PRODUCTION AND LOCALISATION OF HAZE PROTECTIVE MATERIAL FROM *SACCHAROMYCES CEREVISIAE*

by

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DECLARATION

I hereby declare that this work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge and belief, no material described herein has been previously published or written by any other person except when due reference is made in the text (see Acknowledgements for collaborative work described in Chapters 4, 5 and 6).

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ABSTRACT

The effectiveness of methods to extract haze protective material (HPM) from whole yeast cells was tested on three winemaking *Saccharomyces cerevisiae* strains [Maurivin Prise de mousse (PDM) ; flocculent yeast AWRI 65 ; Champagne yeast AWRI 85]. For the three strains, hot extraction with SDS (boiling cells in Tris buffer for 5 min) and the full zymolyase treatment (which combined a pretreatment of the cells with DTE and EDTA followed by an enzymatic digestion of the cell debris with zymolyase) were the most efficient in releasing HPM. In contrast hot citrate extraction (using an autoclave) was far less effective whereas disruption of the cells using a French press device released haze forming mannoproteins. Over all, better results were obtained with Maurivin PDM and AWRI 85 compared to AWRI 65.

Thorough studies of the parameters responsible for HPM release from Maurivin DPM showed that SDS itself was not necessary to extract HPM. Boiling the cells in Tris buffer, without SDS, efficiently extracted HPM. Similarly, the decomposition of the full zymolyase treatment showed that HPM was more specifically extracted during the pretreatment rather than during the enzymatic digestion with zymolyase. Further investigation revealed that HPM was released by EDTA whereas DTE preferentially extracted haze forming mannoproteins. The implications of these data for the location of HPF and its interactions with other cell wall components are discussed.

The extracellular material released during fermentation of Maurivin PDM yeast during storage on yeast lees was recovered either by ultrafiltration or ethanol precipitation. In both cases, the material collected was active in reducing the haze without the enrichment of the extract in mannoproteins being necessary.

Antibodies to a purified wine HPF were used to localise HPF in Maurivin PDM yeast cell. No cross-reactivity against yeast mannans or invertase was detected by the methods of gel double diffusion and western immunoblotting. Agglutination assay indicated that HPF was present on the cell wall surface Successful immunogold labelling followed by transmission electron microscopy showed HPF to be distributed on the cell wall surface, in particular, at the periphery and innermost layers of the cell wall.

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PUBLICATIONS

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ABBREVIATIONS

A absorbance

AWRI Australian Wine Research Institute

BSA bovine serum albumin

°C degree Celsius

Con-A concanavalin-A

DTE dithioerythritol

DTT dithiothreitol

EDTA ethylenediamine tetraacetate

et al. and others

g gram

g acceleration due to gravity

HPF haze protective factor

HPM haze protective material

ihv initial haze value

k kilo (10^3)

kda kilo (10^3) dalton

Kg kilo (10^3) gram

KPa kilo (10^3) Pascal

L litre

2-ME 2-mercaptoethanol

µL micron (10^6) litre

µm micron (10^6) metre

µM micron (10^6) molar

mL milli (10^3) litre

mm milli (10^3) metre

mM milli (10^3) molar

min minute

M_r relative molecular weight

nm nano (10^9) metre

N_2 Nitrogen gas

nd not determined

% percent

psi pounds per square inch (1 psi = 6.89 10^3 Pascals)

PDM Prise de Mousse

PMSF phenylmethylsulfonylfluoride

rpm revolutions per minute

SDS sodium dodecyl sulfate

SDS PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

v/v volume by volume

w/v weight by volume

To my parents,

Chapter 1

INTRODUCTION AND GENERAL LITERATURE REVIEW

1.1 INTRODUCTION

The quality of wine is judged not only by its taste but also by visual appearance. A lack of clarity in a white wine means the bottle is likely to be rejected by the customers and regarded as unsalable by the traders. If a hazy white wine reaches the market, the result can be disastrous for the winemaker in terms of reputation, subsequent treatment and/or disposal.

Due to its complex microbiological content and chemical nature, wine is subject to both microbiological and chemical instabilities. Wine can be spoilt, after processing, by various bacteria or yeasts (Rankine 1989). Microbiological spoilage can result in the production of off-flavours like mousiness or geranium smell whereas yeast spoilage in bottled wine can create a haziness due to yeast cells (Rankine 1989). Non-microbial instabilities also have disastrous consequences. The presence of a few parts per million of copper or iron can induce the formation of an undesirable haze in bottled wine (Dikanovic-Lucan *et al.* 1992). The formation of crystalline deposit when the bottled wine is chilled is due to the precipitation of potassium bitartrate (Moutonet and Escudier 1991).

Protein instability is another example of non-microbial instability and is characterised by the formation of a light amorphous deposit in the wine. This haziness is temperature-dependent and can be observed when the wine is warmed. This instability is due to the slow denaturation and precipitation of heat unstable grape proteins and is, indeed, more critical for the white wines made from protein-rich grapes such as Muscat Blanco Gordo, Traminer and Semillon (Rankine 1989). Some red wines also show this fault if they are over-fined with gelatin (or another proteinaceous fining agent) or if they have a low tannin content (Rankine 1989).

The problem of post bottling haze involving proteins can also occur in beverages other than wine. However haze due only to proteins has rarely been reported (Heatherbell 1976). The formation of phenolic-protein complexes in clarified apple juice can induce haze formation. Association of proteins and phenolic components also represents the most significant source of colloidal turbidity in beer (Kumpel 1990). In lemon juice and tomato products, pectin complexed within a protein matrix leads to the formation of cloudiness in the final product (Takada and Nelson 1983, Klavons and Bennett 1985).

1.2 HEAT UNSTABLE PROTEINS

1.2.1 PROTEIN CONTENT OF WINE

To understand and resolve this problem of protein instability, the protein content of wine has been extensively studied over the last 50 years (Ribéreau-Gayon 1932, Kielhofer 1942) and in particular in the last decades when the protein instability became an important problem for European winemakers (Koch 1959, Moretti and Berg 1965, Cordonnier 1966, Ferenczi 1966). Nowadays, increasingly efficient analytical methods such as gel permeation, electrophoresis, chromatography, electrofocusing and protein blotting make it possible to obtain more accurate information about proteins.

The main sources of proteins in wine are the grapes (Cordonnier 1966). The general protein content in wine can vary from 10 to 300 mg/L (Cordonnier 1966) and depends on the variety and degree of maturity of the grapes. Additionally, the harvesting method (manual or mechanical) and the winemaking procedures, such as adding sulphites, pressing, skin contact and cold settling, can influence the final wine protein content (Tyson *et al.* 1981, Paetzold *et al.* 1990). Furthermore, nitrogenous material can also be released into wine by *Saccharomyces cerevisiae* yeast cells during autolysis (Feuillat and Charpentier 1982).

The high variability in protein content of the wine is also accompanied by a high heterogeneity in size. For instance, Hsu and Heatherbell (1987a) observed the presence of 25 protein fractions in wine with a relative molecular mass (M_r) ranging from 11.2 k to 65 k. The

same group (Ngaba and Heatherbell 1981, Heatherbell *et al.* 1985) reported M_r of wine proteins to be from 16 k to 90 k with pI from 4.5 to 8.

1.2.2 HEAT UNSTABLE PROTEINS

Not all of these protein fractions are, however, involved in wine instability. According to Hsu and Heatherbell (1987b), proteins of lower M_r (around 12.6 k and between 20 k and 30 k) and of lower pI (4.1 to 5.8) are the major fractions contributing to the formation of haze in white table wines. The use of a lectin linked staining procedure suggested that some of these proteins responsible for the haze formation (M_r of 12.6 k, 25 k and 28 k for a Gewürztraminer wine) were glycosylated. Investigations by Waters *et al.* (1991, 1992a) revealed the presence of two major protein fractions in Muscat Gordo wine strongly involved in haze formation. These proteins, with M_r of 24 k and 32 k, were not glycoproteins. As the two groups did not use the same protein isolation procedure, they may not have been studying the same protein pool and thus neither glycosylated nor non glycolsylated proteins can be excluded from playing a role in heat instability. Paetzold *et al.* (1990) on the contrary came to the conclusion that all the proteins in wine were glycosylated and contributed to heat instability. Since the results are based on analyses of fractions isolated from a single chromatographic separation, coelution of proteins with polysaccharides and glycoproteins cannot be excluded. This situation may account for the apparent anomalous result that all wine proteins were glycosylated.

1.3 REMOVAL OF HEAT UNSTABLE PROTEINS

1.3.1 FINING AGENT: BENTONITE

To avoid making wine of variable and unpredictable quality, winemakers need to stabilise the wine for every type of wine instability. For protein instability, haze precursors are known to be grape proteins (Bayly and Berg 1967, Paetzold *et al.* 1990, Waters *et al.* 1991). Thus a method lowering the concentration of proteins before bottling stabilises the wine and prevents the formation of protein haze.

The most commonly used fining agent to remove proteins from wine is the clay bentonite (Minkhorst 1989). Bentonite is a hydrated aluminium silicate which can adsorb wine proteins by electrostatic interactions or hydrogen bonding. Mixed with water, the powdered bentonite swells and is then added to the wine to form a homogeneous colloidal suspension. The clay adsorbs the protein and the whole complex progressively precipitates to the bottom of the tank to form the sediment lees. After settling the wine is racked from the lees.

The ability of this clay to adsorb proteins has been subject to numerous investigations since its first introduction in California (Saywell 1934). Studies have shown that in both laboratory scale and product trials, bentonite is not the perfect wine clarifying agent and has several disadvantages.

The most serious problem is the rather non selective adsorption capacity of this clay. Volatile compounds responsible for the wine aroma can also be adsorbed onto bentonite leading to a loss of aroma in the treated wine and therefore a loss of quality. Miller *et al.* (1985) reported a decrease in the concentration (up to 35%) of some alcohols and esters responsible for the fruity and wine-like aromas in a bentonite-stabilised wine. The investigations of Voilley *et al.* (1990) led to the same conclusion. In addition, bentonite also removes some heat stable proteins (Ngaba and Heatherbell 1981).

A second disadvantage is the excessive accumulation of lees. At full saturation, bentonite can occupy a volume of 12 to 15 times its dry weight and therefore leads to a loss of 5 to 10% of the total wine volume (Zoecklein 1984). For the wineries with adequate equipment, the wine remaining in the lees can be recovered by vacuum drum filtration. During this process the lees are in contact with air and the wine recovered is therefore partly oxidised and hence of lower quality (Minkhorst 1989).

When sodium bentonite is used (the commonly used form), the protein adsorbed is exchanged with sodium ions which are then released into the wine. This sodium concentration increase can affect wine exports where a limited sodium content in wine is allowed (Rankine 1987).

1.3.2 ALTERNATIVES TO BENTONITE

An alternative fining method to bentonite is ultrafiltration. This method is a well-established purification technique in the food industry (Brock 1983) and particularly in the production of fruit juices (Moslang 1984).

Membrane separation has a big advantage over the bentonite treatment as the recovery of wine volume - only partial with bentonite - would be almost total with ultrafiltration (Miller *et al.* 1985). Nevertheless, loss of some aroma compounds (from 30 to 80%) due to fixation onto the membrane or onto the surface of the entire apparatus has been noted by Voilley *et al.* (1990).

As far as the protein removal is concerned, Heatherbell and Flores (1988) have shown that the proteins of M_r close to the molecular weight cut off (MWCO) of the membrane can pass through the membrane and still induce heat instability. Proteins of M_r much higher than MWCO (MWCO is usually 10,000 daltons) are mainly retained during ultrafiltration. In this case, ultrafiltration can not be used as an unique substitute for bentonite as total heat unstable protein removal can not be achieved. If used in association with bentonite, ultrafiltration allowed a reduction of 80 to 95% in bentonite requirement (Heartherbell and Flores 1988).

Other recent investigations in the prevention of haze have focused on proteases, enzymes which can hydrolyse proteins contained in wine and degrade them into peptides which are no longer sensitive to heat. In this way, the application of a physical treatment to remove the heat unstable proteins could be reduced or even completely avoided.

The use of proteolytic yeast strains able to produce extracellular acid protease(s) during fermentation was considered at one time as a potential method to reduce the protein pool and thus stabilise the wine protein. Indeed, the total protein content of wine decreased under the protease activity of some strains (Rosi and Costamagna 1987, Rosi *et al.* 1988) but not all proteins were degraded and the residual protein content was still high. Further research work by Lagace and Bisson (1990) showed that some strains were effective in partially degrading wine proteins. Heatherbell *et al.* (1985) experimented with a fungal acid protease. This enzyme appeared to be efficient in degrading heat unstable proteins only under certain

conditions. Complete removal of wine proteins could not be achieved at wine pH and cellar temperature.

The inability of several other exogenous peptidases to degrade heat unstable proteins has also been demonstrated more recently by Waters *et al.* (1990, 1992a). Although the commercially available peptidases tested were active in must and wine, they did not result in any significant decrease in the wine heat instability. Later studies by this group demonstrated that the grape proteins present in wine were pathogenesis related proteins of the berry, a group of proteins with known proteolytic resistance (Waters *et al.* 1995).

1.4 HAZE PROTECTIVE FACTOR (HPF)

1.4.1 PURIFICATION AND PARTIAL CHARACTERISATION

Based on preliminary work (Waters *et al.* 1990), wine proteins were separated by ammonium sulfate precipitation followed by ultrafiltration (Waters *et al.* 1991). One of the isolated protein fractions did not show any heat instability and even had a protective effect on the heat instability of other protein fractions. The composition analysis revealed this fraction to be rich in carbohydrates.

Further studies (Waters *et al.* 1992b) focused on the characterisation of this fraction and its origin in both white and red wines (Muscat Gordo and Carignan Noir). A number of techniques were applied to fractionate and purify the anti-haze wine protein, i.e. affinity chromatography on lectin Concanavalin-A followed by anion- and cation- exchange chromatographies (Pellerin and Brillouet 1992) and gel permeation. The isolated carbohydrate rich fraction from red wine (Waters *et al.* 1994) proved to be a mannoprotein with a relative molecular mass of 420 k and composed of 70% polysaccharides (98% mannose, 2% glucose) and 30% protein. It represented only 0.007% of the total neutral polysaccharides in the wine. A mannoprotein fraction isolated from white wine with fewer purification steps (the gel permeation step was omitted) (Waters *et al.* 1993) was composed of 96% of carbohydrates with a domination of mannose (78%) over glucose (13%) and only 4% of protein. Its ability to reduce haze was still significant.

In both white and red wines, the main amino acids present in the mannoprotein were serine (31% and 28% of the molar protein content, respectively), threonine (13%, 12%) and glycine (9%, 17%) plus a relatively high proportion of basic amino acids. The mannose units of the carbohydrate moiety were *O*- and *N*- linked to the protein and formed a (1-6) linked backbone to which (1-2)- and (1-3)- linked mannose side chains were attached.

Demonstrations of the haze protective effect of this mannoprotein were carried out with success on the haze induced by bovine serum albumin or by the different wine protein fractions (Waters *et al.* 1993). The study of its mechanism of action revealed that this mannoprotein apparently acted by reducing the particle size of the haze, to as low as 5 microns, which is hardly detectable with the naked eye and considered to be commercially acceptable in the beer industry (Leedham and Carpenter 1977).

1.4.2 POSSIBLE ORIGIN OF HPF

With the predominance of mannose in the Haze Protective Factor, the amino acid composition and the structural links between the carbohydrate moiety and the protein, Waters *et al.* (1993) were able to establish similarities between this wine derived material and a mannoprotein present in the yeast *Saccharomyces* described by Ballou (1982) (see Section 1.5.1.3). This similarity in properties suggested that this wine mannoprotein might have originated from the yeast cell wall.

This hypothesis was validated by an experiment (Waters *et al.* 1992b) in which a *Saccharomyces* yeast strain, widely used by winemakers in Australia, was fermented in a synthetic grape juice medium. Only the mannoprotein fraction isolated from the yeast cell wall by hot citrate extraction showed haze protective ability.

Confirmation of the positive effect of mannoproteins from yeast cell walls on the wine protein stability can also be found in the research work of Ledoux *et al.* (1992). Yeast cell wall mannoproteins prepared according to Villetaz's method (Villetaz *et al.* 1980) were added to filtered white wine. The samples were heated to test the ability to form haze: wines with mannoproteins added developed 50% less haze compared to control wines not containing mannoproteins.

Feuillat *et al.* (1988) observed an increase of the high M_r glycoprotein content in sparkling wine after ageing (8 months) on yeast lees according to the 'méthode champenoise'. The sugar composition of those glycoproteins clearly proved the cell wall origin of these high M_r glycoproteins. Cell wall macromolecules were released into the wine during yeast autolysis and shown to improve the aroma and foam qualities of the sparkling wine (Feuillat and Charpentier 1982, Feuillat *et al.* 1988).

Anecdotal accounts indicate that wines aged on yeast lees seem to show better heat stability than normal wines. As for other cell wall mannoproteins, haze protective mannoprotein could also be released from the yeast cell wall into the wine during autolysis. However, the amount of these mannoproteins released under normal winemaking conditions is obviously too small to prevent the heat unstable proteins from forming haze.

Over all, the research work done so far on the haze protective mannoprotein indicates that it has an excellent ability to reduce heat-induced protein haze. This mannoprotein appears to offer a natural alternative to bentonite to the wine industry as it may be possible to enhance the natural haze protective ability of a wine by deliberately enriching that wine in HPF. This mannoprotein is likely to originate from the yeast cell wall and to be released into the wine during autolysis. In the next Section a description of the structure and organisation of the cell envelope will be given.

1.5 YEAST ENVELOPE AND CELL WALL OF *SACCHAROMYCES*

The cell wall is morphologically the outermost cellular structure of the yeast cell. The cell envelope is defined as the whole zone (cell wall included) situated outside the limits of the cytoplasmic membrane. Due to its most external location, the cell wall and its constituents are involved in the interactions of the cell with its surrounding and are thus of particular interest. Cell walls, mainly from the genus *Saccharomyces,* have been extensively studied and a large amount of literature deals with this subject (Ballou 1982, Catley 1983, Sentandreu *et al.* 1984, Fleet 1991) .

1.5.1 COMPOSITION AND STRUCTURE

The cell wall of yeasts (15-25% of the dry weight of the cell) typically consists of 80-90% of polysaccharide plus a small amount of protein and lipid (Phaff 1971, Bartnicki-Garcia 1968). The main polysaccharides, with some exceptions, are glucans and mannans, chitin is only sporadically present (Ballou 1982, Cabib *et al.* 1982).

1.5.1.1 Glucans

These polymers of glucose account for 30 to 60% of the dry weight of *Saccharomyces* cell wall. Glucans are uniformly distributed throughout the cell wall and show a high heterogeneity in size. Classification according to their differences in extractibility and solubility from the yeast cell walls is still widely used. Harsh extraction with alkali and acid leads to an insoluble glucan fraction accordingly named 'alkali- and acid-insoluble glucan'. The alkali-insoluble glucan is responsible for the cell shape and rigidity (Fleet and Manners 1976). It is composed of ß(1-3) linked glucose (97%) and is poorly branched. An acetic acid extraction (Manners *et al.* 1973a) or a digestion with ß(1-3) glucanase (Manners *et al.* 1973b) on the alkali-insoluble glucan releases the second type of glucan: the ß(1-6) linked glucan fraction which is of low molecular mass and highly branched. The third category of glucan, alkali-soluble, is mainly ß(1-3) linked glucan (85%) complexed with mannans (1-3%) via some ß(1-6) linked glucose residues (Fleet and Manners 1977). This alkali-soluble fraction has an amorphous appearance and is thought to be responsible for the flexibility of the cell wall.

1.5.1.2 Chitin

Chitin is a linear polysaccharide of ß(1-4) linked *N*-acetylglucosamine residues deposited at the budding and scar zone of the mother cell after separation of the daughter cell. This polymer accounts for 1-2% of the total polysaccharide content or total mass of the walls of *Saccharomyces cerevisiae* depending on the budding stage of the cell. However non-budding cells still contain a small amount of chitin (Beran *et al.* 1972). Chitin can be isolated via alkali and acid extractions followed by a ß(1-3) glucanase treatment to remove glucan (Cabib and

Bowers 1971, Beran *et al.* 1972). Even after exhaustive purification it will be still contaminated with traces of glucan and mannan which is partially due to the fact that it is covalently linked to ß(1-6) glucan (Suratit *et al.* 1988).

1.5.1.3 Structural mannoproteins

Mannose polymers represent between 25 to 50% of the dry weight of *Saccharomyces cerevisiae* cell wall (Phaff 1971, Ramsay and Douglas 1979, Ballou 1976) and are the second major component of the yeast cell wall after the glucan fractions. They were found to be covalently linked to proteins (Ballou 1976, Ballou 1982, Sentandreu *et al.* 1984) and are therefore termed mannoproteins. Isolated crude mannoprotein fractions show a high distribution of relative molecular mass from 40 k (Nakajima and Ballou 1974) to more than 300 k (Van Rinsum *et al.* 1991).

Structural mannoproteins participate in the architecture and structure of the cell wall and are distributed all over the surface of the cell wall. Similar to the alkali soluble glucan fraction, mannoproteins do not seem to be responsible for the cell shape (Fleet 1991) and appear as amorphous material during electron microscopy studies. Due to their surface location on the cell wall, mannoproteins are involved in the determination of the cell immunogenicity which means that a host organism of the yeast will produce antibodies against these mannoproteins (Suzuki *et al.* 1968, Ballou 1970).

The basic structure of those cell wall mannoproteins was studied by several groups (Sentandreu and Northcote 1968, Nakajima and Ballou 1974) until Ballou, in 1976, proposed a model which has remained relatively unchanged until today (Ballou 1982, Fleet 1991). According to this model (Figure 1.1), structural mannoproteins are predominantly composed of mannose (about 90%), protein (about 10%) plus a minor amount of phosphorus (0.1-1%) and *N*-acetylglucosamine (GNAc). Short mannose oligosaccharides (up to 5 sugar residues, 10% of the total mannose) are linked to the protein via *O*-glycosidic bonds through the hydroxyl groups of serine and threonine.

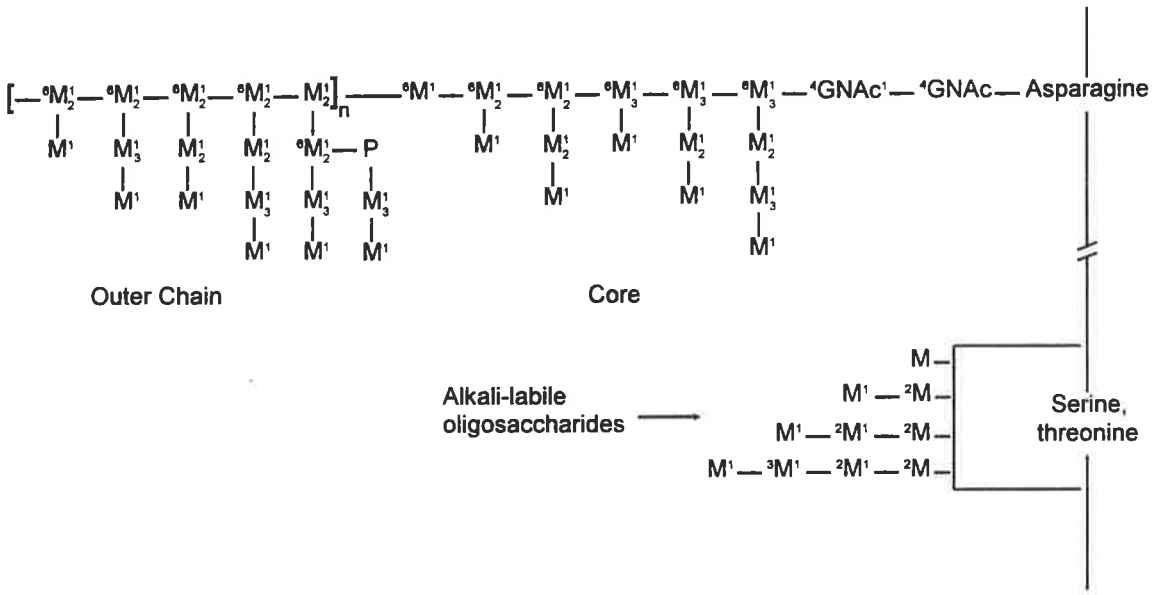


Figure 1.1 Detailed structure of mannoprotein from *Saccharomyces cerevisiae* (from Ballou 1982 and Fleet 1991). M, mannose; GNAc, *N*-acetylglucosamine; P, phosphorus. All linkages are of α configuration except for (1-4) which is of ß configuration. n= 10-15.

The remaining mannose polymers are connected to the protein by *N*-glycosidic bonds between di-*N*-acetylglucosamine and asparagine, and organised in an α(1-6) linked backbone chain of mannose with short lateral α(1-2) and α(1-3) linked mannose branches. The core of this long polymer is characterised by the presence of 2 GlcNAc units to which the first mannose unit is attached by a ß(1-4) linked bond. The outer chain on the contrary contains the α(1-6) linked mannose units of the backbone to which mannose, mannobiose, mannotriose and mannotetraose side chains are linked forming a mannose branched structure. Some of the side chains contain a phosphodiester bond linked to position 6 of the mannose.

1.5.1.4 Cell envelope molecular organisation

The model of yeast envelope molecular organisation proposed by Lampen (1968) or Kidby and Davies (1970) suggests that the outer layer contained a network of mannoproteins cross-linked by phosphodiester bonds between their carbohydrate moieties and by disulfide bridges between their protein part. The existence of the phosphodiester bonds has been questioned by the work of Vrsanska *et al.* (1977). The presence of disulfide bridges was confirmed by the cytochemical studies of Chattaway *et al.* (1976) and by the fact that extraction of cell wall mannoproteins was possible using reducing agents (Valentin *et al.* 1984, Pastor *et al.* 1984). According to the model, the inner layer of the cell wall is composed of a microfibrillar network of glucan polymers associated with some mannoproteins from the upper layer through their proteic parts.

The concept of the cell wall consisting of three layers of different composition has been supported by various electron microscopy and cytochemical studies (Dube *et al.* 1973, Lipke *et al.* 1976). The outer layer, electron dense, is a smooth and amorphous surface layer made of mannans (and supposedly mannoproteins). Immunochemical studies have confirmed this fact (Horisberger *et al.* 1976, Horisberger and Vonlanthen 1977). This structural mannoprotein network is intimately associated with the inner less-dense layer composed of amorphous alkali soluble ß(1-3) and ß(1-6) glucans. The possible extraction of mannoproteins by enzymatic preparations containing ß(1-3) glucanases supported the existence of such a mannoprotein-glucan association through covalent ß(1-3) bonds (Pastor *et al.* 1984, Frevert and Ballou 1985, Elorza *et al.* 1988) or ß(1-6) links (Fleet and Manners 1976, 1977). The alkali insoluble ß(1-3) glucan acts as a mesh for the inner part of the cell wall and appears like a fibrillar network in

cytochemical microscopy studies (Kopecka 1985, see Fleet 1991). The presence of mannoproteins can also be detected in those inner layers of the cell wall and in particular in the periplasmic space ('space' between the plasma membrane and this fibrillar network) which contain some mannosylated enzymes (see Section 1.5.5.1) plus some structural mannoproteins that are in transit through the periplasmic space itself before being integrated into the cell wall (Pastor *et al.* 1984).

The present model of cell wall molecular organisation (Figure 1.2) is based on these putative mannoprotein-glucan associations (Schekman and Novick 1982, Sentandreu *et al.* 1984, Zlotnik *et al.* 1984) but a number of matters remain unclear and the complete organisation is yet to be elucidated.

1.5.2 BIOSYNTHETIC PATHWAY OF THE MAIN CELL WALL COMPONENTS

The biosyntheses of the sugar polymers and mannoproteins have been studied extensively by numerous research groups (Schekman and Novick 1982, Sentandreu *et al.* 1984, Rothblatt and Schekman 1989). The complexity of the implied mechanisms involved has led to only partial success and more studies will be needed to complete the full picture.

Chitin and glucan biosyntheses are catalysed by enzymes found on or associated with the plasma membrane of the yeast cell. The enzyme, glucan synthetase, was found to catalyse the addition of glucose onto a linear ß(1-3) glucan polymer (Shematek and Cabib 1980, Larriba *et al.* 1981) while chitin synthetase 1 and 2 (Sburtlati and Cabib 1986, Orlean 1987) incorporate *N*-acetylglucosamine units into a ß(1-4) linked polymer. The synthesised polymers are probably assembled directly on the cell surface (Schekman and Novick 1982).

Biosynthesis of mannoproteins is done intracellularly. Firstly the protein moiety is synthesised on polyribosomes associated with the endoplasmic reticulum (ER). The nascent protein is afterwards discharged into the ER lumen to follow the secretory pathway, en route to the cell surface. Post-translational protein modifications by enzymatic complexes from the different compartments ensure the assembly and glycosylation of the protein.

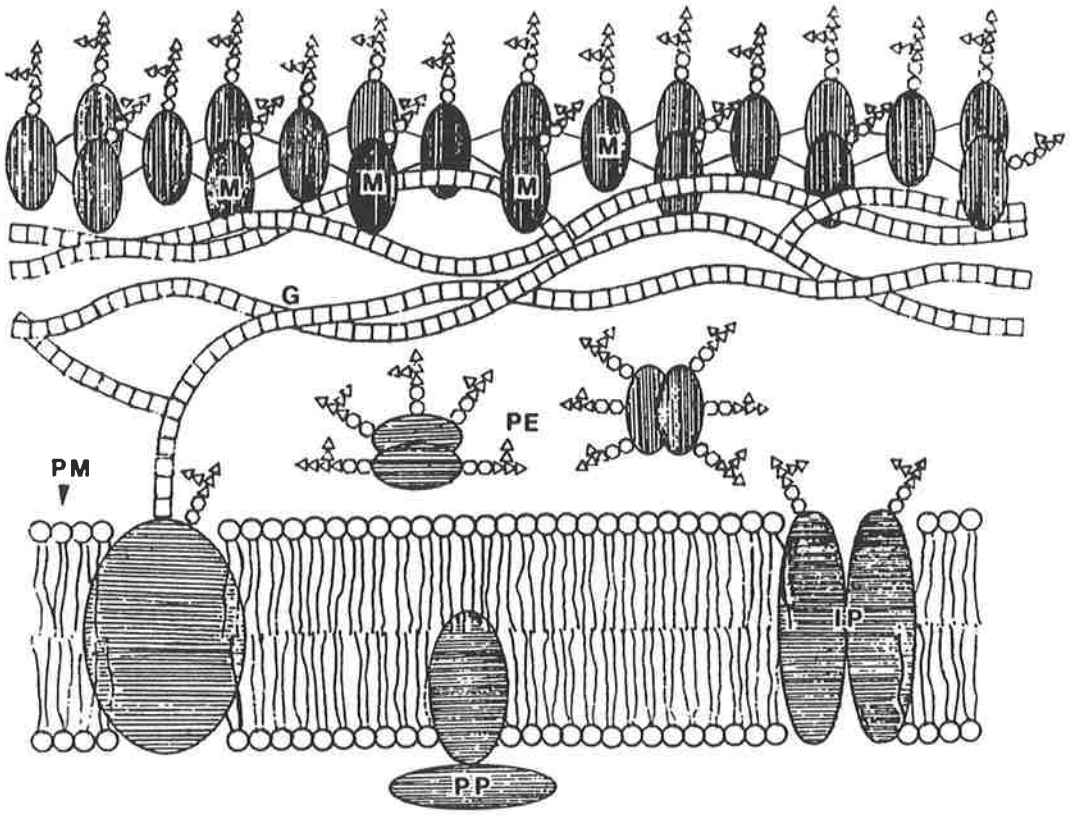


Figure 1.2 Cell wall molecular organisation (from Schekman and Novick 1982). G, glucan; M, mannoprotein; PE periplasmic enzyme; IP, integral membrane protein; PP, peripheral membrane protein.

