440			
50	Normal	270	107		
51	Deformed cells and/or with vesicles	345 328		1.9 5.3	

9.2 Table 2: Phenotypical characterization of the strains conserved on plate or deep frozen during after heat shock of 48h at 37°C

Strain	Low viability (coloration on Erythrosin B medium)	Stability	Autolysis (release of alkaline phosphatase detected on BCIP medium)	Stability
1	Negative	Yes	Negative	Yes
2	Intermediary	Yes	Intermediary	Yes
3	Intermediary	Yes	Negative to intermediary	Yes
4	Intermediary to positive	Yes	Intermediary to positive	Yes
5	Intermediary	Yes	Intermediary	Yes
6	Intermediary	Yes	Intermediary	Yes
7	Intermediary	Yes	Negative to intermediary	Yes
8	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)

Explanations:

Negative	White on both media
Negative to intermediary	One or two replicates only intermediary
Intermediary	Slightly pink on Erythrosin B medium Slightly blue on BCIP medium
Intermediary to positive	One or two replicates only intermediary
Positive	Intensely pink to red on Erythrosin B medium Turquoise on BCIP medium

Table 2: continued

Strain	Low viability (coloration on Erythrosin B medium)	Stability	Autolysis (release of alkaline phosphatase detected on BCIP medium)	Stability
9	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)
10	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)
11	Intermediary	Yes	Negative to intermediary	Yes
12	Intermediary	Yes	Negative to intermediary	Yes
13	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)
14	Intermediary to positive	Yes	Intermediary	No (negative 2/3 cases conserved on plate)
15	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)
16	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)
17	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)

Table 2: continued

Strain	Low viability (coloration on Erythrosin B medium)	Stability	Autolysis (release of alkaline phosphatase detected on BCIP medium)	Stability
18	Intermediary	Yes	Negative to intermediary	Yes
19	Positive	No (conserved on plate only intermediate)	Intermediary to positive	No (intermediary to negative conserved on plate)
20	Intermediary to positive	No (conserved on plate positive)	Intermediary	Yes
21	Intermediary to positive	Yes	Intermediary	No (intermediary to negative conserved on plate)
22	Negative to intermediary	Yes	Intermediary	No (intermediary to negative conserved on plate)
23	Intermediary to positive	Yes	Positive	No (intermediary to negative conserved on plate)
24	Intermediary	Yes	Negative to intermediary	Yes
25	Negative to intermediary	Yes	Negative to intermediary	Yes

Table 2: continued

Strain	Low viability (coloration on Erythrosin B medium)	Stability	Autolysis (release of alkaline phosphatase detected on BCIP medium)	Stability
26	Intermediary	Yes	Intermediary	No (negative to intermediary conserved on plate)
27	Negative to intermediary	Yes	Negative to intermediary	Yes
28	Intermediary	Yes	Intermediary	No (negative to intermediary)
29	Intermediary	No (conserved on plate 2/3 positive)	Intermediary	No (negative 2/3 cases conserved on plate)
30	Intermediary to positive	Yes	Intermediary to positive	No (negative to inter-mediary conserved on plate)
31	Positive	Yes	Positive	No (intermediary to positive conserved on plate)
32	Intermediary	No (conserved on plate positive)	Intermediary	Yes
33	Negative to intermediary	Yes	Negative to intermediary	Yes

Table 2: continued

Strain	Low viability (coloration on Erythrosin B medium)	Stability	Autolysis (release of alkaline phosphatase detected on BCIP medium)	Stability
34	Positive	Yes	Intermediary to positive	Yes
35	Negative to intermediary	Yes	Negative to intermediary	Yes
36	Intermediary	Yes	Intermediary	Yes
37	Intermediary to positive	Yes	Intermediary	Yes
38	Positive	Yes	Positive	No
39	Intermediary	No (conserved on 2/3 positive)	Negative to intermediary	Yes
40	Positive	No (conserved on 2/3 positive)	Intermediary to positive	Yes
41	Intermediary	Yes	Intermediary	No (negative 2/3 cases conserved on plate)
42	Positive	Yes	Positive	No
43	Intermediary to positive	Yes	Intermediary to positive	No (negative 2/3 cases conserved on plate)
44	Intermediary	Yes	Negative	Yes
45	Intermediary to positive	No (positive conserved on plate)	Positive	Yes
46	Intermediary to positive	Yes	Intermediary	Yes

Table 2: continued

Strain	Low viability (coloration on Erythrosin B medium)	Stability	Autolysis (release of alkaline phosphatase detected on BCIP medium)	Stability
47	Intermediary	Yes	Negative to intermediary	Yes
48	Intermediary	Yes	Negative to intermediary	Yes
49	Positive	Yes	Positive	No
50	Intermediary	Yes	Intermediary to positive	Yes
51	Positive	Yes	Positive	Yes

10 Declaration of conformity of the dissertation to the rules of the doctoral examination board of Justus-Liebig-Universität

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."

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Geisenheim, 03.07.2014 Bernd Christoph Lochbühler

(Bernd Christoph Lochbühler)