The *N*- and *O*-glycosylation of the oligosaccharide core occurs during translocation of the nascent protein into the ER, although elongation of the outer chain, with mannose addition catalysed by a set of mannosyltransferases, is completed in the Golgi apparatus during the transit to the cell surface. Phosphorylation as well as protein folding and assembly occur in the ER while proteolytic cleavage takes place in both the ER and the Golgi apparatus (Schekman and Novick 1982, Rothblatt and Schekman 1989).

Mannoproteins are finally secreted into the extracytoplasmic medium by the standard exocrine process: the secretory vesicles fuse with the plasma membrane and deliver the soluble mannoproteins to the cell surface. For instance, invertase and acid phosphatase, both periplasmic mannoproteins, were found to be transported by vesicles to the plasma membrane (Schekman and Novick 1982). Some alternative secretory pathways like a direct extrusion of mannoproteins across the plasma membrane were once proposed but no further evidence tends to support this hypothesis. (Rothblatt and Schekman 1989, Schekman and Novick 1982, Sentandreu *et al.* 1984).

Assembly of the different cell wall components to give the final supramolecular organisation involves mechanisms which still remain obscure today.

1.5.3 CELL WALL EVOLUTION OVER THE YEAST GROWTH CYCLE

Yeast cells, in the vegetative cycle, reproduce exponentially through the budding process. With nutrient depletion, the cells stop the division process to enter the stationary phase. The initial wall in exponential phase is soft and deformable whereas the final wall in the stationary phase is rigid, thicker and less porous (Valentin *et al.* 1987, De Nobel and Barnett. 1991).

During the transition from exponential to stationary phase, deposition of amorphous material at the more external layer of the cell wall was first observed by Northcote and Horn (1952). The molecular mass of mannoproteins extracted from whole cells in the early exponential phase ranged from 120 to 500 k (with a mean of 200 k) whereas they ranged from 250 to 350 k (mean of 300 k) in the late exponential phase (Valentin *et al.* 1987). The size of the protein moiety of this material as well as the amount of *O*-glycosylated mannose were the same throughout the different growth stages. The increase in size was attributed to the *N*-linked

mannose with either an elongation of the outer chains or an increase in the number of *N*-linked mannose chains. The same general observation was reported by Thieme and Ballou (1972): mannoproteins in the exponential phase were found to be more heterogeneous and some of them larger than those in stationary phase. The change in mannoprotein size implies some restructuring that may account, in part, for the cell wall rigidification and increase in thickness. Another explanation for the cell wall rigidification would be the variation of the disulfide bridge content which significantly increases for stationary phase cell walls (De Nobel *et al.* 1990).

During the cell growth, the general content of cell wall glucan stays the same although the amount of alkali insoluble glucan increases to the detriment of the alkali soluble glucan (Katohda *et al.* 1976). Hartland *et al.* (1993) showed that the alkali insoluble glucan is directly derived from an alkali soluble precursor. Formation of covalent linkages between chitin and ß-glucan is also thought to be an important part of the process of cell wall rigidification.

1.5.4 AUTOLYTIC DEGRADATION OF THE CELL WALL

After cell death, the cells undergo a process of self enzymatic degradation of their cellular constituents which is called autolysis. This process is characterised by a few typical changes of the cell wall. Of the three cell envelope layers usually observed under electron microscopy, Piton *et al.* (1988) observed the almost complete disappearance of the most internal mannoprotein layer in the first three months of Champagne wine ageing on yeast lees following the secondary fermentation. The most external mannoprotein layer decreases only slowly over eight years of ageing. Chemical analyses of the cell wall have shown a decrease of the glucan and protein fractions with a subsequent increase in the mannose to glucose ratio (Charpentier and Feuillat 1986). This decrease is attributed to a group of ß(1-3)glucanases (Fleet 1991) which degrade the glucan network and thus disorganise and loosen the cell wall structure. The disorganisation is responsible for an increase in porosity that allows the release of all the intracellular degradation products into the medium. The loosening of the glucan layer could explain the 10% increase in the cell wall thickness observed by Freyssinet *et al.* (1989).

These observations are consistent with those of Feuillat *et al.* (1989) and, Charpentier and Feuillat (1993) who observed an increase in mannose and glucose rich macromolecules in wine during autolysis. Monitoring of the macromolecules released during autolysis in model wine (pH 3, 40°C, 14 days) revealed an early and constant release of glycosidic colloids and, in particular, of a 400 k M_r fraction. The pool of mannoproteins released into the medium increased steadily over 14 days in contrast to the glucan fraction which was degraded by ß(1-3) glucanases present in the medium. The protein pool released in the autolytic medium increased during the initial 48 hours (at pH 3, 40°C) and then decreased due to the action of acid protease A (Charpentier and Freyssinet 1989).

High M_r polysaccharides (2,000 k) of yeast origin were also observed in wine kept on yeast lees (Meursault type wine where the wine stayed in contact with yeasts for 8 to 9 months). A shift towards polysaccharides of lower M_r (150 and 70 k) was observed with time (Feuillat *et al.* 1989). In a similar study, yeast polysaccharides (600 k) present at the beginning of the autolysis were partly hydrolysed into fractions of 60 and 20 k after six months (Freyssinet *et al.* 1989).

1.5.5 KNOWN GLYCOPROTEINS OF THE CELL ENVELOPE OF *SACCHAROMYCES*

Besides the structural cell wall mannoproteins, there are two other categories of mannoproteins which are well defined by their functionality, location and biological properties.

1.5.5.1 Periplasmic enzymes

This first category is that of the inducible hydrolytic enzymes which hydrolyse nutritional substrates (which cannot penetrate the cytoplasmic membrane) in the periplasmic space to enable the cell to metabolise them intracellularly. Two well-known periplasmic enzymes are invertase (ß-fructofuranosidase) and acid phosphatase (Arnold 1991).

Invertase activity in normal growth conditions is virtually zero, it will appear only when the yeast cells are grown on very low concentrations of glucose or in the presence of substrates such as sucrose. Invertase is a mannoprotein composed of approximatively 50% protein and 50% mannose. The carbohydrate moiety consists of a mannose polymer with an α(1-6) linked

mannose backbone with a core and an outer chain as for the structural mannoprotein (Neumann and Lampen 1967, Ballou 1976). The carbohydrate portion is linked to asparagine residues of the protein via the two GNAc units. No *O*-glycosylated mannose oligosaccharides were found to be associated with the peptide moiety, and the peptide moiety was always composed of two identical 60 k subunits (Trimble and Maley 1977). Invertase is usually found as a dimeric form (Gascon and Lampen 1968) with a M_r ranging from 200 to 300 k according to the degree of glycosylation of the sugar moiety (Trimble and Maley 1977, Esmon *et al.* 1981) but can also form oligomers with up to eight units (Chu *et al.* 1983). Invertase is in a free state in the periplasmic space and is barely released into the growth medium by intact *Saccharomyces cerevisiae* cells (Sutton and Lampen 1962, Cordonnier *et al.* 1975).

Acid phosphatase hydrolyses phosphate esters which cannot penetrate the cytoplasmic membrane. In *Saccharomyces cerevisiae*, this mannoprotein is composed of about 50% mannose *N*-linked to the peptide. The relative molecular mass of the mannoprotein ranges from 170 k to 360 k because of the high heterogeneity of its carbohydrate part (Barbaric *et al.* 1984). The native enzyme exists in a dimeric form and is mainly in a free state in the periplasmic space although 25% of it can be found bound to the cytoplasmic membrane (Arnold 1972). Linnemans *et al.* (1977) claimed that acid phosphatase could also be found on the cell wall surface. Arnold and Garrison (1981, see Arnold 1991) showed that this discrepant report was in fact an artifact and was due to surface deposits of lead during the cytochemistry study.

Other periplasmic enzymes like asparaginase, melibiase or trehalase are less well characterised (Arnold 1991).

1.5.5.2 Sexual agglutination factors

The second type of known mannoproteins are the sexual agglutination factors α-agglutinins and a-agglutinins. These mannoproteins are found at the cell surface of haploid yeast cells and mediate the process of agglutination with mating cells (Terrance and Lipke 1981). The first type contains 50% sugar whereas the second has 90%, with both sugar moieties composed of *O*- and *N*-linked carbohydrates. In contrast to the periplasmic enzymes, these mannoproteins

do contain some glucose although the mannose content can reach 94% of the total sugar content (Sijmons *et al.* 1987).

1.5.5.3 Is HPF a known mannoprotein ?

Following the isolation and partial characterisation of HPF, the scientific literature was regularly surveyed for any data available on yeast mannoproteins. No other yeast mannoprotein described in the literature was similar to HPF regarding the size, sugar and amino acid content compositions, linkage type and putative location. This survey was continued throughout this work.

1.6 AIMS OF THIS THESIS

HPF offers the wine industry a potential alternative to the use of bentonite fining to stabilise wines from protein haze. Limited information about HPF is available and there is a dearth of information about means of producing haze protective material (HPM) from yeasts. Thus there is a need to explore ways to produce HPM and to obtain more material for further research work on HPF. Furthermore, the location and interactions of HPF with the cell envelope components are not known. Such fundamental information would be also valuable for an optimisation of HPF production.

This thesis therefore investigated:

* effective treatments to extract HPM from yeast cells,
* information about its release during the process of fermentation and storage on yeast lees,

and,

* how HPF interacts with the cell envelope components and where it is located within the cell envelope.

Chapter 2

GENERAL MATERIAL AND METHODS

2.1 MATERIAL

Ammonium chloride, D-glucose 'Analar' (analytical grade), ethylenediamine tetraacetate (EDTA), sodium dodecyl sulfate (SDS), 2-mercaptoethanol (2-ME), glycine and riboflavin were purchased from BDH or BDH & Merck Laboratory Chemicals Pty Ltd (VIC, Australia). Glycine was sourced from Merck KGA (Germany). Mannose, D-(+)-mannose, methyl-α-D-mannoside, yeast invertase, bovine serum albumin (BSA, fraction V), Coomassie Brilliant Blue R-250, Tris(hydroxymethyl)aminoethane (Tris), ß-D(+) glucose and glycerol, dithioerythritol (DTE) were from Sigma Chemical Company (MO, USA). Myo-inositol was sourced from Fluka Biochemie (Switzerland). Zymolyase 100T was sourced from ICN Pharmaceuticals Inc (NSW, Australia). Concanavalin-A Sepharose 4B and H/R columns (medium pressure tolerance) were purchased from Pharmacia Australia Pty Ltd (NSW, Australia).

Diaflo ultrafilters YM 10 (10 k Molecular Weight Cut-Off) membranes were from Amicon Ltd (MA, USA). Dialysis tubings (around 12 k Molecular Weight Cut-Off) were purchased from Selby Scientific (SA, Australia). Acrylamide, *N'N'*-bis-methylene-acrylamide, ammonium persulfate, *N,N,N',N'*-tetramethylethylene-diamine (TEMED), bromophenol blue, BioRad molecular weight standards, BioRad Silver stain kit and Econo-Pac 10 DG desalting columns were from BioRad Laboratories Pty Ltd (NSW, Australia).

Bacteriological agar was from Amyl company (VIC, Australia). The Yeast Peptone Dextrose medium (YPD medium) contained, per litre: yeast extract (10 g), peptone (20 g) and dextrose (20 g), and was purchased from DIFCO Laboratories (MI, USA). The 0.2 μm autoclavable membranes were sourced from Gelman Sciences (MI, USA).

The D-glucose/D-fructose UV method determination kit and phosphomannose isomerase (PMI) were provided by Boehringer Mannheim GmbH (Mannheim, Germany). The kit contains phosphofructose isomerase, hexokinase and glucose-6-phosphate dehydrogenase as suspensions and triethanolamine buffer in the form of a powder containing ATP, NADP and magnesium sulfate.

All other products were of the highest purity available.

Water used was purified by a Milli-Q reagent water system from Millipore Pty Ltd (NSW, Australia).

2.2 YEAST GROWTH

2.2.1 YEAST STRAIN

The yeast strain used was the 'Prise de mousse' (Maurivin, Mauri Foods yeast group, QLD, Australia) which originated from the Champagne region. This diploid *Saccharomyces cerevisiae* (var. *bayanus*) strain will be referred to in this study as Maurivin PDM.
Yeast stock culture was maintained at 4°C on slopes of YPD medium supplemented with agar (20 g/L) and subcultured every 6 months.

2.2.2 SYNTHETIC GRAPE JUICE MEDIUM (SGJM)

The synthetic grape juice medium (adapted from Henschke and Jiranek, 1993) approximates the composition of a typical grape juice. SGJM contained glucose (200 g), mineral stock solution (1 mL), potassium hydrogen tartrate (2.5 g), L-malic acid (3 g), $MgSO_4.7H_2O$ (1.23 g), K_2HPO_4 (1.14 g), $CaCl_2$ (0.33 g), citric acid (0.2 g), myo-inositol (100 mg), pyridoxine HCl (0.78 mg), nicotinic acid (3.125 mg), calcium pantothenate (1.95 mg), thiamin HCl (1.055 mg), riboflavin (78 μg), biotin (24 μg), NH_4Cl (1.76 g) per litre. This medium was adjusted to pH 3.5 and sterile filtered through a 0.2 μm membrane.

Mineral stock solution (x 1,000) contained $MnCl_2$ $4H_2O$ (198.2 μg), $ZnCl_2$ (135.5 μg), $FeCl_2$ (31.96 μg), $CuCl_2$ (13.6 μg), H_3BO_3 (5.7 μg), $Co(NO_3)_2$ $6H_2O$ (29.1 μg), $NaMoO_4$ $2H_2O$ (24.2 μg), $KClO_3$ (10.8 μg) per litre.

2.2.3 Yeast propagation

Using a microbiological loop, yeast from a YPD slope culture was initially seeded into a sterile flask containing SGJM (2-4 mL). According to the final volume of the fermentation medium (see the experimental Section in the following chapters) and the standard inoculation rate at each step [approximately 5% (v/v)], the volume at each propagation stage varied (see the experimental Section in the following chapters). The yeast propagation cultures were incubated overnight at 27°C with a loosely fitted screwcap lid. When the growing culture was in exponential phase (15-20 hours), the transfer to the next propagation medium was performed.

2.2.4 Yeast growth

According to volume, the fermentation was conducted in different sized vessels as specified in the experimental Section of the following chapters. In all cases, the fermentation vessel was sealed by a rubber bung fitted with a fermentation lock, to minimise the ingress of air, and a hypodermic needle was connected to a floating line to sample the culture.

Unless otherwise indicated, following inoculation with freshly propagated yeast culture, incubation took place at 25°C with agitation on a shaking platform (110 rpm). Yeast growth was monitored by measuring the absorbance at 650nm (A650 nm) of the fermentation culture on a UV max microplate reader (Molecular Device Corp, CA, USA). The cellular morphology of the cells including budding was also assessed by phase contrast microscopy.

2.3 GENERAL METHODS

2.3.1 WET WEIGHT DETERMINATION

A nominal wet weight of yeast cells in grams was determined following the separation from the yeast culture supernatant and washing of harvested cells by centrifugation (18,000 *g*, 10 min, 5°C) with a Beckman centrifuge (USA), Model J2-21M/E, rotor AJ10, capacity 3 L. For technical reasons dry weights of yeast cells could not be determined in all cases and thus only wet weight data are given throughout this study.

2.3.2 SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS PAGE)

SDS PAGE was performed on a pre-prepared, ready to use, 4-15% gradient gel from BioRad Laboratories Pty Ltd (Mini-Protean II Ready Gels in 0.375 M Tris-HCl, pH 8.8) according to the method of Laemmli (1970). BioRad molecular weight standards (BioRad Laboratories Pty Ltd) were used as M_r markers.

2.3.2.1 Sample preparation

Samples were diluted at least fivefold in sample buffer, boiled for four minutes, and then loaded into the wells. Electrophoresis sample buffer was prepared by combining water (400 µL), 0.5M Tris-HCl (pH 6.8; 150 µL), glycerol (100 µL), 10% (w/v) SDS (200 µL), 2-ME (100 µL) and 0.05% (w/v) bromophenol blue (50 µL).

2.3.2.2 Electrophoretic conditions

A BioRad Mini Protean II unit (BioRad Laboratories Pty Ltd) was used to run the gels. They were run at a constant voltage setting of 200 V until bromophenol tracker dye was 5 mm from the bottom of the gel (usually 40 min). The running buffer contained Tris-HCl (3 g), glycine (14.4 g) and SDS (1 g) per litre.

2.3.2.3 Staining for protein

After electrophoresis, the separated proteins were fixed and stained by incubating the gel in fixative (methanol/acetic acid/water 4:1:5) containing 0.1% (w/v) Coomassie Brilliant Blue R-250 for 30 min and then destained with either fixative or water for 30 min.

2.3.2.4 Staining for carbohydrate

Staining to detect carbohydrate material was accomplished with the periodic acid Schiff (PAS) staining procedure of Allen *et al.* (1976). After fixing, gel was incubated in 0.2% aqueous periodic acid at 4°C for 45 min, in Schiff's reagent at 4°C for 45 min and then destained in 10% acetic acid. Schiff's reagent was prepared by dissolving basic fuschin (1 g) in boiling water (200 mL), stirring for 5 min and then cooling to 50°C. 1 M HCl (20 mL) was added and the mixture cooled to 25°C. Sodium metabisulfite (1 g) was added and the solution left in the dark overnight. Activated charcoal (2 g) was added and the mixture was shaken vigorously for 1 min then filtered.

2.3.3 CONCANAVALIN-A AFFINITY CHROMATOGRAPHY

Degassed Concanavalin-A (Con-A) gel suspended in acetate buffer was packed into a HR 16/50 column (16 mm x 500 mm, column volume approximately 100 mL), unless otherwise indicated, and equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 0.5 mM of $CaCl_2$, $MgCl_2$ and $MnCl_2$ (starting buffer). Samples were diluted tenfold in starting buffer and degassed by sonication under vacuum (at least 15 min per litre) before loading (flow rate: 1 mL/min). Collection of the unbound material was achieved with degassed (by sonication under vacuum) starting buffer (around tenfold the column volum, flow rate: 1 mL/min). Elution of the bound material was achieved by adding a 0.1 M solution of methyl-α-D-mannoside in degassed starting buffer (elution buffer) at constant flow rate (1 mL/min). Protein was detected by monitoring the absorbance at 280 nm on a Waters 440 absorbance detector (Waters Millipore, MA, USA). Data were collected and analysed by a Waters Maxima 820 software.

Regeneration of the column was achieved by washing with at least three column volumes of 0.1 M Tris-HCl buffer (pH 8.5) containing 0.5 M NaCl and a further three column volumes of 0.1 M sodium acetate buffer (pH 4.5) containing 0.5 M NaCl. This cycle was repeated three times followed by re-equilibration with at least five column volumes of starting buffer.

2.3.4 ULTRAFILTRATION

Ultrafiltration was performed in 400 mL capacity stirring cells (Waite Campus Engineering Workshop, SA, Australia) at 4°C under a nitrogen pressure of about 400 kPa. Cells were equipped with a Diaflo YM 10 (10 k Molecular Weight Cut-Off) membrane (Amicon Ltd, MA, USA) unless otherwise stated. For sample volumes greater than 400 mL, the cell was refilled with sample until the retentate volume reached 25-50 mL, then the initial volume (400 mL) was reconstituted with water and the sample was re-concentrated again until the estimated salt (or sugar) concentration dropped below 10 μg.

2.3.5 MICROMETHOD FOR THE MEASUREMENT OF THE HEAT INDUCED HAZE (HEAT TEST)

The effects of mannoprotein additions on the protein haze potential were determined by the micromethod described by Waters *et al.* (1991) modified as follows.

Centrifuged aqueous solutions of mannoproteins (0-15 μL made up to 15 μL water) were added to ultrafiltered wine (180 μL) containing a known amount of bovine serum albumin (BSA, 125 μg, 5 μL of 25 μg/μL) following the proportions in Table 2.1 Wine ultrafiltration was performed in a 400 mL capacity ultrafiltration cell (Section 2.3.4) at 4°C under nitrogen pressure of 150 kPa. The filtrate was collected under nitrogen atmosphere to minimise oxidation and stored at -20°C in 250 mL aliquots. After being mixed and sealed, the samples were heated for one hour at 80°C and then left on ice for one hour. After 20 min at room temperature and mixing, 100 μL of each sample was transferred to a 96 flat-bottomed well microplate. The haziness was measured at A490 nm on an UV max microplate reader (Molecular Device Corp, CA, USA).

The A490 nm values for the sample tested at different concentrations (tube series 1-5, Table 2.1) and the control values were corrected for the blank value (wine without BSA added corresponding to 0% of haze formed). The corrected control value (ultrafiltered wine plus BSA without sample added) corresponded to 100% of haze. The difference between the blank and the control value was termed the 'working margin'.

Wines used in this study were Muscat Gordo Blanco (Gordo) from the 1994 vintage and Sauvignon Blanc from the 1996 vintage. Both of them were supplied by Orlando Wyndham Ltd (Barossa Valley, SA, Australia), were not protein stabilised and stored at -20°C. The Sauvignon Blanc wine was supplemented with Riesling grape extract at 10% (v/v) (see next Section 2.3.6).

To obtain comparable heat test results with different wines (ultrafiltered wine or ultrafiltered wine supplemented with grape extract), invertase was used as a reference and was systematically heat tested for each series of heat tests. The average value of haze decrease observed with invertase (around 45-35% of the initial haze value for 0.075 to 0.15 mg of invertase per mL) were used as a reference value. If the heat test values obtained with invertase were too low or too high compared to the reference values mentioned above, results of that series were not considered.

Table 2.1 Protocol for the measurement of the heat-induced protein haze

	Ultrafiltered wine (µL)	BSA (µL)	Sample (µL)	Water (µL)
Blank	180	0	0	0
Control	180	5	0	15
Tubes series 1	180	5	2.5	12.5
Tubes series 2	180	5	5	10
Tubes series 3	180	5	7.5	7.5
Tubes series 4	180	5	10	5
Tubes serise 5	180	5	15	0

2.3.6 PRODUCTION OF GRAPE EXTRACT

When the working margin of the heat test (difference between the blank value and the corrected control value) was considered too low to properly observe the effects of mannoprotein addition on haze, ultrafiltered wine was supplemented with grape extract to increase the working margin.

The grape extract was prepared from whole frozen Riesling berries (cuticules included) collected during the 1995 vintage in Eden Valley (SA, Australia). The thawed berries were pressed and thoroughly rinsed with model wine solution. This cycle was repeated three times, the free run juice and the washings were discarded. The crushed berries [60% (w/w) in model wine solution] were then homogenized with a Ultra-Turrax high speed homogenizer with a F25N dispersing head (Janke and Kunkel GmbH, Germany). The extract was then maintained under agitation and nitrogen atmosphere at room temperature for one hour before being centrifuged (3,000 *g*, 5 min). The supernatant was ultrafiltered on a YM 10 membrane (Section 2.3.4) and then stored in 5 mL aliquots at -20°C or directly added to ultrafiltered wine.

The model wine contained 5 g potassium hydrogen tartrate, 25 mg sodium metabisulfite, 20 mg ascorbic acid per litre and 10% (v/v) ethanol.

2.3.7 METHOD FOR THE DETERMINATION OF MANNOSE AND GLUCOSE CONTENTS

Polymeric forms of mannose and glucose present in the samples were hydrolysed into monomeric sugars. The total amount of monomeric sugars was then determined enzymatically using the D-glucose/D-fructose UV method determination kit (Boehringer Mannheim GmbH, Mannheim, Germany). The initial monomeric sugar content present in the samples was measured enzymatically as above with omission of the hydrolysis step. The content of polymeric sugars was calculated by subtraction of the initial monomeric content from the total content of monomeric sugars after hydrolysis.

2.3.7.1 Hydrolysis

Two volumes of 2.25 M sulphuric acid were added to one volume of sample, the solution was then heated for 90 min at 100°C in glass tubes. Cooled hydrolysed samples (60 µL) were transferred to microplate wells and neutralised with addition of 90 µL NaOH (2 M) and 75 µL triethanolamine buffer (pH 7.6).

2.3.7.2 Enzymatic assay

The principles of the enzymatic reactions are given below (Boehringer Mannheim, 1992). ATP (adenosine-5'-triphosphate) and NADP (nicotinamide-adenine dinucleotide phosphate) appearing in the reactions were present in the triethanolamine buffer used for the enzymatic assay.

1- Measurement of glucose content with the addition of the enzyme set E2

Enzyme set E2: Hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH) catalyse, respectively, the conversion of glucose to glucose-6-phosphate (G-6-P, reaction 1) and to gluconate-6-phosphate (reaction 2). The amount of NADPH formed in reaction 2 is equivalent to the amount of glucose present and is measured by the increase in absorbance at 340nm.

$$\text{D-Glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{G-6-P} + \text{ADP} \qquad \text{(reaction 1)}$$

$$\text{G-6-P} + \text{NADP}^+ \xrightarrow{\text{G6P-DH}} \text{gluconate-6-phosphate} + \text{NADPH} + \text{H}^+ \qquad \text{(reaction 2)}$$

Enzyme E3: Enzyme E3 is phosphoglucose isomerase (PGI). In reaction 4, PGI catalyses the conversion of fructose-6-phosphate (F-6-P, formed in reaction 3) to G-6-P which is then catalysed by G6P-DH (reaction 2). The amount of NADPH obtained in reaction 2 is equivalent to the amount of fructose (reaction 3) present and is measured by the increase in absorbance at 340nm.

$$\text{D-Fructose + ATP} \xrightarrow{\text{HK}} \text{F-6-P + ADP} \qquad \text{(reaction 3)}$$

$$\text{F-6-P} \xrightarrow{\text{PGI}} \text{G-6-P} \qquad \text{(reaction 4)}$$

2- Measurement of the mannose content with the addition of the enzyme phosphomannose isomerase (PMI)

In reaction 6, PMI converts mannose-6-phosphate (M-6-P, formed in reaction 5) to F-6-P which is converted to G-6-P (reaction 4) and to gluconate-6-phosphate (reaction 2). The amount of NADPH formed in reaction 2 is equivalent to the amount of mannose (reaction 5) present and is measured by the increase in absorbance at 340nm.

$$\text{D-Mannose + ATP} \xrightarrow{\text{HK}} \text{M-6-P + ADP} \qquad \text{(reaction 5)}$$

$$\text{M-6-P} \xrightarrow{\text{PMI}} \text{F-6-P} \qquad \text{(reaction 6)}$$

The absorbance (A340 nm) was read prior to the addition of the set of enzymes E2 and E3 (7.5 µL each) and after one hour of incubation at 25°C. As yeast cells do not release any fructose (Usseglio-Tomasset, 1978), the enzymes E2 and E3 were used simultaneously and the corresponding A340 nm reading is the measure of the glucose content only. PMI enzyme (8 µL) was then added and after one hour at 25°C the A340 nm was again taken. The difference between A340 nm before and after PMI addition is a measure of the concentration of mannose in the sample.

Each measurement for the enzymatic assay was repeated at least three times or until an average value with a standard deviation lower than 5% was reached.

Known amounts of monomeric D-(+)-mannose and ß-D(+) glucose were used to determine the standard curves. Values for glucose and mannose concentrations of samples were calculated from the regression equation of the standard curves.

2.3.8 AMINO ACID COMPOSITION

Mannoprotein extracts were hydrolysed in 6 M HCl containing 1% (w/w) phenol at 117°C for 16 hours. The amino acid content was analysed using AminoQuant II amino acid analyser (Hewlett-Packard, CA, USA) according to the manufacturer's instructions.

Chapter 3

EVALUATION OF METHODS FOR EXTRACTING HAZE PROTECTIVE MATERIAL FROM *SACCHAROMYCES CEREVISIAE*

3.1 INTRODUCTION AND LITERATURE REVIEW

Fractionation of cell wall components was developed over many years of empirical work (Fleet 1991) and a range of different methods are now available to extract the mannan fraction. As for glucan or chitin extractions (described in Sections 1.5.1.1 and 1.5.1.2), a partial or complete disruption of the cell wall is necessary to release the cell wall mannans. This can be obtained by physical, chemical or enzymatic means and these methods and their consequences will be discussed in this Section.

Hot citrate extraction by autoclaving whole yeast cells in citrate buffer (pH 7) was one of the first methods to extract cell wall mannoproteins. Extensive autoclaving according to Peat *et al.* [1961, cells autoclaved twice at 140°C (unspecified pressure) for 120 min] led to an almost complete extraction of the mannoprotein fractions. Valentin *et al.* (1984) reported extraction of 80% of the total cell wall proteins by hot citrate treatment. An alkali treatment was needed to extract the autoclave-resistant mannan fraction and to obtain a complete removal of the cell wall mannans (Fleet, 1991).

The main mannoprotein fraction released by autoclaving (135°C, unspecified pressure, 90 min) had a M_r around 133 k (Nakajima and Ballou, 1974). Shibata *et al.* (1983) found that extensive autoclaving (135°C, unspecified pressure, 180 min) released mannoproteins three times smaller than those extracted by zymolyase treatment. This observation supports the suggestion of Van Rinsum *et al.* (1991) that hot citrate treatment does not preserve the structure and composition of the mannoprotein complexes as much as other treatments such as ß-glucanase treatments do and can lead to a breakdown of these complexes. When the cells

are treated at high temperature and pressure for a long period of time, the non-covalent links as well as some covalent links are destroyed, and degraded mannoproteins with lower M_r can be released. The partial degradation of the mannoprotein sugar moiety may also reduce the capacity of the mannoproteins to be ethanol precipitable (Valentin *et al.* 1984).

Treatment of whole yeast cells with ß(1-3) glucanase is a powerful and commonly used tool to form protoplasts and to subsequently release periplasmic material into the medium. For instance, 95% of invertase located in the periplasmic space can be found in the medium after protoplasting cells (Pastor *et al.* 1982). It is conventional to facilitate the action of ß(1-3) glucanase on the glucan network by pretreating the cell walls or whole cells with a protease or a reducing agent solution to open up the cell wall and give the enzyme access to the glucan network. This pretreatment is thought to soften up the upper mannoprotein layer of the cell wall (see Section 1.5.1.4 for cell envelope organisation) whereas, by its enzymatic action, a ß(1-3) glucanase such as zymolyase is likely to release material which is covalently linked to or embedded in the cell wall glucan network (Pastor *et al.* 1984, Elorza *et al.* 1985, Molloy *et al.* 1989).

Treatment with ß(1-3) glucanase has also been used to isolate cell wall surface mannoproteins. A zymolyase treatment on whole cells previously washed with ethylenediamine tetraacetate (EDTA) solution, or simply with water, efficiently extracted α-agglutinins (Pierce and Ballou 1983, Hauser and Tanner 1989).

Pastor *et al.* (1984) observed that treatment of *Saccharomyces cerevisiae* cell walls with zymolyase and laminarinase (both containing ß(1-3) glucanase activity) preferentially released a group of mannoproteins which could be separated, electrophoretically, into a diffuse band around 120 k and a discrete band at 29 k. A multitude of mannoproteins with relative molecular weights below 100 k were released when cell walls were extracted with hot sodium dodecyl sulfate (SDS), including the 29 k material, whereas the 120 k material mentioned above was not extracted (Pastor *et al.* 1984). Valentin *et al.* (1984) also observed the great heterogeneity in size of the mannoprotein fraction solubilised by SDS and concluded that SDS is far less specific in releasing material compared to zymolyase.

The difference in mass of the mannoproteins released either by SDS or zymolyase was also described in the work of Elorza *et al.* (1985) on *Candida albicans* although Molloy *et al.* (1989) argued that the lower M_r material found in SDS extracts was from the cytoplasmic membrane.

In the research work mentioned above, the size of the mannoproteins extracted was determined electrophoretically on SDS polyacrylamide gels. Although it depends on the acrylamide percentage of the gel, only material with M_r less than to 120 k or 200 k could migrate and be observed. Therefore, the results described by Pastor *et al.* (1984), Valentin *et al.* (1984) and Elorza *et al.* (1985) only apply to mannoproteins not exceeding 200 k in mass. Interestingly, material remaining at the top of the separating gel was observed for the various fractions run by Valentin *et al.* (1984) and Pastor *et al.* (1984). Molloy *et al.* (1989) also noticed some material which remained in the stacking gel and at the top of the separating gel for the SDS extracted material from *Candida albicans*. This problem was also encountered by Waters *et al.* (1993) with the separation of purified HPF and by Frevert and Ballou (1985) for the separation of a cell wall mannoprotein from *Saccharomyces cerevisiae*. This behaviour is typical for that of very high M_r mannoproteins which cannot migrate into the gel because of their high mannose content. Considering this fact, it is likely that very high M_r mannoproteins are released by both hot SDS and zymolyase treatments.

Cell wall mannoproteins can also be solubilised by using various chemicals. As mentioned above, SDS, an anionic detergent, is often used in the fundamental studies of the cell wall composition because of its ability to solubilise mannoproteins. Indeed, SDS is thought to release material which is not intrinsic (internal) and not covalently linked to the cell wall (Elorza *et al.* 1985, Molloy *et al.* 1989, Pastor *et al.* 1984).

Urea is another chemical used to solubilise cell wall material because of its strong denaturing ability. Treatment of purified cell walls with urea can release 65% of the total cell wall proteins with a preference for those of low M_r (< 90 k). The band pattern on SDS polyacrylamide gel of urea extracts was somewhat similar to that of SDS extracted mannoproteins (Valentin *et al.* 1984).

Mechanical disruption of the cell wall has not been used often for fundamental studies of cell wall mannoproteins but is a powerful tool to release periplasmic mannoproteins. Sonication of whole cells was used by Kidby and Davies (1970) to release periplasmic material although the physical breakage of the cell by applying shearing forces, with a French press apparatus or with a homogenizer, is more efficient. The cracks formed in the cell wall during this physical breakdown could release 85% of invertase into the medium simultaneously with 75% of acid phosphatase (Arnold, 1972). Mechanical disruption of the cell wall by shaking with fine glass beads with or without a preliminary freeze-thaw cycle was also efficient in releasing α-agglutinins (Yamaguchi *et al.* 1982, Terrance *et al.* 1987).

Thus, mannoproteins of different M_r or location can be extracted from the cell envelope under enzymatic, chemical or mechanical treatment. Extraction of cell wall mannoproteins by enzymatic treatment is advantageous because the solubilisation is gentle and tends to preserve the initial structure of the extracted components. A similar tendency is observed for the chemical treatments where milder methods, such as hot citrate extraction, are preferred to the harsher, hot alkali extraction. Besides the enzymatic and chemical treatments, physical treatments that involve the use of shearing force to disrupt the wall are still widely used. The literature demonstrates that these three categories of methods successfully release high M_r mannoproteins from the cell envelope and thus may be suitable for the release of HPF. Furthermore, because each method disrupts cells and releases material according to its specific properties (SDS has detergent properties, zymolyase is a ß-glucanase, French press releases periplasmic material), the possible release of haze protective material (HPM) with these different methods can provide information on how HPF interacts with other cell components and, possibly, where it is located within the cell envelope.

The four extraction methods reported (SDS, full zymolyase, French press and autoclave treatments) were tested with different yeast strains for the release of HPF ; the results obtained are presented and discussed in this Chapter.

3.2 EXPERIMENTAL

3.2.1 MATERIAL

Phenylmethylsulfonyl fluoride (PMSF) was purchased from Sigma Chemical Company (MO, USA). Other materials were as described in Section 2.1.

3.2.2 YEAST STRAINS

The yeasts used were Maurivin PDM (see Section 2.2.1) plus two *Saccharomyces cerevisiae* strains AWRI 65 and AWRI 85 which were freeze-dried cultures obtained from the Australian Wine Research Institute (AWRI) collection. AWRI 65 (synonyms: AWRI 1A, AWRI 350) is described in the AWRI collection records as a wine yeast, flocculent and non-producer of H_2S. AWRI 85 (synonym: AWRI 275) is described as a French Champagne yeast with no particular characteristics.

Yeast were kept at 4°C on YPD slope cultures and were subcultured every six months.

3.2.3 GROWTH ON GLUCOSE ENRICHED MEDIA

The yeast propagation was conducted as described in Section 2.2.3. The volumes for the propagation stages were 3, 25 and 500 mL, respectively. The final propagated culture (500 mL), upon reaching exponential phase, was transferred to 9.5 L SGJM.

Final growth was carried out in 10 L of SGJM contained in a 25 L plastic fermentation vessel at the conditions mentioned in Section 2.2.4. Cultures were grown up to late exponential phase (7.5-11.5 g/L, wet cell weight) or to stationary phase (16 g/L, wet cell weight) [refer to the growth curves in Figures 1 A), 2, 3, Appendix A].

Cells were harvested by centrifugation (18,000 *g*, 10 min, 5°C) and washed with water (x 3, each time with 10 L). The wet cell pellet was weighed and stored at -20°C in aliquots (10 g). The culture supernatants of Maurivin PDM cultures were collected, filtered through a 0.45 μm membrane and stored at -20°C until further analyses (see Chapter 5).

3.2.4 GROWTH ON MANNOSE ENRICHED MEDIA

The yeast propagation was conducted as described in Section 2.2.3. Consecutive propagation volumes of 5-7 and 150 mL were used. The final propagated culture (150 mL), once reaching exponential phase, was transferred to 2.9 L SGJM.

Growth of Maurivin PDM on mannose was carried out in 3 L of SGJM (contained in a 4 L glass container) as mentioned in Section 2.2.4 except containing 150 g per litre of mannose in plpace of glucose. Culture was grown up to late exponential phase [8-11 g/L, wet cell weight, refer to the growth curve in Figure 1 B), Appendix A].

Cells were harvested by centrifugation (18,000 *g*, 10 min, 5°C), washed with water (x 3, each time with 3 L). The wet cell pellet was weighed and stored at -20°C in aliquots (10 g). The culture supernatants of Maurivin PDM cultures were collected, filtered through a 0.45 μm membrane and stored at -20°C until further analyses (see Chapter 5).

3.2.5 MANNOPROTEIN EXTRACTION FROM WHOLE YEAST CELLS

The cells were thawed at room temperature immediately prior to the following extractions:

3.2.5.1 Mechanical disruption with a French press

Thawed cells (0.2 g wet cell weight per mL of solution) were suspended in a French pressure cell in 70 mL chilled Tris HCl buffer (50 mM, pH 7.5) containing 1 mM of PMSF. The pressure cell was chilled before loading and the yeast cell suspensions were dispensed from and collected into ice-water baths. All manipulations were done in a cold room (4°C). Multiple passages at constant speed (3 mL/min) through the press (cell pressure of 20,000 psi) of the initial suspension and the following disrupted suspensions were necessary to obtain 95% of cell disruption as observed by phase contrast microscopy. The cell debris was recovered by centrifugation (48,000 *g*, 15 min, 5°C), washed with water (x 2, each time with 50 mL) and stored frozen. The supernatants were collected and filtered through a 0.45 μm membrane.

3.2.5.2 Pretreatment and zymolyase digestion of the cell wall (full zymolyase treatment)

Thawed cells (0.3 g wet cell weight per mL of solution) were initially pretreated in 15 mL Tris HCl buffer (100 mM, pH 8) containing 5 mM DTE and 5 mM EDTA at 28°C for 30 min in a shaking water bath (300 rpm, Model OWD 1412, Paton Scientific, SA, Australia). The cell pellet was recovered by centrifugation (48,000 *g*, 10 min, 5°C) and washed with water (2 x, each time with 15 mL). The supernatants from the pretreatment and the washings were pooled (around 50 mL) and dialysed against distilled water (6 L, 3 changes) at 4°C. The cell debris was resuspended in the same buffer as above but also containing zymolyase [2% (w/v)] and incubated in the above conditions for 60 min. After incubation, the cells were centrifuged and washed as mentioned earlier. The supernatants from the zymolyase digestion were collected and pooled with those from the pretreatment before being filtered through a 0.45 μm membrane. The cell debris was discarded.

3.2.5.3 Hot SDS extraction

Thawed cells (0.2 g wet cell weight per mL of solution) were suspended in 70 mL Tris HCl buffer (10 mM, pH 7) containing 2% (w/v) SDS and boiled for 5 min with manual shaking. The suspension was then centrifuged (48,000 *g*, 10 min, 5°C) and the cell pellet washed with water (x 2, each time with 70 mL). The supernatants (around 200 mL) were dialysed against distilled water (20 L, 3 changes) at 4°C and then ultrafiltered (the retentate was kept, see Section 2.3.4) on a YM 10 membrane. The cell debris was discarded.

3.2.5.4 Hot citrate extraction

Thawed cells (0.15 g wet cell weight per mL of solution) were suspended in 100 mL citrate buffer (20 mM, pH 7) and autoclaved at 105°C (19.475 kPa) for 60 min (after Peat *et al.* 1961). The cell debris was recovered by centrifugation (48,000 *g*, 10 min, 5°C) and washed (x 2, each time with 100 mL). The supernatants (around 300 mL) were pooled and filtered through a 0.45 μm membrane. The cell debris was discarded.

3.2.6 MANNOPROTEIN EXTRACT PURIFICATION

Filtered supernatants obtained from the extractions from whole yeast cells as described in Section 3.2.5 were diluted tenfold in starting buffer and loaded onto a Con-A column following the procedure in Section 2.3.3. The retained fraction on Con-A was eluted and desalted by ultrafiltration on YM 10 membrane (see Section 2.3.4). The retentate was collected, freeze-dried using a Dynavac freeze-drying unit and weighed.

3.2.7 HEAT TESTING OF THE MANNOPROTEIN EXTRACTS

The freeze-dried mannoprotein extracts (referred to as French press, autoclave, full zymolyase and SDS extracts) were tested for potential haze formation according to the procedure in Section 2.3.5.

3.3 RESULTS AND DISCUSSION

The three wine making yeast strains tested were grown in a medium rich in carbohydrates. After collection of the cells, the different methods of extraction previously described (Section 3.2.5) were applied to whole cells. Mannoproteins contained in the crude extracts were retained by the lectin Con-A as this lectin has high affinity for mannan polymers (So and Goldstein 1968). The eluted material was ultrafiltered and thus the extract contained only components with a molecular weight higher than 10 k. After freeze-drying, the dried material obtained was usually white and fluffy.

3.3.1 EXTRACTED MANNOPROTEIN YIELDS

The yields of mannoproteins extracted from whole yeast cells with the different methods are given in Table 3.1 and discussed below.

Table 3.1 Mannoprotein yield from whole cells using various extraction methods applied to three yeast strains (cells in late exponential phase)

Yeast strains	Methods of extraction *				
	Full zymolyase	SDS	French press	SDS on French presssed cells	Autoclave
Maurivin PDM	1.28	0.20	0.52	1.20	0.57
AWRI 85	1.65	0.11	0.40	nd	0.61
AWRI 65	0.80	0.10	0.83	nd	0.51

* results are expressed as % (w/w) of mannoprotein material extracted (dry weight) per wet weight of cells used for the extraction

nd not determined

For the three yeasts tested, the full zymolyase treatment was the most effective treatment for releasing mannoprotein material. After the softening treatment with EDTA and DTE, zymolyase attacks the glucan network of the cell wall and releases mannoproteins interspersed within or linked to this network (Pastor *et al.* 1984, Elorza *et al.* 1985, Molloy *et al.* 1989). It cannot be excluded, however, that some mannoproteins would be released during the pretreatment with EDTA and DTE. Under the conditions used here, the extensive action of zymolyase on the cell wall would release periplasmic material and, as the extraction was performed without an osmotic stabiliser, cytoplasmic material was also expected to be present in the extraction supernatant (Andrews *et al.* 1990).

In contrast to the full zymolyase treatment, the treatment with SDS released only small amounts of mannoproteins from the three yeasts tested. SDS used alone has a limited effect on whole cells (Horvath and Riezman 1994) and only the surface directly in contact with SDS would be extracted. Therefore the small amount of material extracted is probably exclusively originating from the cell wall. More material was extracted from the cells when SDS treatment was applied to cells previously disrupted using the French press (see next paragraph). In this case SDS could penetrate more deeply into the cell wall layers through the cracks made during the French press treatment.

Intermediate yields of mannoproteins were obtained after treatment of the cells with a French press. The breakage of the cells into large pieces under high pressure in the French press cell (as observed by phase contrast microscopy) would free cytoplasmic and periplasmic material from the physical barrier of the cell wall. During this breakage only part of the cell wall material and thus mannoproteins is expected to be solubilised (Arnold, 1972) and the French press extract therefore contains mainly periplasmic and cytoplasmic material. A further extraction with SDS treatment of cells previously 'French pressed' released a significant amount of mannoprotein material. This observation confirmed that the French press treatment did not have an extensive action on the cell wall but enhanced the action of SDS on the cells.

Similarly, intermediate yields of mannoproteins were obtained after treatment of the cells by autoclaving. It has been established that treating the cells at high temperature (above 100°C) and high pressure for a long time severely disrupts the cell and that material from the cytoplasm and periplasmic space is released together with material from the cell wall (Fleet, 1991). Under the conditions used here (time of extraction reduced compared to the literature), only partial removal of cell wall mannoproteins was expected and, accordingly, the extraction yields ranged from 0.51 to 0.61% [(w/w) of material extracted (dry weight) per wet weight of cells used for the extraction] for the three strains. Based on the data of Nakajima and Ballou (1974), an extraction rate indicating an almost complete removal of *Saccharomyces cerevisiae* cell wall mannoproteins would be around 4%.

Thus the observed differences in the yields of mannoprotein released by the four methods tested could be explained by differing efficiency of the methods to release mannoproteins. It was expected that mannoproteins located in the periplasmic space and cytoplasm [although the cytoplasmic mannan only accounts for 0.5 to 1.5% of the total cellular manan (Katohda *et al.* 1976)] would be released by all methods except the SDS treatment. The yields of mannoproteins released by either the autoclave, French press or full zymolyase treatment were therefore related to the efficiency of these treatments to release cell wall mannoproteins.

Comparing the differences in extraction yields obtained for the three yeast strains, highest extraction rates were obtained for the two Champagne yeasts (Maurivin PDM and AWRI 85) whereas extractions of AWRI 65 cells gave lower yields, particularly for the full zymolyase extraction. Flocculent yeasts such as AWRI 65 have a cell wall containing mannoproteins differing in size and structure compared with a non-flocculent yeast (see Section 3.3.2). Those differences might explain the lack of efficiency of the full zymolyase treatment in extracting cell wall mannoproteins from AWRI 65.

Table 3.2 Influence of growth phase and carbohydrate source on mannoprotein yield following full zymolyase treatment on Maurivin PDM cells.

State of growth	Source of carbohydrate of the growth medium*	
	Glucose (20 g/L)	Mannose (15 g/L)
Late exponential phase	1.28	2.45
Stationary phase	1.80	nd

* results are expressed as % (w/w) of material extracted (dry weight) per wet weight of cells used for the extraction

nd not determined

In parallel, attempts were made to optimise the extraction rate of the full zymolyase treatment on Maurivin PDM cells by using cells in stationary phase or by changing the carbohydrate source in the growth medium.

The yield of mannoproteins extracted by the full zymolyase treatment increased by 40% when the extraction was conducted on cells in stationary phase compared to that performed on cells in late exponential phase (Table 3.2). Although the walls of cells in stationary phase are reported to be more resistant to ß-glucanase attack (Fleet, 1991), the presence of DTE in the pretreatment eliminates this resistance (Sommer and Lewis 1971, Fleet 1991) and thus the digestion of the wall by zymolyase is possible. This increase in material extracted could be attributed either to the action of DTE and EDTA during the pretreatment or to zymolyase

during the enzymatic treatment. The latter hypothesis would be in agreement with the data of De Nobel *et al.* (1990) who suggested an increase of the glucanase extractable mannoprotein content in the cell walls of stationary phase cells.

Changing the carbohydrate source in the medium from glucose to mannose nearly doubled the amount of mannoproteins extracted by the full zymolyase treatment (Table 3.2). Biely *et al.* (1971) and Krátký *et al.* (1975a) reported that the cell wall mannan content significantly increases when mannose was used as the carbon source in place of glucose. Again, it could not be determined if more mannoproteins were extracted during the treatment with zymolyase or during the pretreatment with EDTA and DTE.

3.3.2 HAZE PROTECTIVE ABILITY OF THE MANNOPROTEIN EXTRACTS

The different mannoprotein extracts obtained were tested for their haze protective abilities using a heat test procedure. Preliminary heat test measurements for the different extracts showed that the haze decrease was best observed when the mannoprotein extract concentration ranged from 0.5 to 2 mg of extract per mL of wine (in the heat test) and therefore all extracts were tested in this concentration range.

The heat test results for the four methods of extractions tested on the three yeast strains are shown in Figures 3.1 to 3.3.

For Maurivin PDM (Figure 3.1), the SDS treatment released a mannoprotein extract which decreased the haze to 30-25% of the initial haze value (ihv) whereas the full zymolyase extract reduced the haze to 35-40% of the ihv. In comparison, a haze decrease only to 55-60% of the ihv was observed for the autoclave extract. On the contrary, mechanical disruption of the cell with the French press freed a mannoprotein extract which increased the haze value to 120% of the ihv.

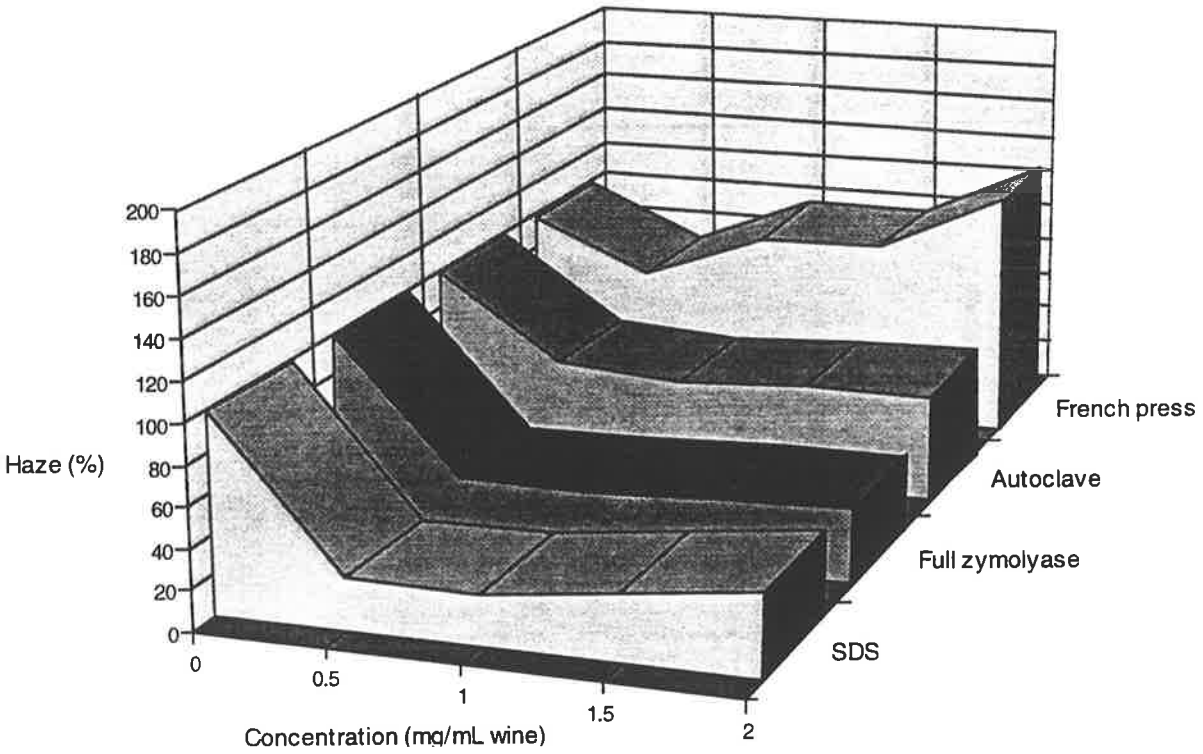


Figure 3.1 The effect of increasing concentration of the mannoprotein extract obtained from thawed late exponential phase Maurivin PDM by the SDS, full zymolyase, autoclave and French press treatments on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 3.2.7. Detailed values are given in Table 1, Appendix B.

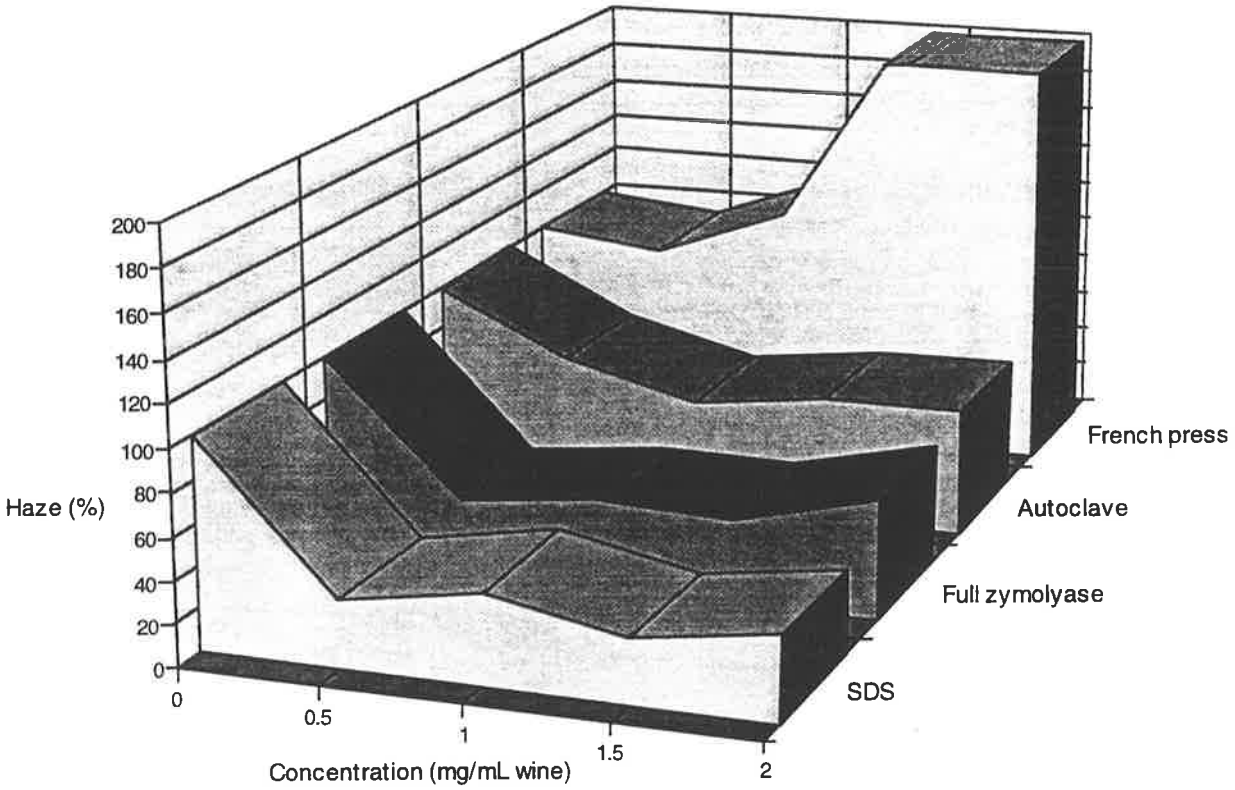


Figure 3.2 Effect of increasing concentration of mannoprotein extract obtained from thawed late exponential phase AWRI 85 by the SDS, full zymolyase, autoclave and French press treatments on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 3.2.7. Detailed values are given in Table 2, Appendix B.

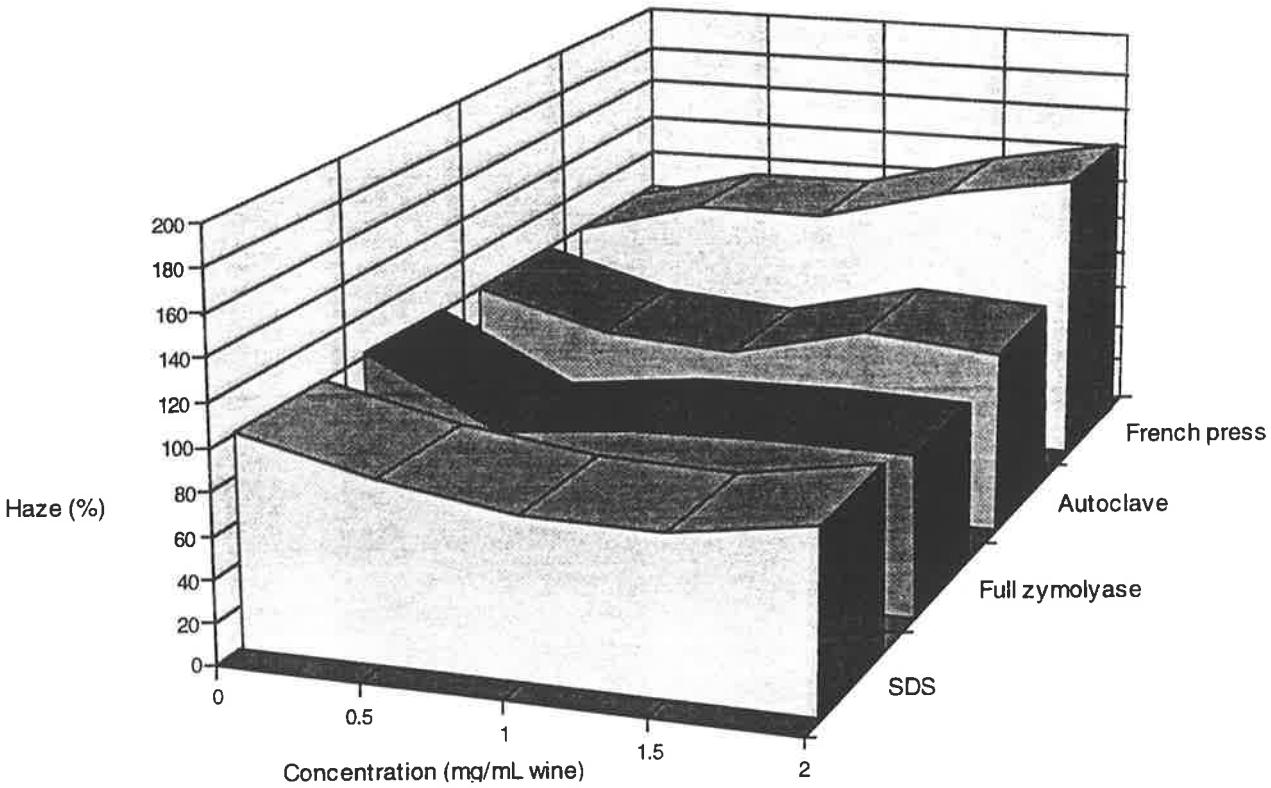


Figure 3.3 Effect of increasing concentration of mannoprotein extract obtained from thawed late exponential phase AWRI 65 by the SDS, full zymolyase, autoclave and French press treatments on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 3.2.7. Detailed values are given in Table 3, Appendix B.

For the other Champagne yeast, AWRI 85, a very similar pattern was obtained for the heat tests (Figure 3.2). SDS and full zymolyase treatments released mannoprotein material with satisfactory haze protective ability as haze values dropped to 30-40% of the ihv. Only an average haze decrease (haze values to 55-60% of the ihv) was observed for the autoclave extract whereas the French press method extracted mannoprotein material which significantly increased the haze as values were as high as 200-250% of the ihv.

No significant decreases in haze were observed for any of the extracts from AWRI 65 (Figure 3.3). It has been noted that flocculent yeast has a cell wall richer in mannoproteins and carbohydrates compared to the same yeast with non flocculating properties (Al-Mahmood *et al.* 1987, Saulnier *et al.* 1991, Bellal *et al.* 1995) and that there are significant differences in structure and molecular weight of the cell wall mannoproteins (Amri *et al.* 1982, Bellal *et al.* 1995). As a result of these changes, it is possible that the haze protective material (HPM) could be either absent or reduced in the mannoprotein extracts from the flocculent yeast AWRI 65.

Comparing the efficiency of the four methods to release HPM, SDS and full zymolyase treatments appeared to be the best treatments for a specific release whereas autoclaving yeast cells resulted in a mannoprotein extract with only average haze protective ability. Opposite to the other methods, the French press releases mannoprotein material strongly increasing the haze.

Other mannoprotein extracts obtained from Maurivin PDM were also tested. The mannoprotein extract from the full zymolyase treatment applied to Maurivin PDM cells grown on mannose gave similar heat test results as for Maurivin PDM grown on glucose (Figure 3.4). Apparently, growth on mannose did not alter the nature of the mannoproteins extracted. This result suggests that other mannoproteins did not develop to the detriment of the haze protective mannoprotein fraction.

The mannoprotein extract obtained from the full zymolyase treatment of Maurivin PDM cells in stationary phase and grown on glucose showed similar haze protective ability to the extract from yeast cells in late exponential phase (Figure 3.4, the experiment related to cells grown on mannose to stationary phase was not carried out). This result shows that the extraction of haze

protective material was also possible from cells in stationary phase suggesting that the presence of this material in the cell wall was not growth dependent.

3.3.3 HAZE FORMING MANNOPROTEIN MATERIAL RELEASED DURING FRENCH PRESS TREATMENT BY MAURIVIN PDM CELLS

The heat test results for the mannoprotein extracts obtained from Maurivin PDM by the full zymolyase or SDS treatments (low percentage of haze formed) are significantly different from those obtained with the French press extract (high percentage of haze formed) (see Figure 3.1). This mechanical method of extraction seemed to preferentially release haze forming mannoproteins. Accordingly, any positive effect of haze reducing mannoprotein material might be swamped by the abundant haze formed and therefore not detectable. This makes it uncertain if any HPM was released during the French press treatment, because the co-release of haze forming material would have prevented its detection by heat testing.

However, the mannoprotein extract isolated from SDS treatment of French pressed Maurivin PDM cells had a strong haze protective ability since the haze went down to 45-50% of the ihv (Figure 3.5). Thus HPM was present, at least in part, in the insoluble cell wall debris obtained after French press treatment and was released during SDS treatment. This result further supports the hypothesis that material with haze protective ability was either not released, or only released to a small extent, by treatment with a French press.

3.3.4 DIFFERENTIATION OF MANNOPROTEIN EXTRACTS ON THE BASIS OF M_R AND AMINO ACID COMPOSITION

To differentiate the mannoprotein extracts from Maurivin PDM yeast according to the M_r of the contained mannoprotein, extracts were separated on a SDS PAGE gradient gel (Figure 3.6). For all extracts, the staining of proteins with Coomassie Blue revealed only a few faint bands in the low molecular range whereas sugar staining revealed the presence of sugar rich proteins at the top of the gel which were initially not detected with Coomassie Blue. This observation is consistent with the presence of high M_r mannoproteins which cannot migrate into the gel (see Section 3.1) and which have a high percentage of mannose residues.

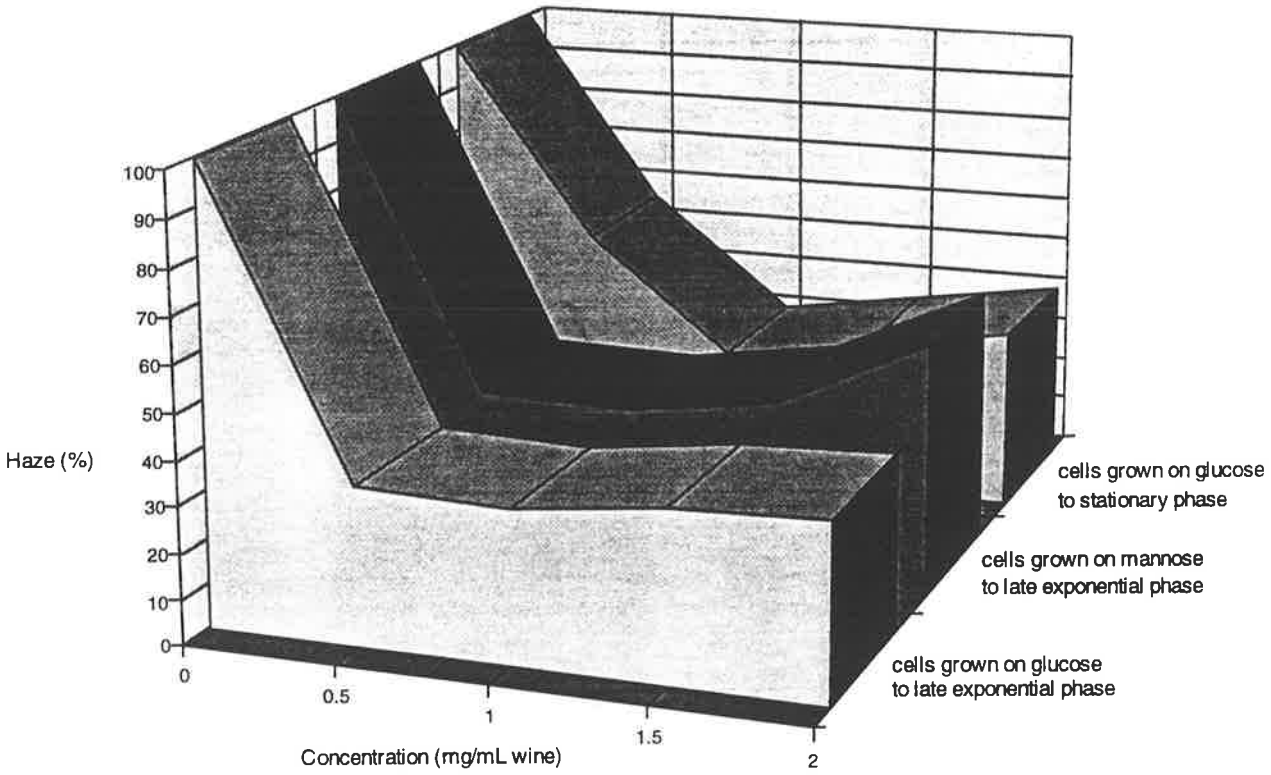


Figure 3.4 Influence of the carbohydrate source contained in the growth medium of Maurivin PDM cells (glucose or mannose) and state of growth of the harvested cells (late exponential or stationary phase) on the extraction of HPM under the full zymolyase treatment. The effect of increasing concentration of mannoprotein extract on the heat-induced protein haze was measured as described in Section 3.2.7. Detailed values are given in Table 4, Appendix B.

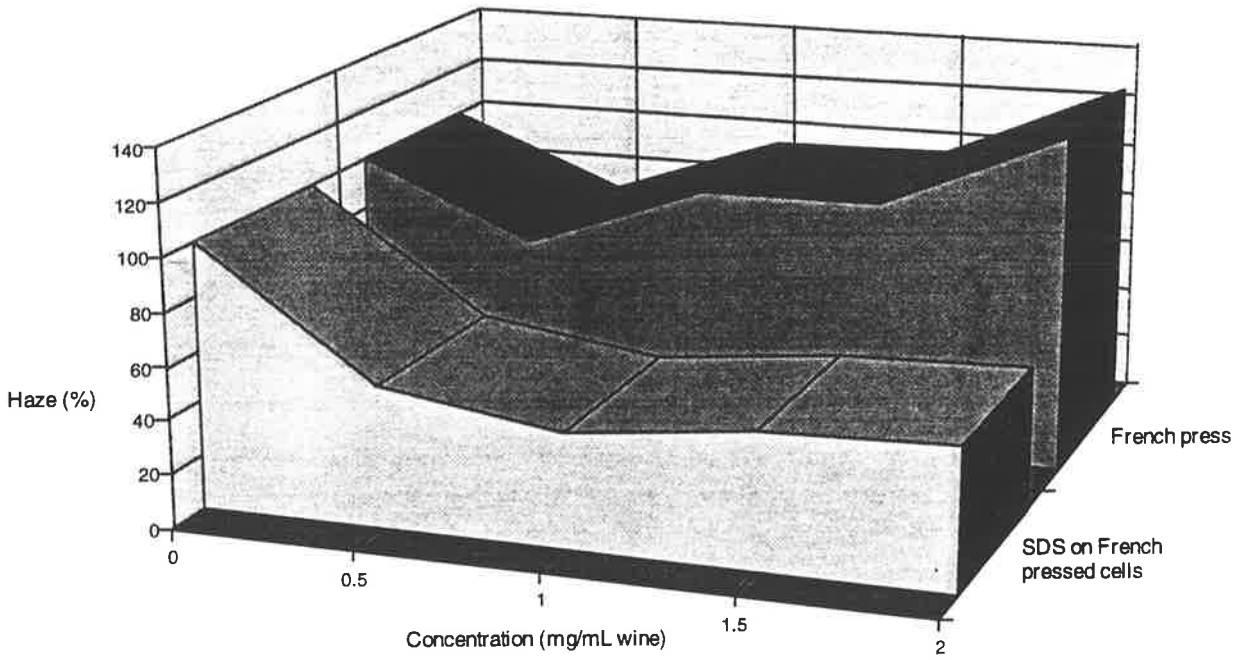


Figure 3.5 Effect of increasing concentration of the mannoprotein extract obtained from thawed late exponential phase Maurivin PDM cells by the French press or the SDS treatment applied to cells previously French pressed on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 3.2.7. Detailed values are given in Table 1, Appendix B.

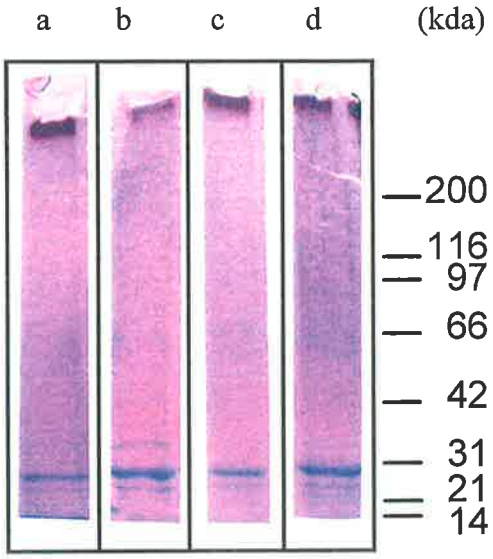


Figure 3.6 Electrophoretic patterns in SDS PAGE gel of (a) full zymolyase, (b) SDS, (c) autoclave and (d) French press mannoprotein extracts obtained from late exponential phase Maurivin PDM cells.

The gel was performed as described in Section 2.3.2. Bands were detected with Coomassie Blue (for protein blue stain on the gel) and PAS stains (for carbohydrate pink stain on the gel). The position of the molecular weight standards is given on the side (expressed in daltons): 200 k, myosin ; 116.2 k *E.coli* β-galactosidase, 97.4 k rabbit muscle phosphorylase b ; 66.2 k bovine serum albumin ; 42.6 k hen egg white ovalbumin ; 31 k bovine carbonic anhydrase ; 21.5 k soybean trypsin inhibitor ; 14.4 k hen egg white lysozyme.

Table 3.3 Amino acids composition of the yeast mannoprotein extracts obtained using four methods of extraction. Analyses were performed as in Section 2.3.8.

Amino acid	Methods of extraction *			
	French press	Autoclave	Full zymolyase	SDS
Aspartic acid / Asparagine	12.4	10.9	11.9	12.0
Glutamic acid / Glutamine	7.1	8.5	7.6	6.8
Serine	12.5	16.4	13.7	13.3
Histidine	1.4	0.5	1.3	1.2
Glycine	6.8	5.9	6.4	7.1
Threonine	9.6	13.1	11.6	12.5
Alanine	9.2	10.2	9.8	9.9
Arginine	6.6	1.5	4.8	3.5
Tyrosine	2.4	1.9	2.5	2.3
Valine	6.6	8.3	7.0	6.8
Methionine	1.0	1.1	1.0	1.2
Phenylalanine	4.6	3.1	3.9	4.0
Isoleucine	4.9	4.4	4.6	4.3
Leucine	6.5	5.4	6.0	5.8
Lysine	3.2	2.6	2.7	2.8
Proline	4.5	5.8	4.8	5.6
Cysteine	nd	nd	nd	nd
Tryptophan	nd	nd	nd	nd

* results are expressed as mole %

nd not determined

These large mannosyl groups present on these mannoporteins prevent the protein moiety being detected with Coomassie Blue (Frevert and Ballou 1985, Shibata *et al.* 1983) and explain the easy detection with the sugar stain.

This experiment confirmed that high M_r mannoproteins were released from Maurivin PDM by the four methods tested. According to the stain intensities, more mannoproteins were extracted during autoclave and full zymolyase treatments than during French press and SDS treatments, respectively. This is broadly consistent with the yields given in Table 3.1

In all those samples, a band at 29 k was detected by Coomassie Blue. This band was observed during analyses of material from the cell wall (Pastor *et al.* 1984, Herrero and Boyd 1986) and was identified as being a cell wall glycoprotein (gp29) which was easily extractable from cell walls by SDS and zymolyase treatments or simply by boiling (Pastor *et al.* 1984, Klebl and Tanner 1989). The ease of extraction may explain why this glycoprotein is also present in the French press and autoclave extracts. In the published research work mentioned above, the detection of material in the electrophoretic gel was always done by autoradiography. It is, nevertheless, very likely that this glycoprotein which is composed of 90% protein and only 10% sugar (Klebl and Tanner 1989) is detectable with a protein stain rather than with a sugar one. This hypothesis would support the staining with Coomassie Blue of this glycoprotein as observed in the extracts tested here.

In parallel, amino acids analyses of the four mannoprotein extracts were carried out and are presented in Table 3.3. The results showed a predominance of hydroxy amino acids for the four extracts as serine and threonine together accounted for 22.1, 29.5, 25.3 and 25.8% (mole %) of the French press, autoclave, full zymolyase and SDS extracts, respectively. The latter value is in agreement with the data of Molloy *et al.* (1989). For the hydrophobic amino acid content, no significant variation among the four extracts was noticed. Valine, methionine, isoleucine, leucine, tyrosine and phenylalanine made up 26.2, 24.4, 25.2 and 24.7% (mole %), respectively, of the French press, autoclave, full zymolyase and SDS extracts. Over all, the total amino acid composition was quite similar for all extracts analysed and could therefore not explain the difference observed in heat test results.

3.4 CONCLUSION

In this Chapter, by the means of the heat test procedure, four different methods were tested for their abilities to release HPM from whole cells of three yeast strains (Table 3.4). The full zymolyase treatment involving a pretreatment with DTE and EDTA followed by a enzymatic break down of the cells with zymolyase was the most effective method in terms of haze protective ability of the mannoprotein extract and yield. The extraction yield of HPM by full zymolyase treatment on Maurivin PDM yeast cells increased when the extraction was carried out on cells grown to stationary phase compared to late exponential phase cells, and when cells were grown in a medium containing mannose in place of glucose.

The SDS treatment also extracted a mannoprotein fraction showing high haze protective ability but the amount extracted was very little. In contrast, hot citrate procedure released a mannoprotein extract with medium haze protective ability and mechanical disruption of the cell under the French press led to the release of a mannoprotein extract significantly increasing the haze.

Of the three *Saccharomyces cerevisiae* yeasts tested as a potential source for HPF, the Champagne yeast Maurivin PDM showed the best ability in releasing HPM. Flocculating properties of AWRI 65 seemed to offset a preferential release of haze protective material as no significant haze decrease was observed whatever treatment used. The other Champagne yeast, AWRI 85, gave results as good as Maurivin PDM. Nevertheless, Maurivin PDM would be the most logical choice of strain for further studies because of its wide use in Australian winemaking for the production of white table wine and bottle fermented sparkling wine.

It is possible to deduce, based on the specific properties of each treatment (SDS has detergent properties, zymolyase is a ß-glucanase, French press releases periplasmic material), what the characteristics of the environment in which the extracted mannoprotein material was located. This information, plus the data for each treatment (heat test results and extraction rate) can provide clues about the interactions of HPM with other cell components and therefore its possible location within the cell envelope.

Table 3.4 Rating of the haze protective ability of the mannoprotein extracts obtained with the four different methods of extraction applied on three yeast strains

Treatment	Haze protective ability *		
	Maurivin Prise de Mousse	AWRI 65	AWRI 85
Full zymolyase treatment	+++	+	+++
SDS treatment	+++	+	+++
Autoclave	++	+	++
French press	+	-	-

* extent of haze decrease:

-: no haze decrease observed. Haze value is equal or higher than the ihv (100%)

+: haze decrease between 100 and 60% of the ihv

++: haze decrease between 60 and 40% of the ihv

+++: haze decrease between 40 and 20% of the ihv

Accordingly, the data for the French press treatment (poor heat test results) and for the SDS treatment of cells previously French pressed (satisfactory heat test results) showed that HPM was not efficiently extracted during the French press treatment and remained in the cell wall debris obtained after this treatment. As the French press method is mainly releasing periplasmic mannoproteins (see Section 3.1, Arnold 1972), this observation suggested that HPM was not of periplasmic origin.

The possible extraction of HPM with SDS from the cell wall debris (obtained after the French press treatment) and from whole cells (satisfactory heat test results, poor extraction rate) supports the assumption that HPM was associated with the cell wall. Extraction with SDS implies the disruption of non-covalent bonds and, in particular, of hydrophobic interactions (Hjelmeland and Chrambach, 1981) and therefore it could be assumed that HPM was not covalently linked to the cell wall.

Nevertheless, HPM was also released during the full zymolyase treatment which means that HPM could have been released either by EDTA and DTE during the pretreatment or by zymolyase during the enzymatic digestion of the cell wall. In the latter case, HPM could be released by zymolyase because it was covalently linked to the glucan network or enmeshed into it.

Therefore no definitive conclusions about the interactions of HPM with other cell wall components and its location(s) can be drawn here other than to conclude that HPF is a cell wall component. Further investigations to clarify these points will be presented in the next Chapter.

Chapter 4

EXTRACTION PROFILE OF HAZE PROTECTIVE MATERIAL FROM *SACCHAROMYCES CEREVISIAE* MAURIVIN PRISE DE MOUSSE

4.1 INTRODUCTION AND LITERATURE REVIEW

Among the methods tested in this study to extract haze protective factor (HPF), sodium dodecyl sulfate (SDS) and zymolyase treatments specifically released haze protective material (HPM, Chapter 3). SDS treatment is a simple method consisting of boiling whole cells in Tris buffer with 2% (w/v) SDS. The full zymolyase treatment is a two step procedure where cells are first pretreated with dithioerythritol (DTE) and ethylenediamine tetraacetate (EDTA) before being enzymatically digested by zymolyase.

The two chemicals, EDTA (chelating agent) and DTE (thiol reducing agent), used in the pretreatment are thought to soften up the cell wall and thereby facilitate the action of ß-glucanase enzymes, like zymolyase, on the glucan network. Accordingly, pretreatment with EDTA and DTE is used with glucanase enzymes to convert cells into spheroplasts and to subsequently release periplasmic material (Pastor *et al.* 1982, 1984).

Thiol reducing agents, like DTE or dithiothreitol (DTT), used on their own as a pretreatment of *Saccharomyces cerevisiae* cells were also shown to facilitate the action of the ß-glucanase enzyme for the release of periplasmic enzymes like acid phosphatase or invertase (Sommer and Lewis 1971, Valentin *et al.* 1984, Herrero and Boyd 1986). It was also observed that a small part of the periplasmic pool could be directly released upon exposure of the cells to the reducing agent. For instance, 5% of the total invertase pool and 3% of the total asparaginase II pool could be released within 30 min of treatment with DTT (50 mM, pH 7, 37°C ; Smith and Ballou 1974, Dunlop *et al.* 1978). Thus thiol reducing agents, used alone, can presumably modify the wall structure so that the release of periplasmic material becomes possible.

The extraction of cell wall material by a thiol reducing agent was visually confirmed in electron microscopy studies of Cassone *et al.* (1978) on *Candida albicans*. Treatment of the cells with DTT (12 mM DTT, 30 min, 37°C) released large amounts of components from the outer layer of the cell wall while the inner layers remained intact. Even after extensive treatment for 3 hours, no spheroplasts could be observed. These observations suggested that treatment of *Candida albicans* with a thiol reducing agent could not extract all the cell wall components and that its action was probably limited to the cell wall surface. Frevert and Ballou (1985), in a study of cell wall components of a mutant of *Saccharomyces cerevisiae*, also concluded that the material extracted by DTE was located on the surface of the cell. Furthermore, this material was strongly coloured by a stain specific for sugars and had a M_r exceeding 200 k. DTE could possibly release high M_r mannoproteins from the cell wall of this *Saccharomyces cerevisiae* mutant. The observations described above suggest that the release of HPF may possibly occur during the pretreatment of the cells with DTE.

Chelating agents, like EDTA, can also be used alone to release material from the yeast cell walls. EDTA extraction of purified cell walls of *Saccharomyces cerevisiae* or *Candida albicans* led to the release of a small amount of mannoproteins (Valentin *et al.* 1984, Elorza *et al.* 1985).

Another chemical compound similar to the core structure of EDTA, ethylenediamine, is also used to extract mannoproteins from yeast cell walls. According to the procedure first mentioned by Northcote and coworkers (Korn and Northcote 1960, Sentandreu and Northcote 1968), extensive treatment with ethylenediamine extracted a large soluble fraction of mannoproteins from the cell wall. With a similar procedure Katohda *et al.* (1976) extracted mannans from the cell walls of baker's yeast. These findings suggest that EDTA may also contribute to the extraction of HPF during the pretreatment of the cells.

The full zymolyase treatment which successfully released HPM included three agents: EDTA, DTE and zymolyase. All three components are potential solubilising agents of mannoproteins (as seen above and in Chapter 3 for zymolyase). It was therefore important to test each agent individually for its capacity to extract HPM. The other treatment shown in Chapter 3 to extract HPM was boiling whole cells in Tris buffer with the detergent, SDS. There were also potentially two components to this treatment: boiling in Tris buffer and SDS, and the effect of each of these treatments on mannoprotein extraction also required examination. Each of these

agents have specific properties (like glucosidase activity for zymolyase, chelating properties for EDTA, reducing properties for DTE or detergent properties for SDS) and extract material from the cells according to these properties. Thus the heat test results for the materials extracted by the different agents could provide information on how HPF is released, how it interacts with other cell components and where it is possibly located in the cell envelope. The results of these investigations are presented in this Chapter.

4.2 EXPERIMENTAL

4.2.1 MATERIAL

Saccharomyces cerevisiae yeast Hulls (dry powder) were provided by Lallemand Pty Ltd (SA, Australia). Other materials used in this Chapter are described in Sections 2.1 and 3.2.1.

4.2.2 YEAST STRAIN

The yeast used was *Saccharomyces cerevisiae* Maurivin PDM (see Section 2.2.1). Yeasts were kept at 4°C on YPD slope cultures and were subcultured every six months.

4.2.3 GROWTH PROTOCOL

The yeast propagation was conducted as described in Section 2.2.3. The volumes for the propagation stages were 3, 25 and 500 mL, respectively. The final propagated culture (500 mL), upon reaching exponential phase, was transferred to 9.5 L SGJM containing glucose.

Final growth was carried out in 10 L of SGJM contained in a 25 L plastic fermentation vessel at the conditions mentioned in Section 2.2.4. Cultures were grown up to late exponential phase (7.5-11.5 g/L, wet cell weight) or to stationary phase [16-17 g/L, wet cell weight, refer to the growth curve in Figure 1A), Appendix A]. Cells were recovered by centrifugation (18,000 *g*, 10 min, 5°C), washed 5 times (each time with 8 L) with water before immediate extraction or 3 times (each time with 10 L) before being stored in aliquots (10 g) at -20°C. The culture supernatant was collected, filtered through a 0.45 μm membrane and stored at -20°C until further analyses (see Chapter 5).

4.2.4 CELL WALL PURIFICATION

Various methods were tested to isolate cell walls. Sonication of the whole cells followed by high pressure treatment (20,000 psi) using a French press (see Section 3.2.5.1) gave a disruption rate which never exceeded 95%. Disruption of the whole cells by shaking using a vortex according to Julius *et al.* (1984) and Zueco *et al.* (1986) led to less than 85% of cells disrupted. The most efficient method (see protocol below) was to isolate cell walls using a homogeniser according to Elorza *et al.* (1985) and Jiranek *et al.* (1995). This method disrupted 99% of cells as observed by phase contrast microscopy.

Freshly harvested cells in late exponential phase were washed with water (x 3, each time with 10 L) and resuspended in chilled Tris HCl buffer (50 mM, pH 7.5, referred to in this Section as Tris buffer) at a concentration of 0.5 g cells (wet weight) per mL of Tris buffer [in order to minimise the effect of endogenous (1-3)-ß-glucanases on the cell wall during this purification (Fleet, 1991), all washing steps were carried out with chilled Tris buffer]. Glass beads (diameter: 0.25-0.5 mm) were added to the suspension [5 g of beads per g cells (wet weight)]. Cells (4 g each run) were then disrupted by shaking for 10 min in a Braun homogeniser with CO_2 cooling.

After each run, the glass beads were removed by filtration through a glass sinter (3G2) and rinsed with chilled Tris buffer. The filtrate was centrifuged at 3,000 *g* (5 min, 2°C) and the cell wall pellet was thoroughly washed with chilled Tris buffer (5 x, each time with 35 mL, the supernatant for the last washing was clear) before being immediately used for extraction.
The initial supernatant collected after cell disruption and all the washings were pooled and further centrifuged at 48,000 *g* (5 min, 2°C). The collected supernatants (referred to as cell disruption washings) were ultrafiltered (see Section 2.3.4).

4.2.5 MANNOPROTEIN EXTRACTIONS

4.2.5.1 Pretreatment and zymolyase digestion

The frozen cells were thawed at room temperature just before being used for the following extractions. Cells [0.3 g wet cell weight per mL of solution] were initially pretreated by resuspending in 15 mL Tris HCl buffer (100 mM, pH 8) containing 5 mM DTE and 5 mM

EDTA and incubating at 28°C for 30 min in a shaking water bath (300 rpm, Model OWD 1412, Paton Scientific, SA, Australia). The cells were recovered by centrifugation (48,000 *g*, 10 min, 5°C) and washed with water (x 2, each time with 15 mL). The supernatants from the pretreatment and the washings were pooled (around 45 mL) and ultrafiltered (the retentate was kept, see Section 2.3.4).

The cell debris was resuspended in 15 mL Tris HCl buffer (100 mM, pH 8) containing zymolyase [2% (w/v)], incubated at 28°C for 60 min in a shaking water bath (300 rpm, Model OWD 1412, Paton Scientific, SA, Australia). The cells were recovered by centrifugation (48,000 *g*, 10 min, 5°C) and washed with water (x 2, each time with 15 mL). The supernatants from the zymolyase digestion and the washings were pooled and ultrafiltered (the retentate was kept, see Section 2.3.4). The cell debris was discarded. The two sets of supernatants (pre- and post-treatment) were kept separate.

4.2.5.2 EDTA and DTE treatments

The following treatment applied to thawed or freshly harvested cells or to cell walls. Cells or cell walls [0.3 g (wet weight) per mL of solution] were incubated in 15 mL Tris HCl buffer (100 mM, pH 8) containing 5 mM EDTA at 28°C for 30 min in a shaking water bath (300 rpm, Model OWD 1412, Paton Scientific, SA, Australia). The cells or cell walls were recovered by centrifugation (48,000 *g*, 10 min, 5°C) and washed with water (x 2, each time with 15 mL). The supernatants from EDTA extraction and the washings were pooled and ultrafiltered (the retentate was kept, see Section 2.3.4). The cell debris was discarded.

The DTE treatment was conducted using the same procedure and conditions as above except that 5 mM DTE replaced EDTA in the Tris HCl buffer.

The controls for both EDTA and DTE treatments were the same as the cells were treated with Tris HCl buffer (100 mM, pH 8) with the exclusion of DTE and EDTA.

4.2.5.3 SDS treatment

The following treatment applied to thawed or freshly harvested cells or to cell walls. Cells or cell walls [0.2 g (wet weight) per mL of solution] were suspended in 25 mL Tris HCl buffer

(10 mM, pH 7) containing 2% (w/v) SDS and boiled for 5 min with shaking by hand. The suspension was then centrifuged (48,000 *g*, 10 min, 5°C) and the cell pellet washed with water (x 2, each time with 25 mL). The supernatants (around 75 mL) were dialysed against water (10 L, 3 changes) (omitted for extraction on cell walls) and then ultrafiltered (the retentate was kept, see Section 2.3.4). The cell debris was discarded.

The SDS control experiment consisted of treating cells in Tris HCl buffer (10 mM, pH 7) under the same procedure except for the dialysis step which was omitted and the exclusion of SDS in the Tris HCl buffer.

4.2.6 PURIFICATION OF MANNOPROTEIN EXTRACT

Filtered extracts obtained from the different treatments were diluted tenfold in starting buffer (see Section 2.3.3) and loaded onto a HR 10/30 column (10 mm x 300 mm) packed with Con-A (volume approximately 25 mL) following the procedure in Section 2.3.3.

The retained fraction on Con-A was eluted and then desalted by ultrafiltration (the retentate was kept, see Section 2.3.4). The retentate was collected and freeze-dried using a Dynavac freeze-drying unit. The freeze-dried mannoprotein extracts were weighed and usually appeared white and fluffy.

4.2.7 MICROMETHOD FOR THE MEASUREMENT OF THE HEAT INDUCED HAZE (HEAT TEST)

The freeze-dried mannoprotein extracts obtained by the different treatments were tested for their haze protective ability according to the procedure described in Section 2.3.5. The mannoprotein extracts that were tested are referred to according to the name of the extraction method, e.g., zymolyase extract from whole cells is the term used for the mannoproteins extracted from whole cells by zymolyase treatment.

Both of the ultrafiltered wines mentioned in Section 2.3.5 (unsupplemented Gordo wine and Sauvignon blanc wine supplemented with Riesling grape extract) were used in this study.

4.3 RESULTS AND DISCUSSION

4.3.1 EXTRACTION OF HPM BY PROTOCOLS RELATED TO THE FULL ZYMOLYASE TREATMENT APPLIED TO THAWED MAURIVIN PDM CELLS IN LATE EXPONENTIAL PHASE

4.3.1.1 Sub-treatments: pretreatment and zymolyase treatments

The full zymolyase treatment (described in Section 3.2.5.2) was a two stage treatment consisting of a pretreatment of the thawed cells with two agents (EDTA and DTE) and a final digestion of the pretreated cells with zymolyase. The full zymolyase treatment thus potentially gave mannoproteins extracted by both the pretreatment and zymolyase.

In this Chapter, the full zymolyase treatment was split up into two sub-treatments. Cells were pretreated with DTE and EDTA, and the mannoprotein fraction collected (referred to as the pretreatment extract) contained only material extracted during the pretreatment. The pretreated cells were afterwards digested with zymolyase and the mannoprotein fraction collected (referred to as the zymolyase extract) thus contained only material extracted by the enzyme.

Table 4.1 The effect of the treatment type on the yield of mannoprotein from thawed Maurivin PDM cells in late exponential phase. Extraction details are described in Sections 4.2.5.1 and 4.2.5.2.

Treatment	Extraction yield *
Pretreatment (DTE + EDTA)	0.87
Zymolyase	1.10
DTE	0.54
EDTA	0.44

* results are expressed as % (w/w) of material extracted (dry weight) per wet weight of cells used for the extraction and are the means of two independent experiments.

The two sub-treatments solubilised mannoproteins to similar extents (Table 4.1). The combined amounts of material extracted by the pretreatment and zymolyase should be similar to the amount extracted during the full zymolyase treatment. Nevertheless, more material was extracted during the two sub-treatments compared to the full zymolyase treatment (compare Table 4.1 to Table 3.1). For the full zymolyase treatment (see Section 3.2.5.2) both dialysis and ultrafiltration were used to prepare the samples whereas only ultrafiltration was used to obtain the extracts herein (see Section 4.2.5.1). This change in technique might explain the differences in yield observed as some material could have been lost during the dialyisis.

The ability of the zymolyase and pretreatment extracts to reduce protein haze in wine was then examined with a heat test. Figure 4.1 shows a decrease to about 50% of the initial haze value (ihv) which was effected by the pretreatment extract. In contrast, 80 to 100% of the ihv was still observed when the zymolyase extract was present. Thus the pretreatment with EDTA and DTE specifically extracted HPM whereas zymolyase treatment of the pretreated cells only released material with no haze protective ability.

The full zymolyase extract (containing both material extracted by the pretreatment plus that given by the following zymolyase treatment) decreased the haze to about 40% of the ihv. This haze decrease was in the same range as the one obtained with the pretreatment extract. These observations suggest that the material released during the pretreatment was largely responsible for the haze decrease observed for the full zymolyase extract.

Concerning the standard deviations observed for the different extracts in Figure 4.1, they varied from 15 to 30% for zymolyase extract, and were 7%, and less than 5% for pretreatment and full zymolyase extract, respectively. It was a general trend over the various heat test results to observe small standard deviations (below 10 %) for extracts showing above average haze reducing properties whereas higher standard deviations (above 10%) were observed for extracts showing average to no haze reducing properties. In other words, extracts with haze reducing properties gave more reproducible heat test results than the ones containing little or no HPM. A possible explanation for the high standard deviations observed for samples with low HPM content could be that all extracts tested were purified only once through a Con-A column. Therefore samples were likely to contain a wide range of mannoproteins which might react in a non defined way with the wine components during the heat test and create variable amounts of haze.

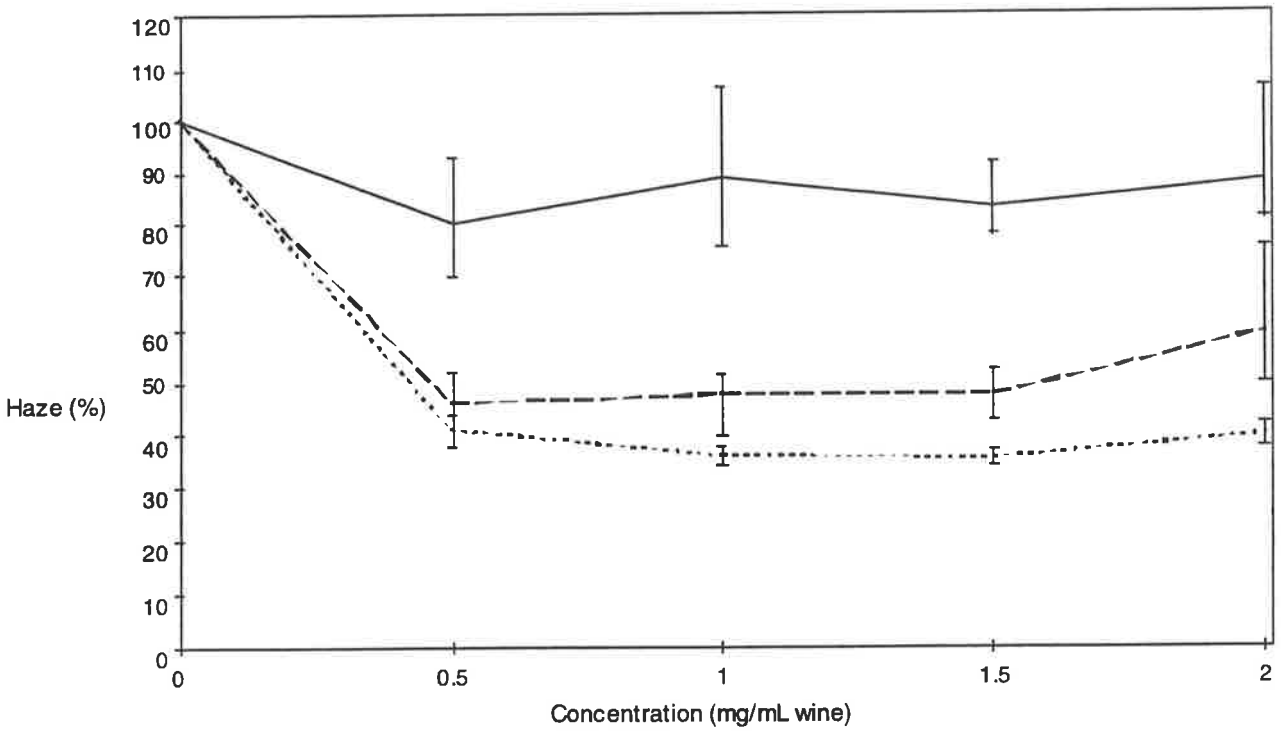


Figure 4.1 The effect of increasing concentration of mannoprotein extract obtained from thawed late exponential phase Maurivin PDM cells by the pretreatment (dashed line), the zymolyase treatment following the pretreatment (full line), or the combined pretreatment and zymolyase treatment (full zymolyase treatment, dotted line) on the heat-induced protein haze. Haze was measured in the heat test as described in Section 4.2.6. Values are the means of two independent experiments. The vertical bars represent the standard deviation for each value.

On the contrary, extracts with a high amount of HPM were rich in active mannoprotein material and responded to the heat test in a more defined way and gave more reproducible results.

4.3.1.2 Extraction of HPM by protocols related to the subtreatment 'pretreatment'

Individual extractions with EDTA and DTE were then carried out to test whether both or only one of the agents contained in the pretreatment was responsible for the release of HPM. Both treatments released a similar amount of mannoproteins which in sum approximated the total amount of mannoproteins released during the pretreatment (Table 4.1).

The ability of the mannoprotein extracts obtained by EDTA and DTE treatments (referred to as the EDTA or DTE extracts respectively) to reduce protein haze in wine was then assessed in a heat test (Figure 4.2). The results of the heat tests revealed that the DTE extract dramatically increased the level of haze (more than 150% of the ihv for the lowest concentration tested) suggesting that DTE did not extract HPM but some material with a strong haze formation ability.

On the contrary, the EDTA extract reduced haze in the same proportions as that obtained by the pretreatment (about 50% of the ihv). These results clearly show that EDTA, as opposed to DTE, specifically released HPM and it can be assumed that EDTA was also responsible for the release of the haze protective material present in the pretreatment extract.

Accordingly, of the three agents (EDTA, DTE and zymolyase) used in the full zymolyase treatment, DTE and zymolyase did not release HPM and EDTA was therefore the sole agent releasing HPM.

These results suggest that HPF was neither released under the enzymatic digestion of zymolyase nor by the reducing properties of DTE. Thus HPF is neither covalently linked through ß(1-3) bonds to the glucan network nor linked through disulfide bridges to other components. Furthermore the specific release of HPM by EDTA implies that HPF is only loosely associated with the cell wall.

4.3.2 EFFECT OF FREEZE-THAWING WHOLE MAURIVIN PDM CELLS IN LATE EXPONENTIAL OR STATIONARY PHASE ON THE RELEASE OF HPM.

In Section 4.3.1, the cells used for the treatments were frozen at -20°C for a minimum of one month. After thawing at room temperaure, the cells were immediately extracted. It was therefore of interest to know whether the process of freezing and thawing released HPM or effected the subsequent extraction of HPM by other treatments.

4.3.2.1 Nature and yield of the carbohydrates released during thawing of whole Maurivin PDM cells in late exponential phase

In addition to determining the dry weight of material bound to Con-A, as has been done for all extractive work described in this thesis, the content of monomeric and polymeric glucose and mannose was also determined before Con-A. Thawed cells were washed twice with water and the washing liquids were collected and directly analysed for sugar content. The results are presented in Table 4.2

The washing liquid contained relatively high levels of polymeric glucose and mannose (27% and 48%, respectively, of the total sugar content). Thus, to freeze-thaw cells released a fraction rich in polymeric mannose (and supposedly mannoproteins) which might contain HPM.

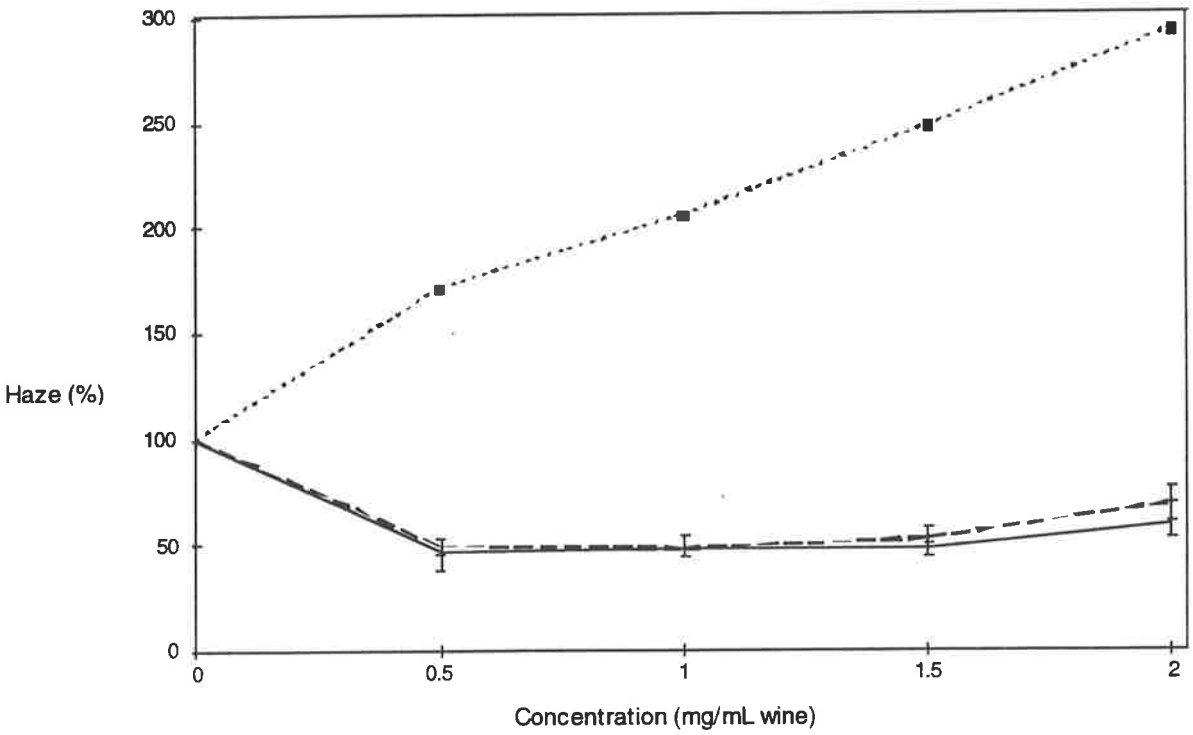


Figure 4.2 The effect of increasing concentration of mannoprotein extract obtained from thawed late exponential phase Maurivin PDM cells by the DTE treatment (dotted line), the EDTA treatment (dashed line), or the combined DTE and EDTA treatments (pretreatment, full line) on the heat-induced protein haze. Haze was measured in the heat test as described in Section 4.2.7. Values are the means of three independent experiments (except for DTE treatment which was from one experiment). The vertical bar represents the standard deviation for each value.

Table 4.2 Yields of polymeric and monomeric sugars in the washing liquid before Con-A procedure, from thawed whole Maurivin PDM cells in late exponential phase. The procedure for sugar measurements are given in Section 2.3.7.

Type of sugar	washing liquid from thawed cells *
Glucose polymeric	0.11
Glucose monomeric	0.08
Mannose polymeric	0.20
Mannose monomeric	0.02
Total sugar content	0.41

* results are expressed as % (w/w) of measured sugar per wet weight of cells used for the sugar measurement. Values are the means of at least three independent experiments. The standard deviation of the samples varied from 7 to 10%. Measurements were not performed for cells in stationary phase

4.3.2.2 Haze protective ability of the mannoprotein extract obtained after thawing whole Maurivin PDM cells in late exponential or stationary phase

The mannoproteins in the washing liquid were then isolated by affinity chromatography on Con-A. The average yield of mannoproteins released by freeze-thawing of the yeast cells (late exponential phase) was 0.07% [(w/w) of material extracted (dry weight) per wet weight of cells used for the extraction] compared to 0.44% and 0.54%, respectively, for EDTA and DTE treatments on thawed cells (see Table 4.1). This represented 13-16% of the DTE and EDTA extractable mannoproteins mentioned earlier and it was therefore essential to estimate the haze protective ability of this fraction.

According to the heat test results (Figure 4.3), the mannoprotein extract obtained by freeze-thawing cells in late exponential phase did not significantly decrease the haze (values down to 75-80% of the ihv) whereas the comparable mannoprotein extract from cells in stationary phase gave a fraction showing satisfactory haze protective ability (haze values down to 35-40% of the ihv).

To freeze and thaw damages the cells, and the extent and type of damage depends on the cooling rate (Griffiths and Beldon, 1978). At slow cooling rates (less than 1°C/min, as per this study), ice is formed outside the cell at the expense of the intracellular water (dehydration) leading to an accumulation of solute ions and cell shrinkage. Due to the increase of solute ion concentration in the cell during freezing, hydrophobic and ionic interactions in the different cell compartments are altered. These changes can lead to the release of non-covalently linked material. Such an effect was recognised by Morris and Clarke (1981) who observed a release of water soluble peripheral proteins from a cytoplasmic membrane after freeze-thawing. In general, the process of freezing is correlated with the release of cytoplasmic and cell wall material as well as cations into the medium (Morris and Clarke, 1981). Therefore it is not surprising that non-covalently linked material of the surface of the cell, as HPF could be, was released from the cell wall under the freeze-thaw stress imposed in this study.

The release of HPM was observed for cells in stationary phase whereas mannoprotein fractions with poor haze protective ability were released during thawing of cells grown to late exponential phase. Since HPM is present in the cell walls at both growth stages (see Section 3.3.2), the difference in haze protective ability between freeze-thawed extracts from the two growth phases was possibly due to the rearrangement of the cell wall components during cell maturation [for cells in stationary phase the cell wall is thicker and less porous (De Nobel *et al.* 1990, Valentin *et al.* 1987)]. This rearrangement, characterised by less mannoprotein extract released after freeze-thawing cells in stationary phase [yield of 0.02% (w/w) of material extracted (dry weight) per wet weight of cells used for the extraction compared to 0.07% for cells in late exponential phase] might prevent haze forming material being released and possibly allow HPM to be more specifically released.

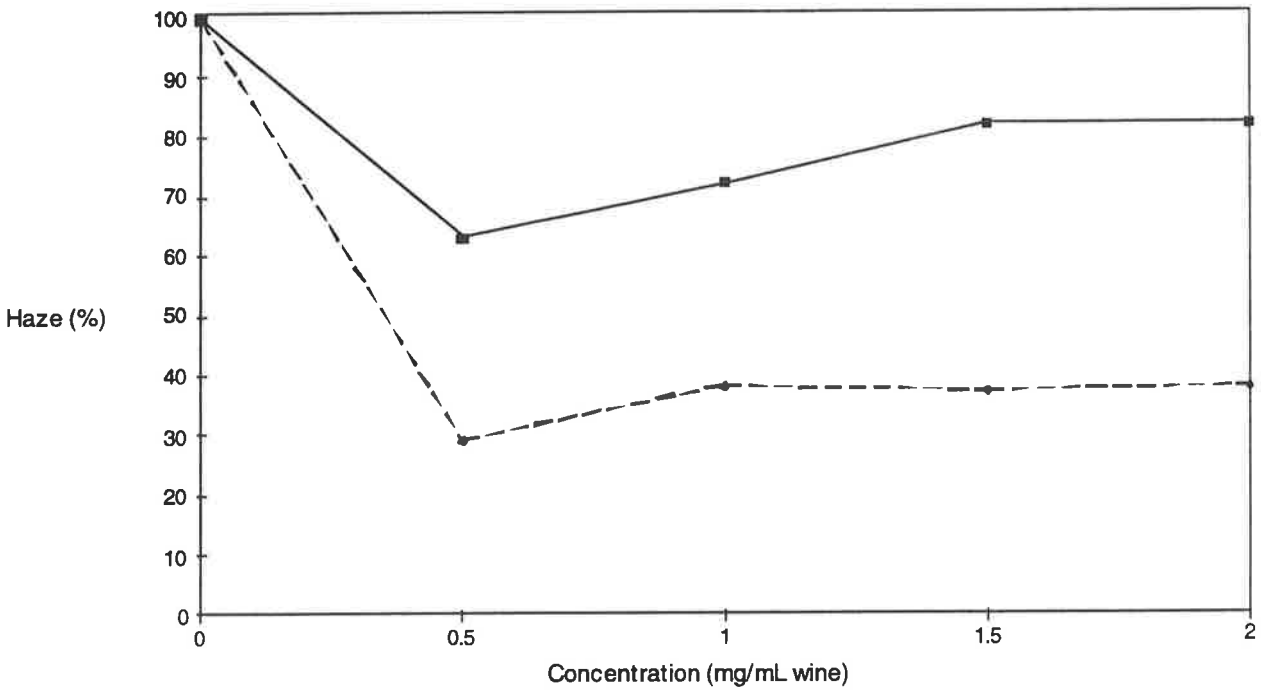


Figure 4.3 The effect of increasing concentration of mannoprotein extract obtained from the thawing process of Maurivin PDM cells in late exponential (full line) or stationary (dashed line) phase on the heat-induced protein haze. Haze was measured in the heat test as described in Section 4.2.7.

4.3.3 EXTRACTION OF HPM BY SDS OR EDTA TREATMENT APPLIED TO FRESH MAURIVIN PDM WHOLE CELLS IN LATE EXPONENTIAL OR STATIONARY PHASE

Previously, Maurivin PDM cells used for the experiments were frozen and then thawed before extraction. To exclude any possible effect of using freeze-thawed cells on the release of HPM, further experiments with EDTA and SDS were carried out on freshly harvested cells in late exponential phase or in stationary phase.

At this stage it was known that HPM could be specifically extracted from Maurivin PDM cells either by treatment with SDS (anionic detergent, see Section 3.3.2) in Tris buffer (pH 7) and with EDTA (chelating agent) in Tris buffer (pH 8). The two sets of treatments were therefore further examined to determine which components in each treatment were responsible for the release of HPM.

4.3.3.1 Yield of extraction of the mannoprotein material obtained by SDS or EDTA treatment of fresh whole cells in late exponential or stationary phase

Table 4.3 The effect of the SDS and EDTA treatments on the yield of mannoproteins from fresh Maurivin PDM cells in late exponential or stationary phase. Extractions were carried out as described in Sections 4.2.5.2 and 4.2.5.3.

Treatment	Mannoprotein extraction yield *	
	Late exponential phase	Stationary phase
SDS	0.20	0.28
SDS control	0.26	0.26
EDTA	0.05	0.03
EDTA control	0.04	0.03

* results are expressed as % (w/w) of material extracted (dry weight) per wet weight of cells used for the extraction and are the means of two independent experiments. The standard deviations of all samples were less than 5%.

The mannoprotein extraction yield for the SDS treatment of whole cells in late exponential phase, given in Table 4.3, was in the same range of values as that obtained from freeze-thawed late exponential phase cells (compare Table 4.3 to Table 3.1). This means that the state (fresh or thawed) of the cells did not alter the efficiency of the treatment with SDS. Similarly, the stage of growth (late exponential or stationary) of the cells did not significantly change the yield of the extraction.

For the control experiments (boiling cells in Tris buffer without SDS) using cells in late exponential or stationary phase, similar or even higher amounts of mannoprotein extract were collected compared to the experiments with SDS. This unexpected finding may be explained by the fact that in the control experiment the dialysis step during sample preparation was omitted. As discussed previously (see Section 4.3.1.1), loss of material may have occurred during dialysis.

The mannoprotein extraction yield of the EDTA treatment of fresh cells was dramatically lower (nine times) than that performed on freeze-thawed cells (compare Table 4.3 to Table 4.1). Presumably, the freezing and then thawing on the cells facilitated the action of EDTA in extracting material from these cells.

In the treatment of fresh late exponential phase cells in the absence of EDTA (EDTA control), around 75% of the material extracted with EDTA was still achieved. This means that EDTA accounted for only 25% of the total extracted material. For cells in stationary phase, the extraction yields of the EDTA treatment and the control decreased compared to those for cells in late exponential phase. This result suggests that EDTA was less efficient in extracting material from cells in stationary phase. As the yields for EDTA and EDTA control for cells in stationary phase were similar, that suggests that EDTA on its own was inefficient at extracting a lot of material from cells in stationary phase.

Compared to the poor yield obtained with EDTA, SDS was more efficient by far in extracting material. This observation is in agreement with Valentin *et al.* (1984) and Elorza *et al.* (1985) who observed a better extraction yield of cell wall mannoproteins with SDS compared to EDTA treatment. The small amount of material extracted in both treatments emphasises the mild action of the two agents on whole cells. This result suggests that both treatments mainly released material from the external side of the cell wall and that the pool of intrinsic (internal)

or periplasmic mannoproteins was not released under these conditions. De Nobel *et al.* (1989) could show that even with 20 times more EDTA than used here (100 mM, 30 min) less than 2% of invertase was released during the extraction. This published data supports the assumption that no periplasmic material was released with the mild EDTA treatment used in this work.

4.3.3.2 Haze protective ability of the mannoprotein extracts obtained by SDS or EDTA treatment of fresh cells in late exponential or stationary phase

After studying the extraction yields, the potential haze reduction ability of the mannoprotein extracts obtained from fresh cells were tested and the results were compared with those obtained from thawed cells.

4.3.3.2.1 Heat test results obtained for SDS treatment applied to fresh late exponential or stationary cells.

For the SDS treatment of fresh cells in late exponential phase (Figure 4.4), a similar strong haze decrease was observed compared to that obtained from thawed cells when tested in the concentration range of 0.5 to 1.5 mg/L. This result shows that the use of thawed or fresh cells had no influence on the final haze protective activity of the extract.

The mannoprotein extract obtained from fresh cells in late exponential phase in the absence of SDS (referred to as the SDS control extract) showed haze protective ability as strong as those shown by the SDS extracts (Figure 4.4). This result means that boiling fresh cells in Tris buffer was apparently sufficient to extract HPM. Since SDS was not needed to release HPM it cannot be considered as a solubilising agent of HPM.

For cells in stationary phase (Figure 4.5), similar results were obtained. Although the material extracted with SDS showed satisfactory haze reducing properties (values down to 25-30% of the ihv for all concentrations tested), heat tests with SDS control extract resulted in a slightly better haze decrease (20 - 25% of the ihv). Thus SDS was neither needed to extract HPM from cells in stationary phase nor in late exponential phase.

Over all, it seemed to be sufficient to boil cells, from either late exponential or stationary phase, to release HPM. This showed, as already suggested in Section 3.3.2, that HPF was present in the cell at two different phases of Maurivin PDM yeast cycle might suggest that HPF is a constitutive cell wall mannoprotein. The specific release of HPM over the yeast growth cycle by boiling the cells in Tris buffer implies that HPF was only interacting with other cell components by non-covalent linkages. This is because heat treatment destabilises all types of non-covalent interactions from ionic or hydrogen bonds to hydrophobic interactions (Schwatzberg and Hartel 1990).

4.3.3.2.2 Heat test results obtained for EDTA treatment applied to fresh late exponential or stationary cells

The heat test results of EDTA extractions of fresh cells in late exponential phase compared to those of thawed cells are summarised in Figure 4.6. For both treatments on thawed or fresh cells, a haze decrease around 45-50% of the ihv (for extract concentrations from 0.5 to 1.5 mg/L) was observed confirming that the use of fresh or thawed cells did not influence the final heat test results even though the yield was higher for frozen cells. The extract of the EDTA control experiment only slightly reduced the haze (at the most 65-70% of the ihv). Because this decrease in haze was always 25 to 30% above the decrease obtained with EDTA present, the chelating agent itself may have some role in the release of HPM.

The presence of metal ions in the cell wall is reported to compensate for the negative charges of the phosphate groups present in the outer core of the structural mannoproteins, as well as those of the peptide moieties of the mannoproteins, and thus stabilise the whole cell wall (De Nobel *et al.* 1989, Valentin *et al.* 1984). The formation of ionic bridges contributes to the cell wall cohesion. Due to its good chelating properties, EDTA can extract metal ions and thereby disorganise the ionic interactions within the cell wall leading to the release of cell wall components in the medium. This might explain the release of HPM during the extraction process and suggests that ionic bridges play a significant role in maintaining HPF within the cell wall. In addition an extraction experiment using EDTA at pH 3 and 5.5, a range of pH where the chelating abilities of EDTA are reduced or absent (Janson and Ryden, 1989) did not lead to the release of HPM (data shown in Table 1, Appendix C). This result further supports the hypothesis that the release of HPM by EDTA at pH 8 was due to the depletion of ions from the cell wall by EDTA.

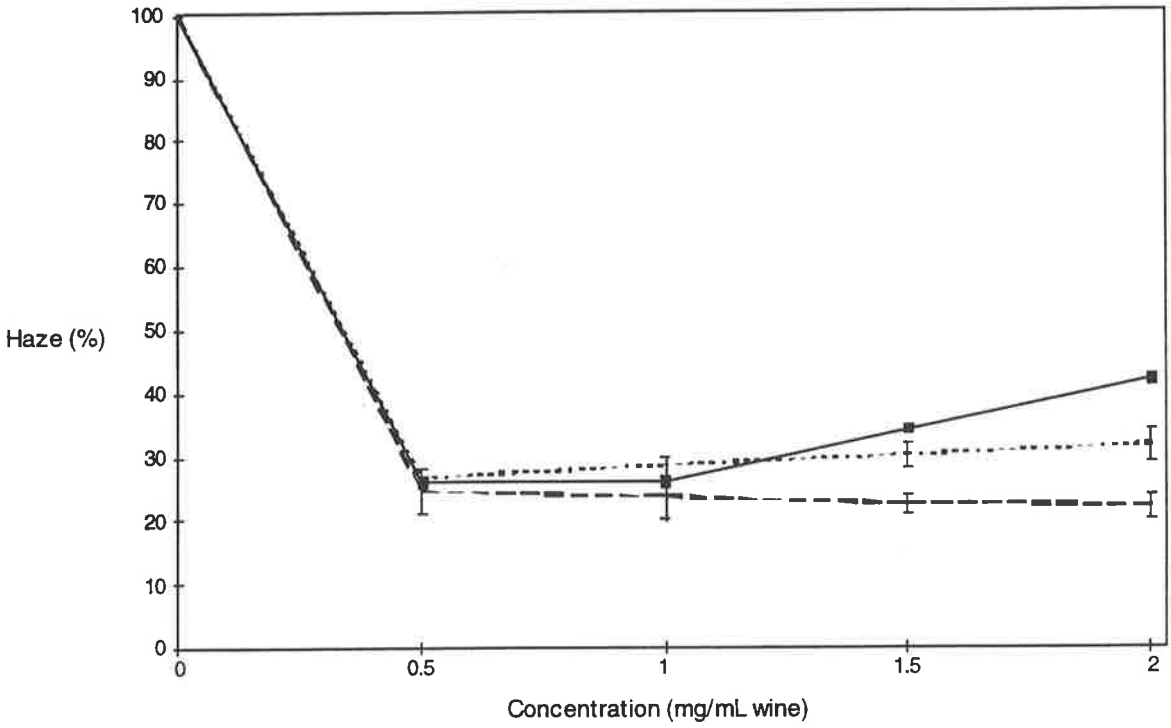


Figure 4.4 The effect of increasing concentration of mannoprotein extract obtained from fresh late exponential phase Maurivin PDM cells by the SDS treatment (dotted line), the SDS control treatment (dashed line), or from thawed late exponential phase Maurivin PDM cells by the SDS treatment (data from Figure 3.1, full line) on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 4.2.7. Values are the means of three independent experiments (except for SDS treatment on thawed cells which was from one experiment). The vertical bars represent the standard deviation for each value.

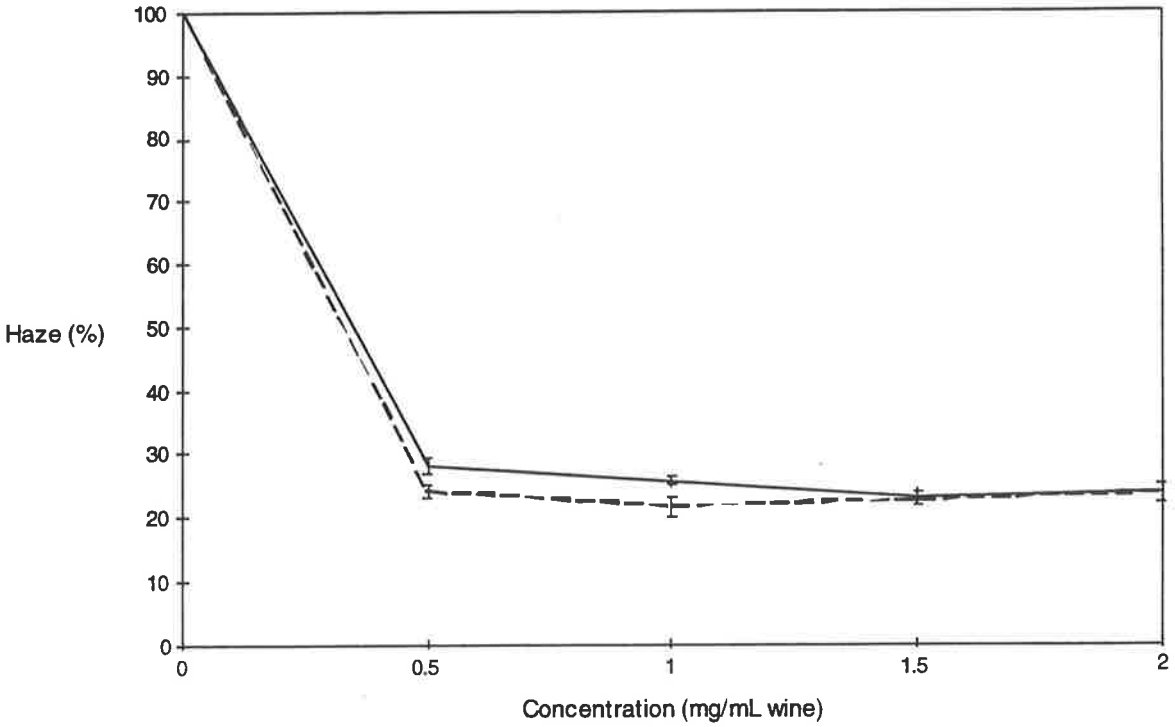


Figure 4.5 The effect of increasing concentration of mannoprotein extract obtained from fresh stationary phase Maurivin PDM cells by the SDS treatment (full line) or the SDS control treatment (dashed line) on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 4.2.7. Values are the means of three independent experiments. The vertical bars represent the standard deviation for each value.

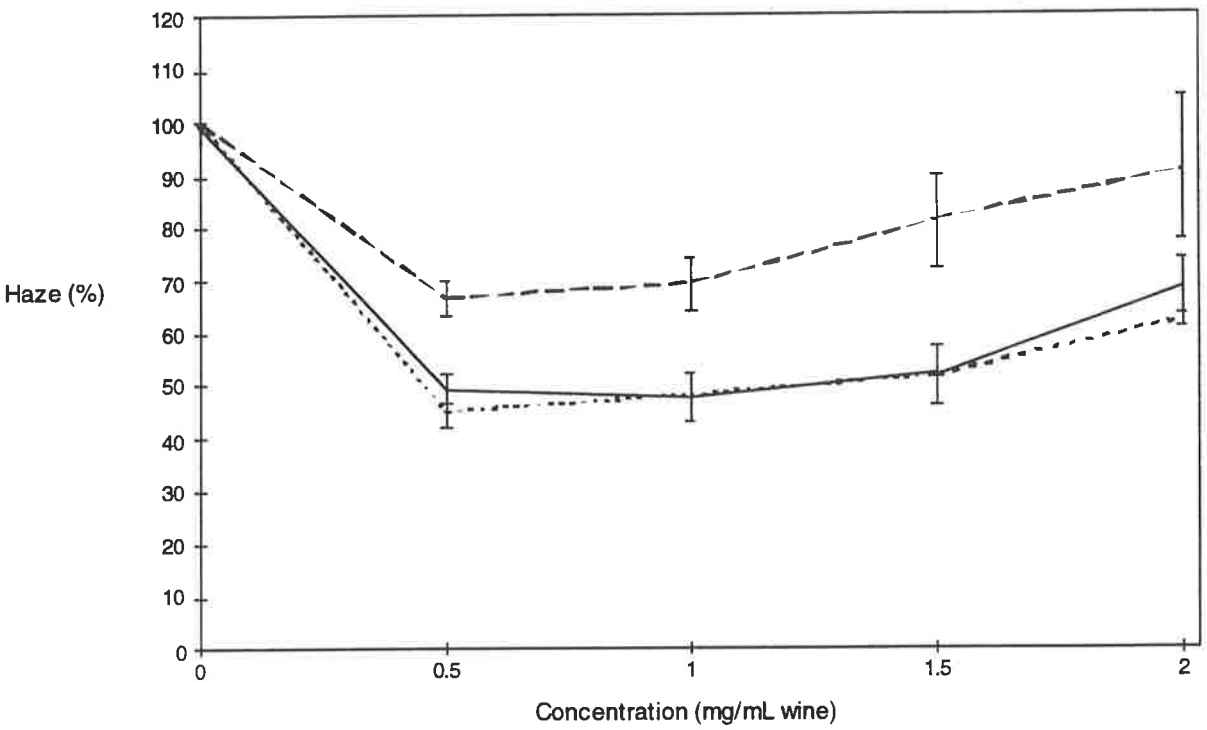


Figure 4.6 The effect of increasing concentration of mannoprotein extract obtained from fresh late exponential phase Maurivin PDM cells by the EDTA treatment (dotted line), the EDTA control treatment (dashed line) or from thawed late exponential phase Maurivin PDM cells by the EDTA treatment (data from Figure 4.2, full line) on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 4.2.7. Values are the means of three independent experiments (except for EDTA treatment on thawed cells). The vertical bar represents the standard deviation for each value.

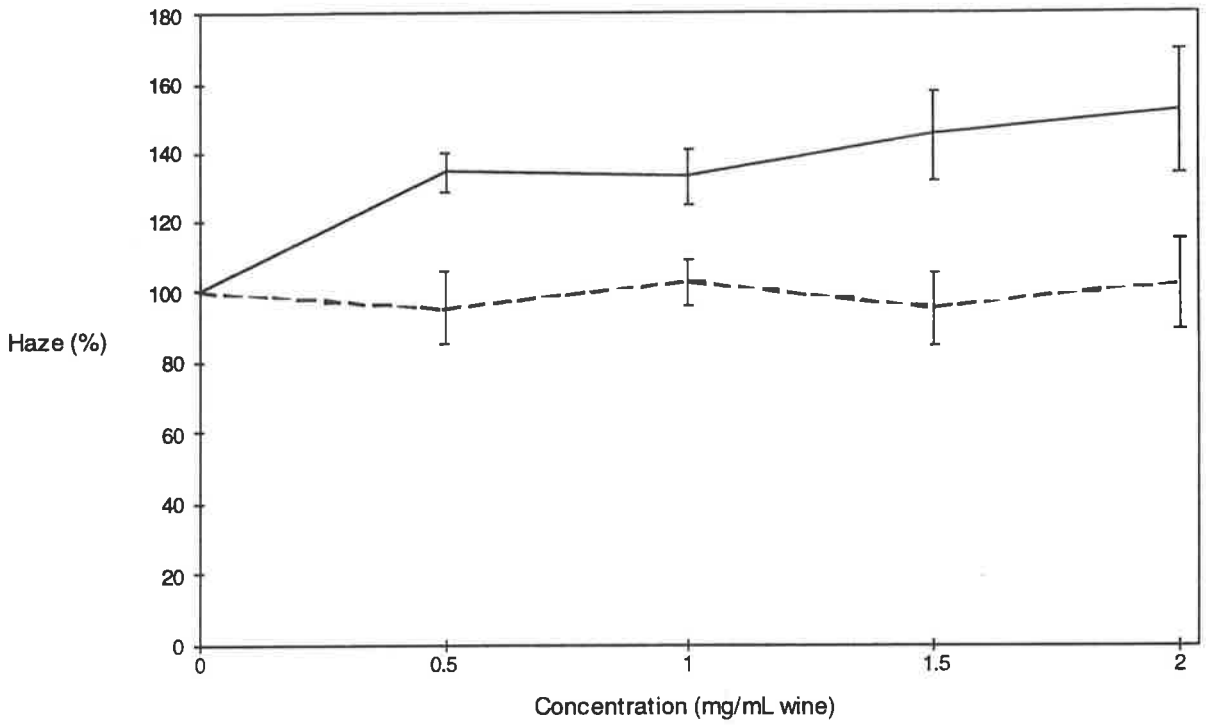


Figure 4.7 The effect of increasing concentration of mannoprotein extract obtained from fresh stationary phase Maurivin PDM cells by the EDTA treatment (dashed line) or the EDTA control treatment (full line) on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 4.2.7. Values are the means of three independent experiments. The vertical bars represent the standard deviation for each value.

In contrast to the results obtained with cells in late exponential phase, material extracted by EDTA from cells in stationary phase did not show any haze reducing properties (the haze values varied only between 90 to 100% of the ihv, Figure 4.7). The corresponding control experiment gave haze values higher than 130% of the ihv. Since HPM is known to be present in the cells in stationary phase (as it was extracted by the SDS treatment), the lack of any haze protective ability of the EDTA extract suggests that EDTA was not able to extract HPM from cells in stationary phase. This could be explained by the fact that the architecture of the cell wall evolves during cell maturation leading to a cell wall in stationary phase that is more structured, rigid and less porous (De Nobel *et al.* 1990, Valentin *et al.* 1987). Due to these changes in architecture, the cell wall was probably more stable and resistant to the extraction of metal ions by EDTA, thus preventing the release of HPM.

For fresh cells in late exponential as well as in stationary phase, the haze protective ability of HPM released by boiling, with or without SDS, was higher compared to that released by EDTA at 28°C. A better haze decrease was obtained for the SDS extract (and the SDS control) compared to EDTA extract and, for the SDS and SDS control extracts, there was no increase of haze observed at higher concentrations of extract (2 mg/mL - see Figure 4.4 and 4.5- or 3 mg/mL - data not shown). That means that SDS or SDS control extracts contained far less haze forming material compared to EDTA extract.

4.3.4 Extraction of HPM by SDS or EDTA treatments applied to Maurivin PDM cell walls isolated from cells in late exponential phase

In parallel to the study of their action on whole fresh cells, SDS and EDTA treatments were applied to cell walls isolated from fresh cells in late exponential phase. This experiment was essential to confirm, as suggested in Section 3.3.3, that HPM was extractable from the cell wall and therefore that HPF is a mannoprotein from the cell wall.

Cell walls were isolated from fresh whole Maurivin PDM cells according to the method described in Section 4.2.4. After thorough washings, extractions with EDTA and SDS were conducted as previously described for whole cells. The mannoprotein material extracted from the cells during the cell wall isolation process was also collected (referred to as cell disruption extract). Due to the difficulty of the method to isolate cell walls and the paucity of the

mannoprotein material obtained, extractions and heat tests were carried out only once. Heat test results are given in Figure 4.8.

HPM was not detected in the cell disruption washings as no significant decrease of haze was observed (80% of the ihv). Katohda *et al.* (1976) estimated that a large part of the mannan found in the cell disruption washings was of cell wall origin and concluded that the ballistic action of the glass beads might dislodge mannoproteins loosely attached to the cell wall. Since no decrease of haze was observed with the cell disruption extract, it can be assumed that if HPM was partly released during this process, its haze protective effect was swamped by other mannoprotein fractions co-released during this process (cytoplasmic and periplasmic materials) resulting in a negative heat test.

For the SDS extract, a haze decrease of around 40-45% of the ihv was observed for all concentrations (except at the concentration 1.5 mg/mL where the haze went down to 30% of the ihv). The haze reduction was not as significant as for the extracts from whole cells (compare Figure 4.8 to Figure 4.4). Nevertheless, the data shows that SDS successfully released HPM from isolated cell walls confirming that HPM is indeed associated with the cell wall.

SDS treatment on isolated cell walls is thought to remove not only cell wall material but other material, in particular proteins, from the cytoplasmic membrane which are present as contaminants in the cell wall preparation (Molloy *et al.* 1989, Zlotnik *et al.* 1984). The hypothesis that HPM might originate from a cytoplasmic membrane contaminant of the cell wall preparation is not supported by the fact that HPM was successfully extracted from whole cells by SDS or EDTA treatment. Both treatments on whole cells were thought not to release any periplasmic material (see Section 4.3.3) and hence any cytoplasmic membrane components.

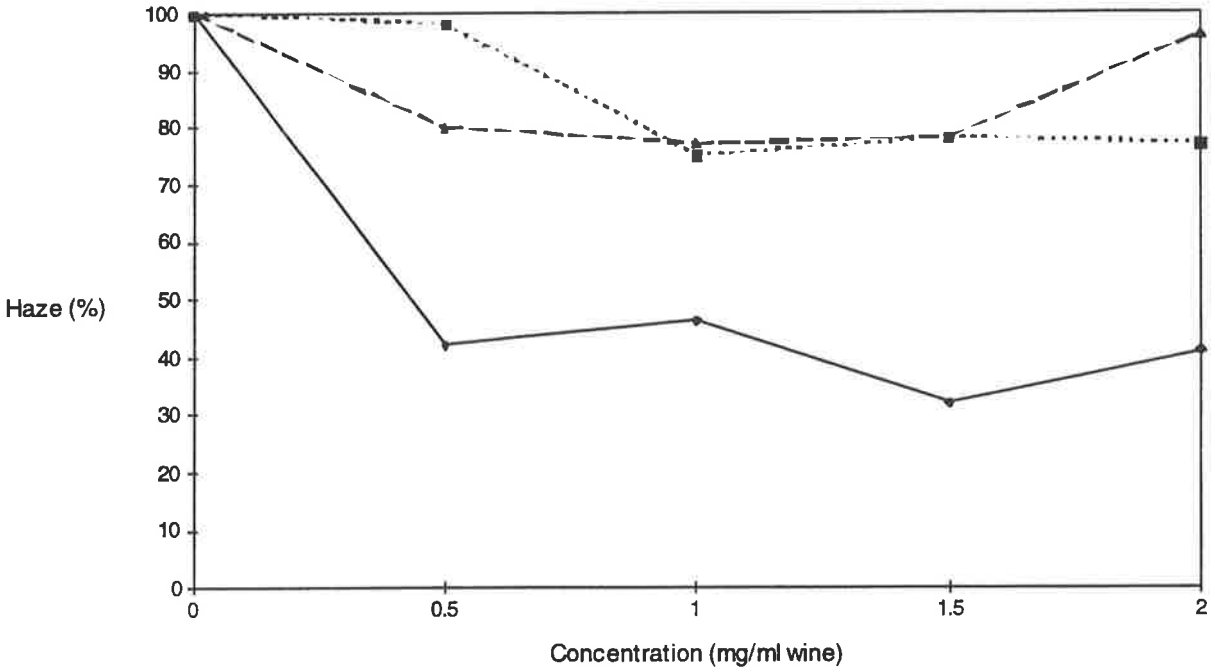


Figure 4.8 The effect of increasing concentration of mannoprotein extract obtained from cell walls from fresh late exponential phase Maurivin PDM cells by the EDTA treatment (dotted line), the SDS treatment (full line) or during the process of cell disruption (cell disruption washings, dashed line) on the heat-induced protein haze in wine. Haze was measured in the heat test as described in Section 4.2.7.

Concerning the EDTA extraction of the purified cell walls, only a slight haze reduction (up to 75-80% of the ihv) was observed whereas a haze decrease to around 45-50% of the ihv was noticed for the extraction of whole cells. For the isolated cell walls, EDTA or SDS might have solubilised not only material at the external side of the cell wall, as for extraction of whole cells, but also at the inner wall side now exposed after the cell disruption process. Thus new and different mannoprotein material having a high haze formation ability might have been released and partly swamped the positive effect of HPM.

4.3.5 EXTRACTION OF HPM BY SDS TREATMENT APPLIED TO COMMERCIAL CELL WALL MATERIAL (YEAST HULLS)

SDS extraction was performed directly on a preparation of yeast hulls (Lallemand, SA, Australia) as described in Section 4.2.5.3. On the contrary to SDS extraction on purified Maurivin PDM cell walls, the material obtained after Con-A was not white and fluffy but powdery and dark in colour. The heat test revealed that the material extracted did not contain HPM as the haze values did not significantly decrease (data shown in Table 2, Appendix C).

The method of preparation of yeast hulls on the industrial scale was not made available. One can speculate that HPM was already released during the industrial process of making yeast hulls and that it was therefore not anymore present in substantial amounts in the commercial cell wall material.

4.4 CONCLUSION

In Chapter 3, SDS and full zymolyase treatments were found to be the most effective methods to release HPM from whole Maurivin PDM yeast cells. In the current Chapter, comparative analyses of the component parts of the full zymolyase treatment revealed that neither zymolyase nor DTE were in fact releasing HPM and that EDTA alone was able to extract HPM. Similar comparative investigation of the component parts of the SDS treatment showed that SDS was not necessary to extract HPM and that boiling cells in Tris buffer (100 mM, pH 8) for 5 min was sufficient. Table 4.4 gives a summary of the haze protective ability of different mannoprotein extracts obtained from treatments applied to whole Maurivin PDM cells.

Treating cells with EDTA or boiling them in Tris buffer (100 mM, pH 8) were found to be the most effective methods to specifically release HPM - the latter treatment being the most advantageous with respect to purity and yield. Nevertheless, since the maximum yields were still relatively low (less than 3 g mannoprotein per kg wet wet cells) neither of these extraction methods would be suitable for scaled-up production.

Extractions with DTE or zymolyase were not specific for the release of HPM and most probably released other mannoproteins that interfered with the haze protective ability of HPF. With the methods which released HPM in a specific way (like EDTA or SDS treatments, or by just boiling cells), HPM was released easily from the yeast cells and this result indicated that HPM was well accessible to the solubilising agents. It can be concluded that HPF is probably present on an external location of the cell. Direct extraction of HPM from purified cell walls confirmed that this material was indeed associated with the cell wall. Thus, HPF can be considered as a cell wall mannoprotein.

The specific methods releasing HPM mentioned above are characterised by the disruption of non-covalent bonds whereas zymolyase or DTE treatments imply the destruction of covalent bonds. Therefore HPF was retained in the cell wall by non-covalent interactions and was not bound to the glucan network by ß(1-3) bonds or to other cell wall components by disulfide bridges. This hypothesis was also supported by the release of HPM under freeze-thaw stress since this technique also involves destruction of the non-covalent bonds like ionic or hydrophobic interactions.

Several types of non-covalent bonds (ionic to hydrophobic interactions) seem to play a role in maintaining HPF in the cell wall during cell growth. A change of the extraction profile of HPM for cells in stationary cells (extractable by boiling and under freeze-thawing but not by EDTA) probably reflects the rearrangement of the cell wall components during maturation. More detailed studies would be necessary to determine the contribution of each type of non-covalent bond in retaining HPF in the cell wall to explain the change of the extraction profile of HPM for cells in stationary phase.

As HPF is only loosely associated with the cell wall, HPF might be present in small quantities in all extracts tested. A cell wall glycoprotein of 29 k (gp29) was reported as extractable by boiling cell walls in Tris buffer or by EDTA treatment (Klebl and Tanner. 1989) but was also detected in extracts obtained by zymolyase or SDS treatments (Pastor *et al.* 1984, Herrero and Boyd 1986). As observed for gp29, HPF was probably present in all extracts even if it was not specifically released by the solubilising agent in the treatment.

This suggestion reveals the limits of the heat test procedure. Since HPF was not detected when present in small amounts, the haze values observed depended on the haze formation ability of other mannoprotein fractions. To observe a haze decrease implied that HPM was present in substantial amounts in the extract. Therefore a new method in place of the heat test would need to be developed to detect small amount of HPF as well as to quantify it.

Cell wall mannoproteins are often classified in two categories in the literature (Elorza *et al.* 1993, Van der Vaart *et al.* 1995). The first category is that of mannoproteins loosely associated with the cell wall (SDS extractable) whereas the second refers to the mannoproteins covalently linked to the cell wall (zymolyase extractable). According to the extraction profile, HPF like gp29 was SDS extractable and would belong to the first category of mannoproteins. Nevertheless both components were also extractable by boiling in Tris buffer. As the SDS treatment and the boiling have a similar impact on the cell wall (disruption of non-covalent bonds, in particular, hydrophobic interactions), it can be assumed that at least part of the SDS extractable mannoproteins are, indeed, extractable by simple boiling, too. With this hypothesis, a third category of mannoproteins should be introduced and referred to as mannoproteins only loosely asociated with the cell wall and extractable by boiling in Tris buffer.

Table 4.4 Rating of the haze protective ability of the mannoprotein extracts obtained with different treatments applied to fresh or frozen .Maurivin PDM whole cells in late exponential (LEP) or stationary phase (SP) according to the haze decrease observed in the heat test. No extraction was carried out on frozen cells in stationary phase.

* detailed descriptions are given in Section 4.2.5

** extent of haze decrease obtained for each extract:

-: no haze decrease observed. Haze value is equal or higher than the ihv (100%)

+: haze decrease between 100 and 60% of the ihv

++: haze decrease between 60 and 40% of the ihv

+++: haze decrease between 40 and 20% of the ihv

nd not determined

Description of the treatment*	Haze protective ability **			Sections where results are discussed
	Thawed whole cells	Fresh whole cells		
	LEP	LEP	SP	
Full zymolyase treatment: combined pretreatment and zymolyase treatment	+++	nd	nd	4.3.1.1
Pretreatment: combined DTE and EDTA treatments	++	nd	nd	4.3.1.1
DTE treatment: 5 mM DTE, Tris HCl buffer, 28°C	-	nd	nd	4.3.1.2
EDTA treatment: 5 mM EDTA, Tris HCl buffer, 28°C	++	++	-	4.3.1.2 4.3.3.2.2
EDTA control: Tris HCl buffer, 28°C	nd	+	-	4.3.3.2.2
Zymolyase treatment: 2%(w/v) zymolyase on pretreated cells, 28°C	+	nd	nd	4.3.1.1
SDS treatment: boiling in 2%(v/v) SDS, Tris HCl buffer	+++	+++	+++	4.3.3.2.1
SDS control: boiling in Tris HCl buffer	nd	+++	+++	4.3.3.2.1

Chapter 5

RELEASE OF HPM FROM *SACCHAROMYCES CEREVISIAE* MAURIVIN PRISE DE MOUSSE DURING FERMENTATION AND STORAGE ON YEAST LEES

5.1 INTRODUCTION AND LITERATURE REVIEW

During the growth of *Saccharomyces* yeast under winemaking or laboratory conditions, the release of mannoprotein material into the growth culture can be observed. The release of such material occurs at different stages of winemaking. At the stage of alcoholic fermentation, Llaubères *et al.* (1987) showed that, for all *Saccharomyces* strains tested, polysaccharides and cell wall mannoproteins, were progressively released into the growth culture. The authors speculated that this phenomenon was associated with the cell wall synthesis of the newly formed yeast cells and that the mannoproteins released were surplus. The mannoproteins released into the fermentation culture have similar composition and structural features to those of the cell wall, however, with a lower protein content and a higher M_r heterogeneity (Saulnier *et al.* 1991). The relative molecular mass of the released mannoprotein fractions ranged from 600 k to 50 k and the amounts of all fractions steadily increased during alcoholic fermentation (Feuillat *et al.* 1989).

After fermentation, some white and sparkling wines are left on yeast lees for periods extending from months to years. During this time frame, the yeast cells enter the stage of autolysis and the amount of mannoproteins released into the growth culture drastically increases (Section 1.5.4, Llaubères *et al.* 1987, Feuillat *et al.* 1989). From a biochemical view point, autolysis is characterised by an extensive degradation of the cell wall glucan by yeast's own enzymes, β(1-3) glucanases which releases mannoproteins covalently linked to the glucan network or embedded into it (Charpentier and Feuillat 1993). Periplasmic mannoprotein material is also released into the growth culture as it is freed from the cell wall. The different pools of mannoproteins are further degraded in the growth culture by proteolytic enzymes (which attack the protein moiety) and α-mannosidases (which attack the mannan

moiety). Any glucan still linked to the mannoproteins is also further degraded by exo β(1-3) glucanases (Charpentier and Feuillat 1993).

The release of mannoproteins can also be observed during the growth cycle of laboratory yeast strains. Biely *et al.* (1974) observed a continuous release of mannoproteins during the whole growth cycle for a *Saccharomyces* strain. Further studies (Krátký *et al.* 1975b) showed that this mannoprotein material, once synthesised, was not inserted into the cell wall but released into the culture. However, it could not be determined if this material was either material left over from the cell wall building or material with no structural role and thus not integrated into the wall. On the other hand, the mannoprotein material involved in the cell wall architecture, once incorporated in the cell wall, was metabolically stable and not released into the growth culture. This was confirmed by Pastor *et al.* (1982) who found that the fraction of mannoproteins integrated in the cell wall were characterised by a very low turnover and thus were only released in small amounts.

Among the known cell envelope mannoproteins some are released into the growth culture whereas others are not. For instance, α-agglutinins (mannoproteins from the cell wall surface which participate in the mating of haploid cells, see Section 1.5.5.2) could be detected in the growth culture of *Saccharomyces cerevisiae* cells (Sijmons *et al.* 1987). Agglutinating factors (involved in the flocculation process) for a *Saccharomyces cerevisiae* brewer's strain were released in small amounts during exponential phase and in greater amounts during stationary phase (Straver *et al.* 1994). On the contrary, inducible enzymes from the periplasmic space like invertase or acid phosphatase (see Section 1.5.5.1) are rarely released into the growth culture from *Saccharomyces cerevisiae* cells. They are thought to be physically restrained by the cell wall (Arnold 1991) although De Nobel *et al.* (1989) suggested that interactions with the electrical charges of the cell wall mannoproteins were also responsible for their retention in the periplasmic space.

The above observations demonstrate that some cell wall mannoproteins are possibly released into the growth culture during yeast growth (cell wall building) or autolysis (cell wall degradation). Thus it was of interest to examine if Maurivin Prise de Mousse (PDM) yeast cells released HPM (haze protective material) during growth and autolysis, and if such a release could be considered as a possible source of HPF.

5.2 EXPERIMENTAL

5.2.1 MATERIAL

Peptone was from Oxoid, Australia Pty. LTD (VIC, Australia). Yeast Medium (YM) broth was purchased from Amyl company (VIC, Australia). Econo-Pac 10DG disposable desalting columns (nominal exclusion limit of 6 kDa) were sourced from BioRad laboratories Pty Ltd (NSW, Australia). The counting chamber Neubauer improved Bright-line (depth: 0.1 mm, area of grid 1 mm^2) was purchased from Weber Scientific International Ltd (Sussex, England). Other materials were as described in Section 2.1.

5.2.2 FERMENTATION TRIALS CONDUCTED AT 25°C WITH AGITATION - COLLECTION OF CULTURE SUPERNATANTS. RECOVERY OF THE EXTRACELLULAR MATERIAL BY ULTRAFILTRATION OR ETHANOL PRECIPITATION

In Chapters 3 and 4, Maurivin PDM yeast cells were grown at 25°C with agitation to late exponential or stationary phase either in a glucose or mannose enriched media (see Sections 3.2.3, 3.2.4 and 4.2.3). The culture supernatant from each fermentation experiment was recovered after centrifugation (18,000 *g*, 10 min, 5°C), filtered through a 0.45 µm membrane and stored at -20°C before use.

The culture supernatants (thawed at room temperature) except one (see next paragraph) were ultrafiltered according to the procedure described in Section 2.3.4. The retentate was collected, freeze-dried using a Dynavac freeze-drying unit and weighed. The material collected will be referred to as the extracellular material released from the agitated 25°C fermentation trials.

The mannoprotein extracellular material of a single culture supernatant from cells Maurivin grown on glucose to stationary phase was recovered by ethanol precipitation. The culture supernatant (10 L) was mixed with 3 volumes of 96% (v/v) ethanol and left at -20°C for 48 hours. The mannoprotein precipitate was recovered by centrifugation (18,000 *g*, 15 min, -10°C) and washed with 75% (v/v) ethanol (x 2, each time with 500 mL). The precipitate was

afterwards dissolved in water and concentrated by ultrafiltration (see Section 2.3.4) before being freeze-dried using a Dynavac freeze-drying unit and weighed.

5.2.3 LARGE SCALE FERMENTATION CONDUCTED AT 25°C WITH AGITATION - YEAST PROPAGATION, MONITORING AND SAMPLING OF CULTURE. RECOVERY OF EXTRACELLULAR MATERIAL BY DIALYSIS

Yeast propagation was conducted as described in Section 2.2.3 in consecutive propagation volumes of 10, 50 mL and 1 L. The final propagated culture (1 L), once reaching exponential phase, was transferred to 19 L SGJM containing glucose (see Section 2.2.2).

Growth of Maurivin PDM was carried out in 20 L SGJM contained in a 28 L vessel under incubation conditions of 25°C with agitation on an orbital shaker (110 rpm, Paton Scientific, Model OP 3422). Cell growth was followed by measuring the absorbance at 650 nm (see Section 2.2.4).

The culture was sampled under N_2 cover at (the value in parentheses is the volume of culture sampled) 0 hour (h) (1.5 L) ; 3h, 6h, 8h, 10h, 13h (1 L) ; 17h (750 mL) ; 20h, 23h (500 mL) ; 28h, 31h, 34h, 37h (300 mL) ; 41h, 50h, 60h, 83h, 104h, 126h, 150h (250 mL) after inoculation.

A 10 mL aliquot of the culture was kept for the total and viable cell counts (see Section 5.2.6). The remaining culture was centrifuged (18,000 *g*, 10 min, 5°C). The culture supernatant was recovered, filtered through a 0.45 µm membrane and stored at -20°C before use. The cells were discarded.

The sampled culture supernatants (thawed at room temperature) were split in 175 mL aliquots which were dialysed against distilled water (10 L, 4 changes). The final monomeric glucose (from the SGJM) concentration in the culture supernatant after dialysis was estimated to be below 10 µg. The collected samples were freeze-dried using a Dynavac freeze-drying unit and weighed. The material collected will be referred to as the extracellular material released from the large scale agitated 25°C fermentation.

5.2.4 FERMENTATION AT 18°C WITHOUT AGITATION - YEAST PROPAGATION, MONITORING AND SAMPLING OF CULTURE. RECOVERY OF THE EXTRACELLULAR MATERIAL BY ULTRAFILTRATION

Yeast propagation was conducted as described in Section 2.2.3 in consecutive propagation volumes of 10, 40 and 750 mL. The final propagated culture (750 mL), once reaching exponential phase, was transferred to 14.25 L SGJM containing glucose.

Growth of Maurivin PDM was carried out in 15 L SGJM contained in a 20 L vessel under the incubation conditions of 18°C without agitation. Cell growth was followed by measuring the absorbance at 650 nm (see Section 2.2.4).

The culture was sampled daily (1 L, after resuspension of the cells) under N_2 cover and the culture supernatant was recovered by centrifugation (4,000 *g*, 10 min, 10°C), filtered through a 0.45 μm membrane and stored at -20°C before use. The cells were discarded.

The sampled culture supernatants (thawed at room temperature) were ultrafiltered according to the procedure described in Section 2.3.4. The retentate was collected, freeze-dried using a Dynavac freeze-drying unit and weighed. The material collected will be referred to as the extracellular material released from the non-agitated 18°C fermentation.

5.2.5 STORAGE ON YEAST LEES AT 18°C - SAMPLING OF CULTURE. RECOVERY OF THE EXTRACELLULAR MATERIAL BY ULTRAFILTRATION

At the end of the fermentation (see Section 5.2.4), the yeast cells were separated from the culture supernatant by centrifugation (4,000 *g*, 10 min, 10°C). The supernatant was ultrafiltered through a YM 30 membrane (see Section 2.3.4) whereas the yeast pellet was kept in a small volume of medium at 4°C.

The retentate from the ultrafiltration contained the extracellular mannoprotein material released into the culture during fermentation and was kept for further analyses. The ultrafiltered medium (mannoprotein-free medium, 10 L) was sterile filtered and transferred into a sterile, air tight storage vessel (15 L). The yeast pellet was resuspended and back added

to the mannoprotein-free medium under aseptic conditions. The storage vessel was sealed, purged with N_2 and stored unstirred at 18°C.

Sampling (1 L, after resuspension of yeast lees) was performed every two weeks for a two month period under N_2 cover. These samples were centrifuged (4,000 *g*, 10 min, 10°C) and the recovered supernatants were stored at -20°C before use.

The supernatants (thawed at room temperature) were ultrafiltered according to the procedure described in Section 2.3.4. The retentate was collected, freeze-dried using a Dynavac freeze-drying unit and weighed. The material collected will be referred to as the extracellular material released during storage on lees.

5.2.6 DETERMINATION OF TOTAL AND VIABLE CELL COUNTS

The total cell density in the growth culture was determined using a Neubauer counting chamber (minimum of 600 cells counted when possible, accuracy 99%).

The viable cell density was determined using the spread plate counting method. Ten fold serial dilutions of the samples were made in sterile 0.1 % (w/v) peptone solution. The diluted samples (two dilutions tested, three replicates for each) were spread on plates made of YM broth (21 g/L) and Agar (20 g/L). The number of viable cells, expressed in colony forming units (CFU) corresponded to the number of colonies counted after 24 hours of incubation at 25°C.

5.2.7 DETERMINATION OF POLYMERIC MANNOSE AND GLUCOSE CONTENTS

Prior to sugar measurements, the culture supernatants sampled during the large scale agitated 25C fermentation (Section 5.2.3) were desalted on Econo-Pac 10 DG columns (BioRad laboratories) to eliminate the monomeric glucose from the SGJM. Culture supernatants sampled during fermentation from 0h to 28h00 were desalted twice whereas the following samples were desalted only once. After desalting the samples were freeze-dried using a Dynavac freeze-drying unit and the freeze-dried material resuspended in water. The freeze-dried extracellular material obtained in Sections 5.2.4 and 5.2.5 were directly used for sugar measurements.

The hydrolysis as well as the enzymatic assays were conducted as described in Section 2.3.7. Each measurement for the enzymatic assay was repeated at least five times.

5.2.8 MICROMETHOD FOR THE MEASUREMENT OF THE HEAT INDUCED HAZE (HEAT TEST)

The extracellular materials recovered after either ultrafiltration, ethanol precipitation or dialysis were tested for their haze protective abilities according to the procedure described in Section 2.3.5. The two ultrafiltered wines described in Section 2.3.5 were used in this study.

5.3 RESULTS AND DISCUSSION

5.3.1 RELEASE OF HAZE PROTECTIVE MATERIAL (HPM) BY MAURIVIN PDM CELLS IN THE AGITATED 25°C FERMENTATION TRIALS

5.3.1.1 Differences in yield of extracellular material released from cells according to the phase of cell growth and method of recovery

The extracellular material released by Maurivin PDM yeast cells was recovered at different growth phases either by ultrafiltration or by ethanol precipitation of the culture supernatants before being freeze-dried (Table 5.1).

With the SGJM containing glucose as a carbon source, the average yield of extracellular material released by cells in late exponential phase into the growth culture was 0.67%. [(w/w) of extracellular material released (dry weight) per wet weight of yeast cells collected] (Table 5.1). When cells grew on SGJM containing mannose, more material was released into the culture and might be attributed to a higher release of extracellular mannoproteins.

For both glucose or mannose as a carbohydrate source, the yields of extracellular material released by yeast cells in stationary phase increased compared to the yields obtained for late exponential phase cells (Table 5.1). Approximately, 60 mg of extracellular material per litre of culture supernatant were recovered at the stage of late exponential phase whereas 145 mg/L were collected from the culture supernatant of cells in stationary phase (recovery by

ultrafiltration, data not presented in Table 5.1). Since the extracellular material released during fermentation mainly consists of cell wall mannoproteins (Llaubères *et al.* 1987, Feuillat *et al.* 1989), it could be considered that the increase in extracellular material yield was connected to a higher release of the extracellular mannoproteins from cells in stationary phase compared to cells in late exponential phase. This observation suggests that the rate of mannoproteins released into the culture increased as the cells matured.

During the extraction trials (see Section 3.3.1), it was observed that more mannoproteins were extractable firstly from cells grown on mannose instead of glucose and secondly from cells in stationary phase as opposed to cells in exponential phase. As described earlier, the same observations applied for the release of extracellular material. Thus the increase of extracellular material (and supposedly mannoproteins) released into the culture was probably directly correlated to the increase of the mannoprotein content of the cell walls.

Ultrafiltration was a simple means to recover the extracellular material and to eliminate the monomer sugars from the SGJM. When the ultrafiltered extracellular material was loaded onto a Con-A column, the UV absorbing peak for the void volume fraction (which contains material unbound to the lectin) was almost non-existent (data not shown). That meant that the majority of protein material released into the culture was bound to the lectin and was thus linked to mannose. This result is in agreement with the literature which reported that mannoproteins are the major components (70 to 80%) of the extracellular material released into the culture by winemaking yeasts (Usseglio-Tomasset 1976, Llaubères *et al.* 1987, Feuillat *et al.* 1989).

Besides ultrafiltration, ethanol precipitation was used to recover the mannoprotein material as it is a well-established method to precipitate mannoproteins (Pastor *et al.* 1984, Elorza *et al.* 1985). The yield of extracellular material after ethanol precipitation was 30% lower compared to that obtained after ultrafiltration (Table 5.1). Thus at least 70% of the extracellular material released into the culture and collected by ultrafiltration consisted of mannoproteins. This percentage was consistent with the values given in the literature (Usseglio-Tomasset 1976, Wucherpfennig *et al.* 1984, Llaubères *et al.* 1987).

Table 5.1 Yields of extracellular material released into the growth culture by Maurivin PDM cells at different stage of growth.

Carbohydrate source of the SGJM	Yield of extracellular material released into the culture supernatant at	
	Late exponential phase	Stationary phase
Glucose	0.67*	1.07 (0.74)
Mannose	0.83	1.58

* results are expressed as % (w/w) of extracellular material released (dry weight) per wet weight of yeast cells collected and recovered by ultrafiltration.

Data in parentheses is the yield of extracellular mannoprotein material recovered by ethanol precipitation. The values given for extracellular material released from cultures containing glucose and recovered by ultrafiltration are the means of at least four experiments. The values given for extracellular materials collected from cultures containing mannose or recovered by ethanol precipitation are from a single experiment.

5.3.1.2 Haze protective ability of the extracellular materials collected

The different extracellular materials obtained were tested for their ability to reduce haze. On the contrary to the extractions from whole cells or cell walls (see Chapters 3 and 4), it was not necessary to load the extracellular material onto a Con-A column as extracellular materials showed satisfactory haze protective ability without this purification step.

For the cells grown on glucose (Figure 5.1) the extracellular material released by the late exponential phase cells and recovered by ultrafiltration showed high haze reducing ability [haze values around 30 % of the initial haze value (ihv)] for all concentrations of samples tested. Therefore haze protective material (HPM) was indeed released by cells during late exponential growth into the culture.

Similarly, a significant haze decrease was observed for the extracellular material collected from culture supernatant of cells in stationary phase (and recovered by ultrafiltration) although the values were slightly less satisfactory than those from cells in late exponential phase (haze values at 35-40% of the ihv for all concentrations of samples tested, Figure 5.1). The difference of 5 to 10% of these values as opposed to those for cells in late exponential phase was not considered as significant since the standard deviation approximated 5 %. The extracellular material recovered from the culture supernatant of cells in stationary phase already contained the extracellular material released earlier during exponential phase. Therefore based on these data it could not be concluded that HPM was also released from cells in stationary phase although it was present in the culture supernatant recovered at this stage of growth.

The extracellular material collected from the culture supernatant of cells in stationary phase and precipitated by ethanol showed better heat test results compared to that obtained by ultrafiltration (haze values around 25% of the ihv, Figure 5.1). Thus ethanol precipitation of the material released into the culture seemed to be more appropriate to recover an extracellular mannoprotein material with strong haze protective ability compared to the ultrafiltration process.

When cells were grown with mannose in place of glucose as a carbohydrate source, a significant haze decrease was observed for the extracellular material from the culture supernatants (Figure 5.2). To utilise mannose seemed to favour the release of mannoproteins with a strong haze protective ability as the haze values decreased to 20 to 25 % of the ihv. The same significant haze decrease was observed for extracellular material collected from culture supernatants of cells either in exponential or stationary phase.

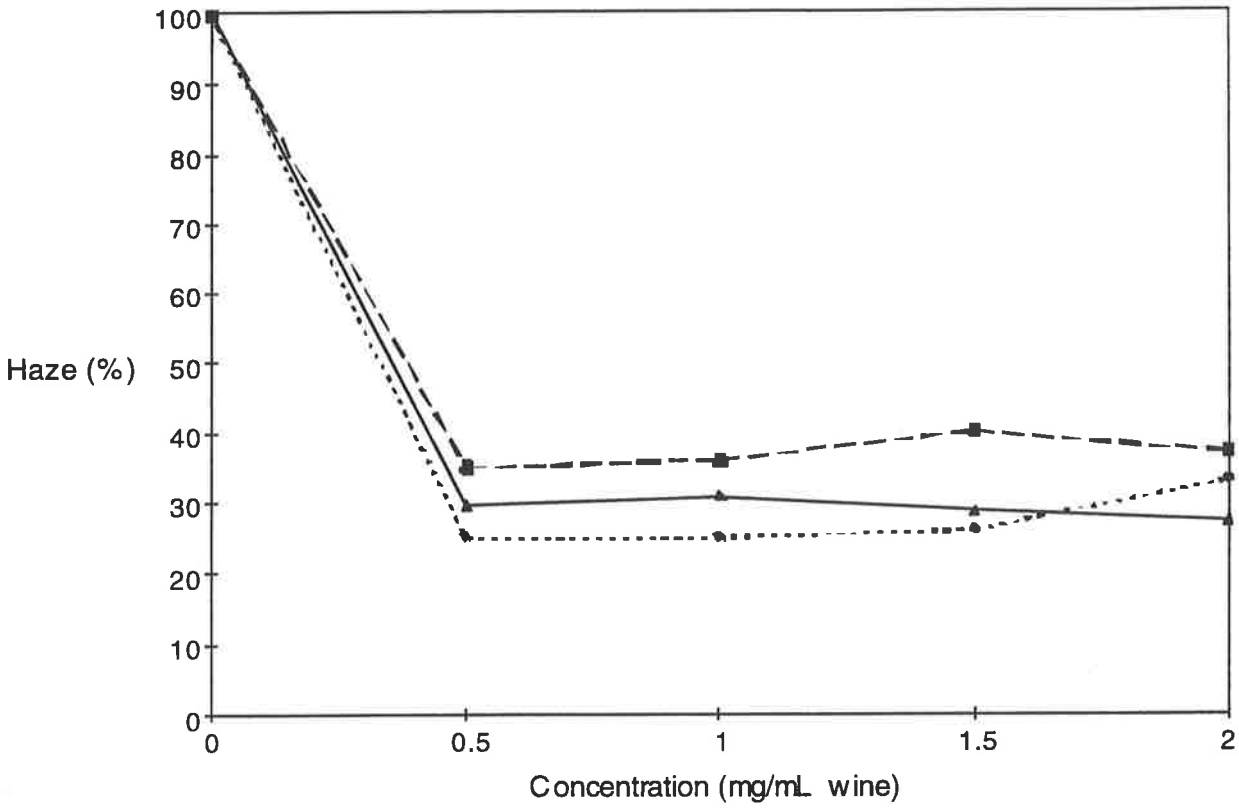


Figure 5.1 The effect of increasing concentration of extracellular material released from Maurivin PDM cells grown on glucose to exponential phase and recovered by ultrafiltration (full line) or to stationary phase and recovered by either ultrafiltration (dashed line) or ethanol precipitation (dotted line) on the heat-induced protein haze in wine. Measurement of the haze was carried out as described in Section 5.2.8. Values are the means of at least two independent experiments. The standard deviation for all values was lower than 5% (not shown on the graph).

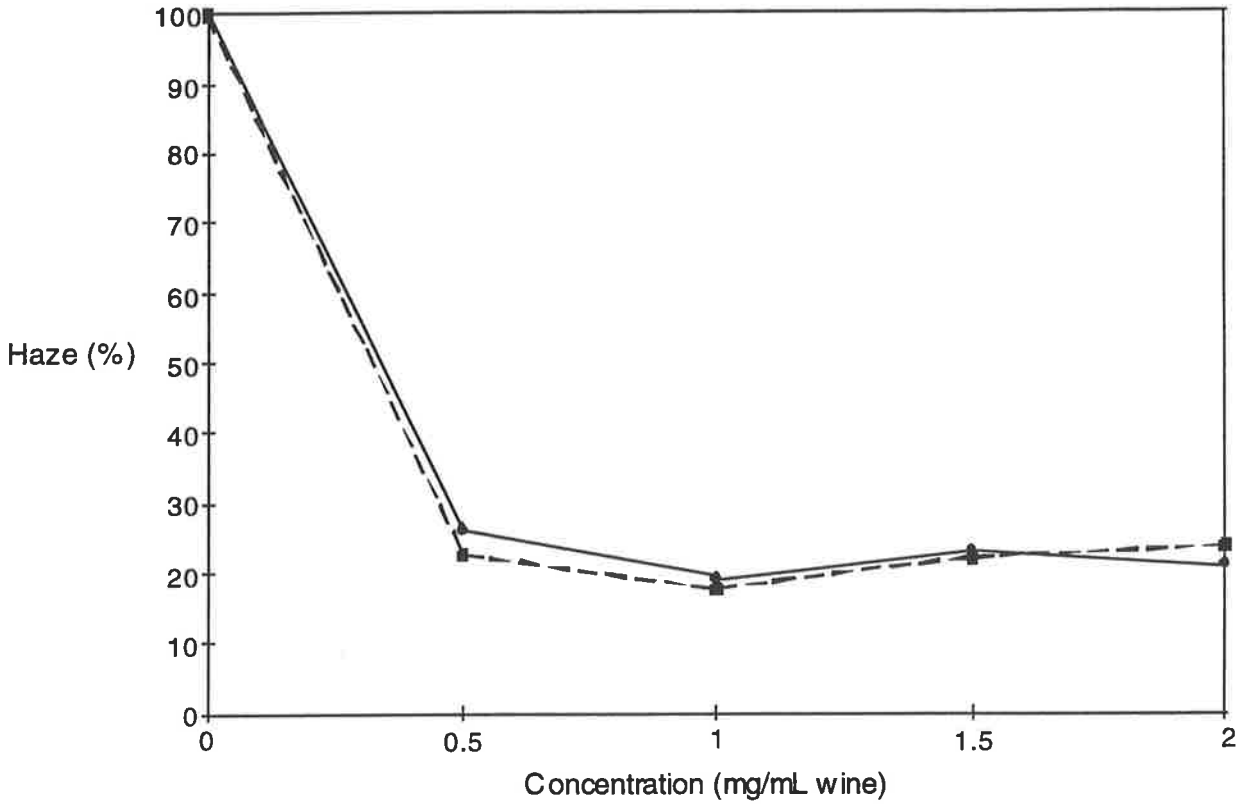


Figure 5.2 The effect of increasing concentration of extracellular material released from Maurivin PDM cells grown on mannose to exponential (full line) or stationary (dashed line) phase on the heat-induced protein haze in wine. Measurement of the haze was carried out as described in Section 5.2.8. Values are the means of at least two independent experiments. The standard deviation for all values was lower than 5% (not shown on the graph).

5.3.2 RELEASE OF HPM BY MAURIVIN PDM CELLS IN THE LARGE SCALE AGITATED 25°C FERMENTATION OR IN THE NON-AGITATED 18°C FERMENTATION

The data given in Section 5.3.1 (from the fermentation trials conducted at 25°C with agitation) showed that HPM was present in the culture supernatants of cells in late exponential or stationary phase. These fermentation trials were not designed to determine whether HPM was released continuously during both exponential and stationary phase or at a specific point during the growth cycle. Therefore two other fermentations were carried out where sampling of growth culture was done through the whole growth cycle. One experiment was conducted under the conditions used so far (25°C, fermentation vessel agitated) but on a large scale (20 L instead of 10 L, see Section 5.2.3). The second fermentation was conducted in an environment simulating the winemaking conditions (18°C, no agitation of the fermentation vessel, see Section 5.2.4). In addition, the release of mannoproteins into the culture supernatant was monitored by measuring the amounts of polymeric mannose released into the culture supernatant (see Section 5.2.7).

5.3.2.1 Monitoring of the release of polymeric mannose and glucose during the large scale agitated 25°C fermentation

For the large scale agitated 25°C fermentation, both the amounts of polymeric mannose and glucose released into the culture supernatant as well as total and viable cell counts were performed (see Sections 5.2.6). The data are summarised in Figure 5.3 and discussed in this Section.

According to the total number of cells (Figure 5.3), the different phases of Maurivin PDM growth cycle could be defined as follows. After the adaptation phase (from zero to around eight hours after inoculation), the cells started to grow exponentially (budding cells, exponential phase) for 14 hours until about 22 hours of incubation. The late exponential which is the second half of the exponential phase started after approximately 15 hours and finished at about 22 hour incubation. After this, the cells entered the transition period from exponential to stationary phase (maturation period). About 40 hours after inoculation, the cells were in stationary phase (non-budding cells) and then entered the decline phase (approximately 83 hours after inoculation).

During the adaptation phase and the first part of exponential growth, the quantity of polymeric mannose as well as polymeric glucose seemed to increase. If the amount of polymeric sugars released as a percentage of the cell biomass was plotted against time (Figure 5.4), the yield of polymeric sugar released during the adaptation phase was strongly emphasised. This increase of polymeric sugars released could be an indirect consequence of the adaptation to osmotic stress (Blomberg and Adler 1992). In the last propagation culture, the cells were in a medium depleted in monomer sugars (part of the glucose was already metabolised). When the cells were transferred to the final growth medium higher in monomer sugars, the cells had to adapt to the new conditions of sugar concentration. The cell shrinkage due to the loss of intracellular water might have induced a loss of macromolecules from the cell or cell wall. That could explain the high release of polymeric glucose and mannose observed during the adaptation phase.

Sugar measurements on samples from the late exponential phase revealed a decrease in the polymeric mannose and glucose contents of the culture. A possible explanation for this decrease could be that the polymeric sugars initially released into the culture were degraded into monomers during the subsequent growth. It is known that a controlled hydrolysis of the walls occurs during cell budding since the wall of the mother cells has to be softened-up locally to allow the emergence of the bud (Phaff 1977, see Fleet 1991). In addition, the total extracellular ß(1-3) glucanase activity increases during *Saccharomyces cerevisiae* budding period and is directly involved in the hydrolysis of the cell wall glucan (Cortat *et al.* 1972). Hien and Fleet (1983) showed that a set of six ß (1-3) glucanases (exo- and endo-glucanases) were produced only in exponential growth of cells. Therefore polymeric glucose present in the culture might be partly hydrolysed during budding by the ß(1-3) glucanases. This suggestion would explain the decrease of the polymeric glucose in exponential phase.

Although the reports about the presence of mannanases (which degrade mannan) in yeast are inconclusive (Fleet 1991), the decrease of the polymeric mannose content in the exponential phase could suggest that mannanases were also released during cell budding. A close examination of the data of Biely *et al.* (1974) revealed a similar trend as the amount of mannans released in the first hours of the exponential growth of *Saccharomyces* yeast did not regularly follow the cell multiplication pattern and were lower than expected.

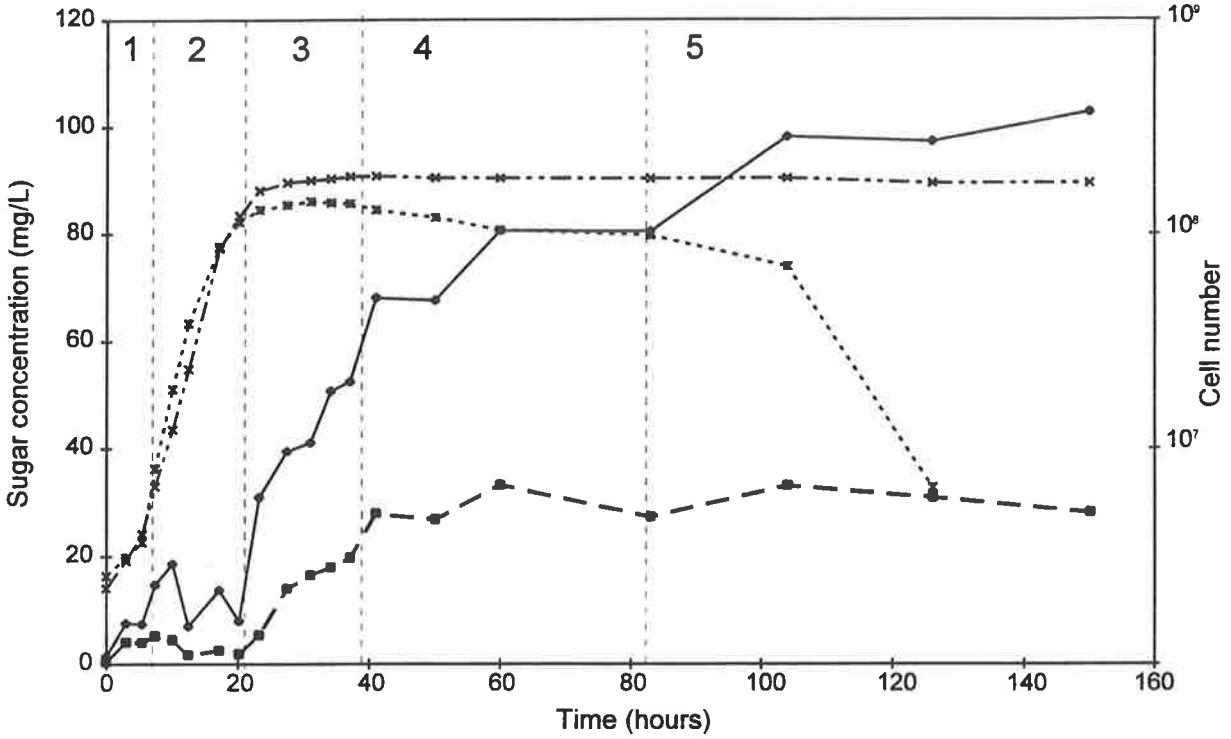


Figure 5.3 Evolution of the polymeric mannose (full line) and glucose (dash line) contents in the culture supernatant over the growth cycle of Maurivin PDM cells. Sugar measurements were carried out as described in Section 5.2.7. The values are the mean of at least three independent experiments. Total cell number (—·—·—) or viable cell number (·····) were determined as described in Section 5.2.6.

1: adaptation phase, 2: exponential phase, 3: transition phase, 4: stationary phase, 5: decline phase

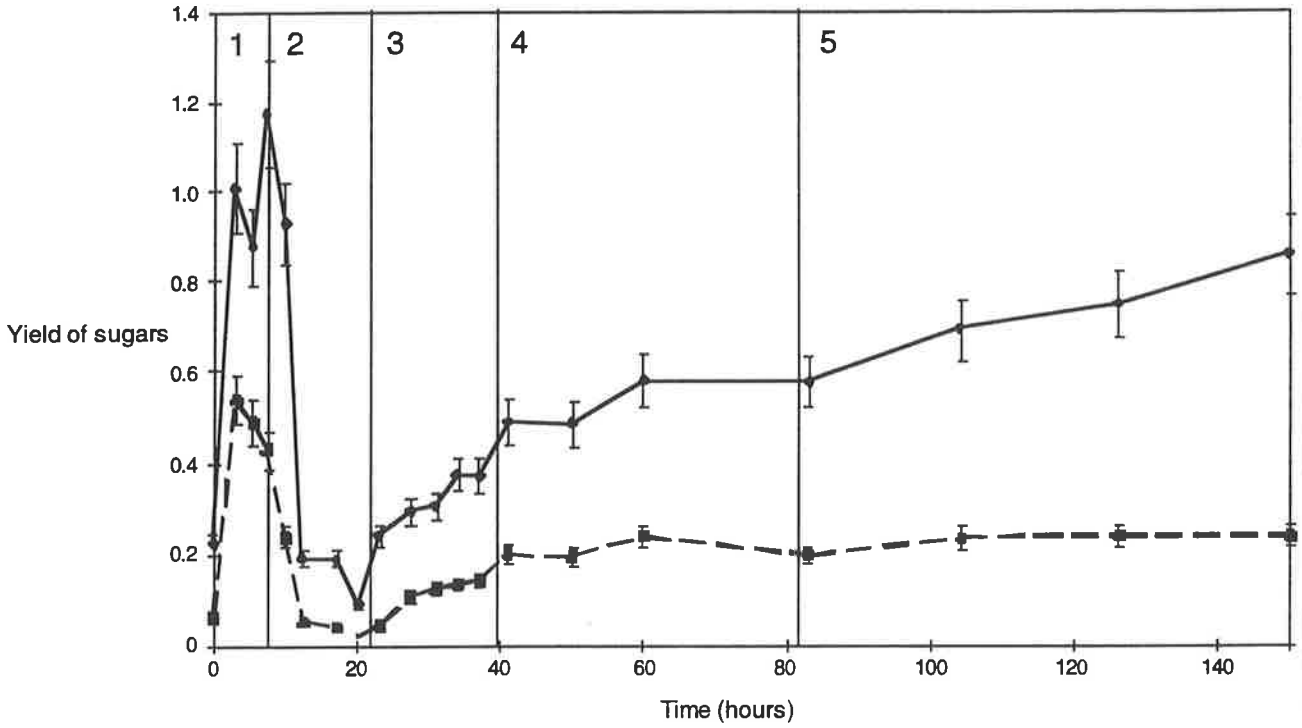


Figure 5.4 Variation of the yield of polymeric manose (full line) or glucose (dashed line) released into the culture supernatant per wet weight of yeast cells collected during the large scale agitated 25°C fermentation (sampling from 0h to 150h)

Results are expressed in % of sugars released (measured as described in Section 5.2.7) per wet weight of yeast cells collected. The vertical bars represented the standard deviation which was estimated at 10%.

1: adaptation phase ; 2: exponential phase ; 3: transition phase ; 4: stationary phase ; 5: decline phase

The polymeric sugar contents in the culture supernatant increased again during the transition from exponential to stationary phase and seemed to parallel the cell growth curve. This observation is consistent with the results of Biely *et al.* (1974) and the data in Section 5.3.1 (the release of mannoprotein material was greater for cells in stationary compared to that in late exponential phase). As suggested in Section 5.3.1, the increase of material released into the culture might be correlated to the increase of the mannoprotein content of the cell walls. Numerous authors showed that during growth and maturation (from the exponential to stationary phase), the cell walls become richer in mannoproteins which accumulate, in particular, at the external layer of the cell wall (see Section 1.5.3, Cassone *et al.* 1978, Valentin *et al.* 1987). As a direct consequence of these changes in the cell wall architecture, some mannoprotein material and, hence, some polymeric mannose might be released into the culture supernatant during the transition phase. The release of polymeric glucose was concomitant with the release of polymeric mannose and seemed to be partially correlated to it as the mannose/glucose ratio over transition and stationary phase (from 28 hours to 60 hours of incubation) ranged from 2.4 to 2.8.

However, a change occured after 83 hours of incubation as the mannose/glucose ratio steadily increased to reach 3.6 at 150 hours. The amount of polymeric glucose did not decrease over this period (values varied between 27 and 33 mg/L) whereas the amount of polymeric mannose increased from 80 to 102 mg/L. As seen in Figure 5.3, the number of viable cells decreased dramatically over this period with the subsequent increase in the number of dead cells (decline phase). Therefore the increase of polymeric mannose in the culture supernatant observed after 83 hours might be a direct consequence of cell death. At this early stage of cell death, the process of autolysis and enzymatic cell wall degradation had presumably not started (1.5.4, Charpentier and Feuillat 1993) and thus is unlikely to explain this increasing release of polymeric mannose. However, the dying or dead cells might undergo a passive release or leakage of polymeric mannose into the culture as this material was no longer needed for the cell wall building or development. The slope of the curve (Figure 5.3) indicated that this release appeared to continue beyond the cessation time of the experiment.

In this large scale agitated 25°C fermentation, the yield of polymeric mannose released in stationary phase (incubation time of about 95 hours, Figure 5.4) per wet weight of yeast cells collected was 0.6%. In the fermentation done in similar conditions but in a smaller volume (see Section 5.3.1, Table 5.1), the yield of mannoprotein material (dry weight) recovered by

ethanol precipitation was 0.74% per wet weight of yeast cells collected. The two yields are comparable although the one for the large scale fermentation (0.6%) was lower than the one for the small scale fermentation (0.74%). The conditions of the experiment described in this Section (large growth volume, large volumes of samples withdrawn, change of size and shape of the fermentation vessel) might have influenced the growth of Maurivin PDM cells and thus induced a lowering of the amount of polymeric sugars released.

5.3.2.2 Monitoring of the release of polymeric mannose during the non-agitated 18°C fermentation

Measurements of the polymeric mannose were also carried out during the fermentation conducted at 18°C without shaking. Sampling was performed regularly during the exponential growth of the yeast cells and the following stationary phase. The amount of polymeric mannose released into the culture was determined for a few samples only. The data are summarised in Table 5.2.

The polymeric mannose content measured at late exponential phase was 104 mg/L of culture supernatant and increased to 175 mg/L at mid stationary phase. Thus the transition between exponential and stationary phase was characterised by an increase of polymeric mannose present in the culture supernatant. The latter concentration measured (175 mg/L at the end of fermentation) was in agreement with the data obtained by Llaubères *et al.* (1987) who observed a released of 180 mg/L of polysaccharides by *Saccharomyces* at the same period. Similarly, Feuillat *et al.* (1989) reported the release of 270 mg/L of colloids by *Saccharomyces*, of which 60% (around 160 mg) were containing mannose.

Compared to the data in Section 5.3.2.1 (large scale agitated 25°C fermentation), the amounts of polymeric mannose measured here were significantly higher. As already suggested, the experimental conditions in Section 5.3.2.1 might account for a lowering of the amount of polymeric mannose released and would thus explain the different yields observed.

The conditions of fermentation such as temperature or agitation seemed to influence on the amount of extracellular material released into the culture supernatant. In the agitated 25°C fermentation trials (Section 5.3.1), approximately 60 mg/L of extracellular material were released into the culture supernatant for cells in late exponential phase compared to 104 mg/L

of polymeric mannose (and supposedly at least the same amount or more of extracellular material) released into the culture supernatant for the non-agitated 18°C fermentation (Table 5.2). For cells in stationary phase in the agitated 25°C fermentation trials, approximately 145 mg/L of extracellular material were released into the culture supernatant whereas 175 mg/L of polymeric mannose were measured for the non-agitated 18°C fermentation. Therefore, more extracellular material was released into the culture supernatant of a fermentation conducted at a lower temperature and without agitation. Llaubères *et al.* (1987) showed that the temperature during the alcoholic fermentation did not influence on the amounts of material released into the culture, therefore the same amount of extracellular material could be expected for the fermentations conducted here at 18°C or 25°C. The differences in yields observed here could be due to differences in fermentation duration. At 25°C with agitation, the fermentation was finished in less than 2 days whereas for the fermentation at 18°C without agitation, the fermentation lasted 6 days. Thus more material could have been released as the time of fermentation lengthened.

Table 5.2 Evolution of the concentration of polymeric mannose in the culture supernatant, and the haze protective activity of the extracellular material released at different stages of fermentation.

Fermentation stage	Polymeric mannose in the culture supernatant (mg/L)*	Haze obtained for the heat test (% of the ihv)**
Mid exponential	nd	80
Late exponential	104	40
Mid stationary	175	30

* Measurements of the polymeric mannose were performed on freeze-dried extracellular material obtained after ultrafiltration (see Section 5.2.4). Hydrolysis and enzymatic assays for the sugar measurement are described in Section 2.3.7.

** Heat tests were carried out as described in Section 5.2.8. The results herein are those obtained with a sample concentration of 0.5 mg/mL of wine in the heat test.

nd not determined

5.3.2.3 Release of HPM during the different growth phases for the large scale agitated 25°C fermentation or for the non-agitated 18°C fermentation

Different samples collected during the growth cycle of the large scale agitated 25°C fermentation were heat tested. None of the samples tested showed any haze protective ability as the haze values were all above the ihv (100%, Figure 5.5). These results are not consistent with those presented in Section 5.3.1 where a significant haze decrease was observed for culture supernatants collected for cells in late exponential and stationary phase.

A possible explanation for this discrepancy could be the difference in preparation of the samples. For the large scale agitated 25°C fermentation, samples were dialysed (see Section 5.2.3) whereas those of the agitated 25°C fermentation trials (see Section 5.2.2) were ultrafiltered. The process of desalting by dialysis, as already suggested in Sections 4.3.1 and 4.3.2.1, might have induced a loss of mannoprotein material (and hence HPM) and led to the absence of haze protective ability for the extracellular material tested.

The appearance of the samples after freeze-drying from the large scale agitated 25°C fermentation was also different to that of the ones of Section 5.3.1 (agitated 25°C fermentation trials). For the latter experiment the extracellular materials released by cells in late exponential phase were consistently of fluffy appearance after freeze-drying (characteristic of the presence of mannoproteins) whereas the ones of the large scale agitated 25°C fermentation appeared gummy (samples from 0 hours to 17 hours) and only started to be be partially fluffy at the end of the late exponential phase (sample 20 hours).

The change in the appearance of the samples of the large scale 25°C fermentation correlated well with the general trend of the heat test results (Figure 5.5). Samples from the adaptation phase (6 hours) or early exponential phase (10 hours) induced a strong haze whereas the haze progressively decreased for the samples of the late exponential (20 hours), transition (31 hours) or stationary phase (41 hours). Although the heat test results were all negative, the gradual haze decrease suggests that more and more HPM was present in the culture supernatant meaning that more and more HPM was released during maturation. This was consistent with the increasing release of polymeric mannose observed for the same period.

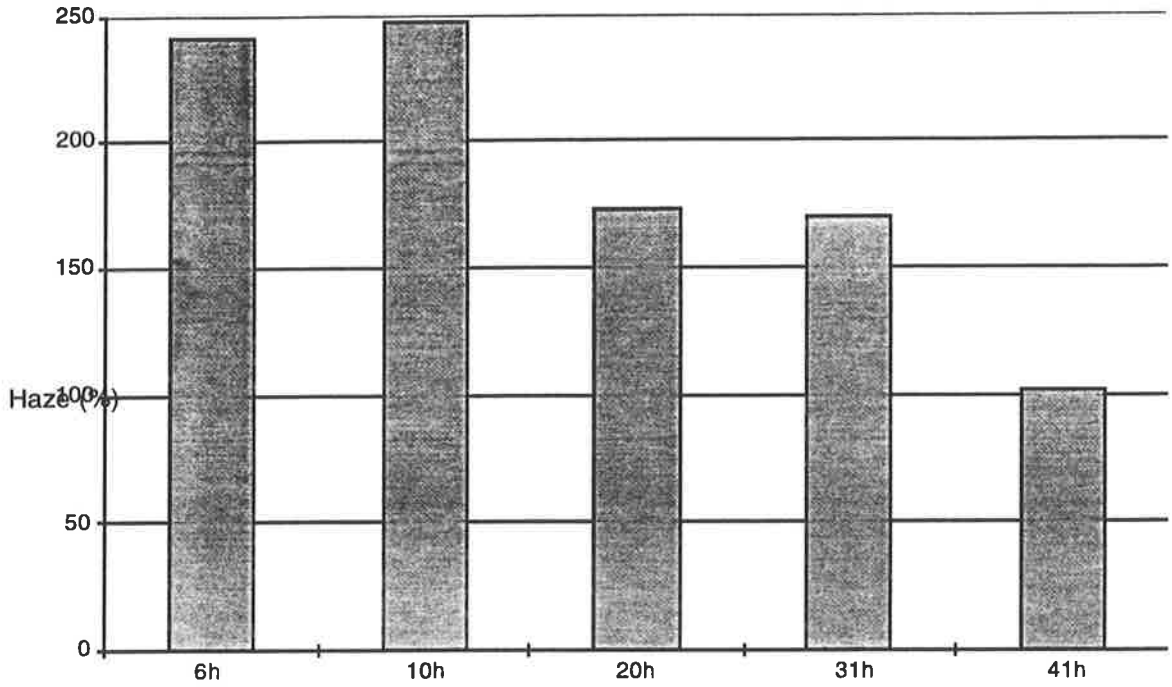


Figure 5.5 Trend of the production of haze observed for extracellular materials taken through the agitated 25°C fermentation at 6 hours (6h), 10 hours (10h), 20 hours (20h), 31 hours (31h) and 41 hours (41h).

Haze was measured with the heat test for a sample concentration of 1.5 mg/mL as described in Section 5.2.8. The other concentrations of samples tested showed a similar trend.

For the non-agitated 18°C fermentation, the heat test of the extracellular material collected at mid exponential phase did not give a positive heat test (haze decrease of only 80% of the ihv) (Table 5.2). However, heat tests of extracellular material released at late exponential or stationary phase revealed the presence of HPM as the haze values went down to 40% and 30% of the ihv, respectively. That means that HPM was not present in the culture supernatant in the early exponential phase and that it was released during cell growth. Thus these results were in good agreement with those of the agitated 25°C fermentation (see Section 5.3.1) where significant haze decrease was observed for extracellular material released by cells in late exponential or stationary phase.

The mannoprotein content of the cell wall increased during cell maturation (see Section 1.5.3 and 3.3.1) and it is known that mannoproteins accumulate on the external layer of the cell wall (Valentin *et al.* 1987). Thus the increasing release of HPM might be connected to these changes occuring in the cell wall. As more mannoproteins were integrated in the cell wall, more HPF was released into the culture.

Since data collected in Chapters 3 and 4 suggests that HPF is only anchored to the cell wall by non-covalent links, it could be assumed that during its integration in the cell wall, excess HPF or HPF not properly anchored to the cell wall was released into the culture. This would suggest that the process of HPF release is proceeding in a passive way and could not be considered as a process of active secretion.

5.3.3 RELEASE OF HPM BY MAURIVIN PDM CELLS DURING STORAGE ON YEAST LEES

To measure the effects of storage on yeast lees, cells were harvested after the non-agitated fermentation at 18°C and resuspended in a medium free of mannoproteins and polymeric sugars. Therefore the amounts of polymeric mannose measured and the results of the heat tests performed were related to the extracellular material released exclusively during storage on yeast lees. The data are presented in Table 5.3.

During storage on yeast lees, polymeric mannose was progressively released into the culture supernatant to reach a concentration of 15 mg/L after 8 weeks. In parallel, the heat tests revealed that the extracellular material released had increasing haze protective ability as a greater haze decrease was observed over time. The first samples tested (from 0 to 6 weeks) did not cause a haze decrease lower than 70% of the ihv whereas the last one decreased the haze to 50% of the ihv.

These observations suggested that HPM was present in the cell wall of resting and/or dead cells, and was progressively released into the culture. After cell death, the process of autolysis is responsible for a gradual enzymatic cell wall degradation (see Section 1.5.4). After 8 weeks on yeast lees at 18°C, the process of autolysis might have just begun and could explain the release of HPM observed at this stage.

Table 5.3 Evolution of the concentration of polymeric mannose released in the culture supernatant, and the haze protective ability of the extracellular material during storage on Maurivin PDM yeast lees.

Time on yeast lees (weeks)	Polymeric mannose in the culture supernatant (mg/L)*	Haze obtained for the heat test (% of the ihv)**
0	0	100
2	4	90
4	10	80
6	11	70
8	15	50

* Measurements of the polymeric mannose were performed on freeze-dried extracellular material obtained after ultrafiltration (see Section 5.2.5). Hydrolysis and enzymatic assays for the sugar measurements are described in Section 2.2.7.

** Heat tests were carried out as described in Section 5.2.8. The results herein are those obtained with an extract concentration of 0.5 mg/mL of wine in the heat test.

5.4 CONCLUSION

Material showing high haze protective ability was isolated from the culture of Maurivin PDM cells by ultrafitration or ethanol precipitation. Neither the ultrafiltered nor the precipitated material needed further purification on Con-A to give satisfactory heat test results. This was in contrast to the extractions on whole cells or cell walls described in previous Chapters where it was necessary to enrich the extracts in mannoproteins to detect HPM. The haze decrease obtained with the extracellular material isolated from the culture supernatant was comparable to those obtained with SDS or full zymolyase treatments (see Section 3.3.2). Thus collection of HPM from the culture supernatant, especially in stationary phase where more extracellular material was released, could be the most suitable method for a scaled-up production of haze protective factor (HPF). The use of cells grown on mannose instead of glucose resulted in an

increased yield of HPM. Nevertheless, the yields of mannoproteins from the fermentation were still relatively low (less than 20 g per kg of wet cells).

HPM was not detected in the culture supernatants of fermentations conducted under incubation conditions of either 25°C with agitation or 18°C without agitation in the first half of the exponential cell growth but was detected during the late exponential phase. The amount of extracellular HPM released in the early exponential phase might have been too low to be detected by the heat test procedure. For this, a more sensitive method of detection will be needed. The release of extracellular HPM into the culture during the cell growth might be associated with the cell wall synthesis of the budding cells. As the amount of HPM released into the culture increased during the transition period between the exponential and stationary phases, the release of extracellular HPM could also be related to cell wall maturation.

As HPF is loosely associated with the cell wall (HPM was extractable by boiling cells or with SDS or EDTA treatments which implied a release of the non-covalently linked material only, see Section 3.4 and 4.4), it could be easily detachable from the wall and thus released into the culture. The presence of other high M_r cell wall mannoproteins in the culture such as α-agglutinins was also been reported (Sijmons *et al.* 1987) even though these mannoproteins which are involved in sexual mating are classified as cell wall mannoproteins. The ease of release of extracellular HPM also suggests that HPF like α-agglutinins might be located on the wall surface.

The data described here do not allow us to clearly determine if extracellular HPM was released during stationary phase, since extracellular HPM released during exponential phase was already present in the culture supernatant at stationary phase. However, as HPM was also found to be present in the cell wall of cells in stationary phase (see Sections 3.3.2 and 4.3.3), it was most probably released from living cells at this stage as well.

The release of HPM was observed from dead cells during storage on lees. This was attributed to the autolytic cell wall degradation of dead cells. Therefore enrichment of extracellular HPM in the wine would occur during both fermentation and storage on yeast lees.

Further studies will be needed to determine if the material responsible for the haze decrease that was isolated from the growth culture (this Chapter) was the same as that being extracted

from the cell wall (Chapters 3 and 4). This information will clarify the mechanism involved in the release of extracellular HPM (release with or without hydrolytic modifications) and determine the function, if any, of such a release.

Chapter 6

IMMUNOLOCALISATION OF HPF IN THE CELL WALL OF *SACCHAROMYCES CEREVISIAE* MAURIVIN PRISE DE MOUSSE

6.1 INTRODUCTION AND LITERATURE REVIEW

Due to their external distribution on the yeast cell wall (see Section 1.5.1.4), mannoproteins are directly involved in the immunogenic response to yeast cell in vertebrates. An antiserum raised against dead whole yeast cells contains antibodies directed against the mannan components (Ballou 1970, Suzuki *et al.* 1968).

The most common antigenic determinant (specific site on an antigen that reacts with an antibody raised against it) for these mannoproteins are the mannose side chains of the outer core (Figure 6.1). The terminal mannose units of the side chains such as α(1-3)-D-mannopyranosyl are the most exposed to the surrounding medium and thus the most involved in the immunogenic response among wild *Saccharomyces cerevisiae* strains (Ballou and Raschke 1974, Ballou 1976). Other groups such as (1-2)-linked or (1-6)-linked mannose units are less immunogenic because of their more internal position. That means that antibodies raised against mannoproteins are mainly directed against the α(1-3)-D-mannopyranosyl and not the (1-2)- or (1-6)-linked mannose units.

Since mannoproteins have the same basic sugar moiety structure as depicted in Figure 6.1, cross-reactivity is expected between different mannoproteins (Ballou, 1976). Antibodies directed against the mannan part of one mannoprotein can recognise and interact with the mannan part of another mannoprotein. However, since the protein part of different mannoproteins is unique, antibodies raised against the protein part are far more specific and cross-reactivity is not expected.

The use of polyclonal or monoclonal antibodies raised against a specific yeast mannoprotein is a powerful tool to further detect this mannoprotein during its synthesis or at different stages of yeast cell growth. For instance, Watzele *et al.* (1988) followed the expression of a 22 k α-agglutinin during cell growth and showed that the presence of this mannoprotein on the growing bud was inducible. Similarly, Marcilla *et al.* (1993) assessed the role of two types of mannoproteins in wall formation of *Candida albicans* by using antibodies raised against them. Sanz *et al.* (1987) detected different forms of a 33 k mannoprotein in *Saccharomyces cerevisiae* by raising antibodies against the protein moiety isolated from the mannoprotein.

Cytochemical techniques using polyclonal or monoclonal antibodies as labels together with detection tools like transmission or scanning electron microscopy can help to visualise particular cell wall mannoproteins in the different compartments of the yeast cell and hence assess their distribution or secretion pathways. Elorza *et al.* (1993) showed, by immunodetection, that some mannoproteins were randomly distributed through the different *Candida albicans* cell wall layers. For the same yeast, Molinari *et al.* (1993) were able to visualise the incorporation of mannoproteins in the inner layers of the cell wall (periplasmic space) and their final export to the outermost layer. Similarly in *Candida albicans*, preferential secretion of mannoproteins from the periplasmic space into the medium was detected by Poulain *et al.* (1989).

In regard to the research work cited above, the localisation of a specific mannoprotein in the *Saccharomyces cerevisiae* cell wall seemed to be feasible. The localisation in the yeast cell wall of the haze protective factor (HPF) was studied by combining immunochemistry and cytochemical techniques. The results of this investigation are presented in this Chapter.

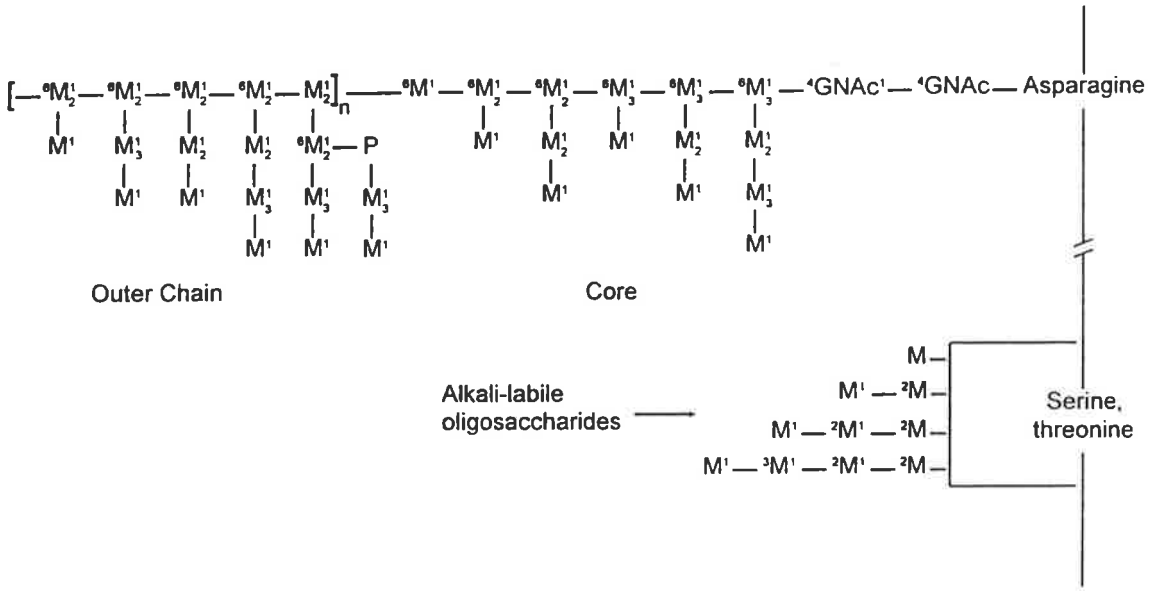


Figure 6.1 Detailed structure of mannoprotein from *Saccharomyces cerevisiae* (from Ballou 1982 and Fleet 1991). M, mannose; GNAc, *N*-acetylglucosamine; P, phosphorus. All linkages are of α configuration except for (1-4) which is of ß configuration. n= 10-15. This figure is the same as Figure 1.1.

6.2 MATERIAL AND METHODS

6.2.1 MATERIAL

Saccharomyces cerevisiae mannans [prepared by the Cetalvon method (Barker *et al*, 1957)], agarose (Type II: medium EEO) and protein-A beads were provided by Sigma Chemical Company (MO, USA). Glycine (Anal R grade) was sourced from Merck KGA (Germany). Calcium lactate, sodium azide and absolute ethanol were purchased from Ajax Chemicals (NSW, Australia). Filter paper (N°1) was from Whatman (W&R Balson Ltd, Great Britain). Nitrocellulose membrane (pore size: 0.45 μm) was provided by Schleicher & Schuell (Dassel, Germany). LR White Resin was from Probing & Structure (QLD, Australia). Autoprobe EM protein A G10 (colloidal gold: 10 nm mean diameter) was provided by Amersham International (Great Britain). Parafilm was sourced from American Can Company (CT, USA).

BioRad Immuno-Blot Assay Kit was purchased from BioRad Laboratories Pty Ltd (NSW, Australia). The kit contained Tris-buffered saline (x 10, pH 7.5), gelatine (blotting grade), Tween-20 (blotting grade), the second antibody conjugate solution [alkaline phosphatase (AP) conjugated to goat anti-rabbit IgG], AP colour reagent A containing nitroblue tetrazolium (NBT) and magnesium chloride in aqueous dimethylformamide, AP colour reagent B containing 5-bromo-4-chloro-3-indoyl phosphate (BCIP) in dimethylformamide and AP colour development buffer (x 25).

Other materials were as described in Section 2.1.

6.2.2 SOURCE OF HAZE PROTECTIVE FACTOR (HPF)

Purified HPF from red wine (Waters *et al*. 1994) was donated by Dr Elizabeth Waters (AWRI, Australia).

6.2.3 PRODUCTION OF POLYCLONAL ANTIBODIES

6.2.3.1 Immunisation and test procedure

Antibodies against purifed HPF were obtained from New Zealand White rabbits. HPF (80 μg) in sterile saline solution (1 mL) was combined with 0.5 mL of PBS (0.15 M NaCl, 0.1 M phosphate, pH 7.2). The immunogen solution (1.5 mL) was mixed at 1:1 dilution with complete Freund's adjuvant. The water in oil emulsion was injected intramuscularly at two separate sites. After 3 weeks, the same protocol was applied using incomplete Freund's adjuvant (dilution 1:1). The emulsion was administered subcutaneously at six separate sites. Four weeks after the last injection, 1 mL of the immunogen solution without adjuvant was injected intravenously. One week later, the rabbit was test bled and the serum separated to check the specificity of the antibodies produced and to detect any cross-reactivity. As the test procedure was positive, the rabbit was sacrified and the serum collected was re-tested for specificity and cross-reactivity. The serum was stored in 10 mL aliquots at -20°C before use.

6.2.3.2 Partial immunoglobulin fractionation

Part of the rabbit serum collected was loaded on a protein-A column according to the procedure of Ey *et al.* (1978). This process is known to partially separate the immunoglobulin G fraction (IgG) from the remaining immunoglobulins M and A.

The serum was adjusted to pH 8 by adding 1/10 volume of Tris HCl (1 M, pH 8) and loaded on a protein A bead column. The column was successively washed with 10 column volumes of Tris HCl (100 mM, pH 8) and 10 column volumes of Tris HCl (10 mM, pH 8). The bound material was eluted with glycine buffer (100 mM, pH 3) and the pH of the fractions collected was adjusted to neutral pH. The fractions containing most of the IgGs were detected by reading the absorbance at 280 nm. The IgG fraction was stored in 1 mL aliquots at -20°C before use.

6.2.4 TEST OF IMMUNOSPECIFICITY AND CROSS-REACTIVITY BY OUCHTERLONY'S IMMUNODIFFUSION ASSAY

A gel double diffusion assay was performed according to Ouchterlony (1949). HPF plus some potential cross-reacting antigens (mannans, invertase, BSA) were placed on wells cut into a horizontal 1% (w/v) agarose gel. Samples on the gel diffusion plate were placed as follows: the antiserum was in the centre well whereas the antigenic solutions to be tested were in separated wells of the outer ring. The test at the stage of pre-bleeding was performed for rabbit antiserum at 1:5 dilution. HPF and the potential cross-reacting antigens tested were used in concentration of around 1mg/mL. The plate was left overnight at room temperature before being interpreted. The test carried out after the final bleeding was performed as above except that the dilution of the antiserum used was 1:2.

6.2.5 TEST OF IMMUNOSPECIFICITY AND CROSS-REACTIVITY BY ELECTROPHORESIS IN AGAROSE GELS AND IMMUNOBLOTTING

Gels containing 1.8% or 1.4% of agarose were used in this study and run as horizontal submerged slabs.

6.2.5.1 Gel polymerisation

The agarose gel solution consisted of 0.97 M Tris, 0.28 M glycine, 58 mM calcium lactate, 0.01% (w/v) SDS with the pH adjusted to 8.6. Agarose [1.4% or 1.8 % (w/v)] was added to the solution (20 mL) and dissolved by heating. The solution was then poured into the gel casting, and an eight well comb was placed in the top of the gel. The gels were allowed to polymerise for 30 min. The gel dimensions were 95 x 75 x 2 mm.

6.2.5.2 Sample preparation

Samples were diluted in sample buffer and then loaded into the wells. The amount of sample loaded was 10 µg. Sample buffer was prepared by combining water (400 µL), Tris-glycine calcium lactate buffer [same concentration as for the gel polymerisation buffer (see Section 6.2.5.1), pH 8.6, 150 µL], glycerol (100 µL) and 0.2% (w/v) bromophenol blue (50 µL).

6.2.5.3 Electrophoretic conditions

Gels were run at a constant current of 70 mA until the bromophenol tracker dye was 5 mm from the bottom of the gel. The running time was around 4 hours for both 1.8% and 1.4% agarose gels. The running buffer contained Tris HCl (6 g), glycine (28.8 g), SDS (1 g) per litre.

6.2.5.4 Transfer to nitrocellulose membrane (immunoblotting)

Transfer of the material which migrated on the agarose gel to a nitrocellulose membrane was done using BioRad Mini Trans Blot Electrophoretic Transfer Cell following the manufacturer's instructions and as described here.

After electrophoresis the agarose gel was incubated in a fixative (methanol/acetic acid/water 4:1:5) for a few minutes and then equilibrated in transfer buffer for 60 min. The transfer buffer consisted of 25 mM Tris and 192 mM glycine. Fibre pads (supplied with Biorad mini Trans Blot cell), filter paper and nitrocellulose membrane were soaked in transfer buffer for 30 min. A gel sandwich was constructed by placing a pre-soaked fibre pad on one panel of the gel holder cassette. A piece of saturated filter paper was placed on top of the fibre pad and further saturated with transfer buffer (3 mL). The equilibrated gel was placed on top of the paper and aligned in the centre of the cassette. The surface of the gel was flooded with transfer buffer (3 mL), and the pre-soaked nitrocellulose membrane was placed onto the gel surface. Any bubbles were removed by rolling a test tube over the top of the membrane. The membrane was flooded with transfer buffer (3 mL) and then the sandwich completed by placing a piece of saturated filter paper on top of the membrane and a saturated fibre pad on top of the filter paper. The cassette was closed and placed in the buffer tank. The tank was filled with chilled transfer buffer and the blotting was achieved at constant voltage of 100 V for 60 min. The heat generated during transfer was absorbed by a block of ice contained in the BioIce (as supplied by BioRad).

6.2.5.5 Immunodetection on the nitrocellulose membrane

After completion of the transfer to nitrocellulose, the membrane was immunologically tested using the Bio Rad Immuno Blot Assay Kit. The principle of the immunodetection is given in Figure 6.2. All steps were performed with the nitrocellulose membrane on a shaking table at room temperature except during the colour development procedure. Products mentioned below were supplied with the Bio Rad Kit (see Section 6.1). All incubation and washing steps were performed in a 5 mL solution for a membrane strip of 7.5 x 95 mm.

The membrane was incubated in a blocking solution for 60 min to cover any sites on the membrane that did not contain transferred material. The blocking solution was prepared by dissolving gelatine (3 g) in 100 mL Tris-buffered saline (TBS, 20 mM Tris, 500 mM NaCl, pH 7.5) and heating it to 50°C in a microwave oven. The membrane was washed for 10 min with a TBS solution containing Tween-20 [0.05% (v/v)] (TTBS) to remove excess blocking protein. The strip was incubated in the primary antibody solution [rabbit antiserum diluted to 1:500 or 1:1000 in antibody buffer (TTBS, 1% (w/v) gelatine)] for three hours. The strip was washed in TTBS (5 min) and in TBS (5 min) to remove the unbound primary antibodies and then incubated with the conjugate antibody solution (33 µL conjugate solution dissolved in 100 mL antibody buffer) for two hours. The strip was washed in TTBS (x 2, 5 min each time) andin TBS (x 1, 5 min) prior to colour development to remove residual Tween-20 from the membrane.

The strip was then immersed in the colour developer solution [1mL colour reagent A and 1 mL colour reagent B in 100 mL Alkaline Phosphatase (AP) colour development buffer [4 mL AP development buffer (x 25) diluted in 96 mL water]. The binding of the enzyme AP to the secondary antibodies became visible through the development of purple bands on the membrane. The reaction was stopped by immersing the membrane in water for 10 min. In the absence of primary antibodies on the membrane, the binding of the secondary antibodies did not take place and no band was observed on the membrane.

6.2.6 HIGH PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHY

Molecular size distribution of purified HPF after storage was assessed as described by Waters *et al.* (1994). Two serial Shodex OHpak KB-803 and KB-805 columns (Showa Denko, Japan, 0.8 x 30 cm) with an OHpak KB-800P guard column (0.6 x 5 cm) equilibrated in $LiNO_3$ were used at room temperature. HPF (20 μg) purified from red wine (Waters *et al.* 1994) was loaded and the eluant was monitored with a Waters 410 differential refractometer and a Waters 440 absorbance detector (A280 nm, Waters Millipore, MA, USA) in combination with Maxima 820 software (Waters Millipore, MA, USA). The molecular weights of components in the sample were estimated with a calibration curve of the two serial columns established at room temperautre using a pullulan calibration kit (Showa Denko, Japan).

6.2.7 DIRECT AGGLUTINATION ASSAY

Table 6.1 Protocol for the agglutination assay

	Volume added (μL)				
	Antiserum	Preimmune serum	PBS	2-ME (0.1 M)	Yeast suspension [0.5%(v/v)]
Row A, test antiserum	50	-	65	-	50
Row B, test IgG agglutination	50	-	50	15	50
Row C, preimmune serum control	-	50	65	-	50
Row D, serum-free control	-	-	115	-	50

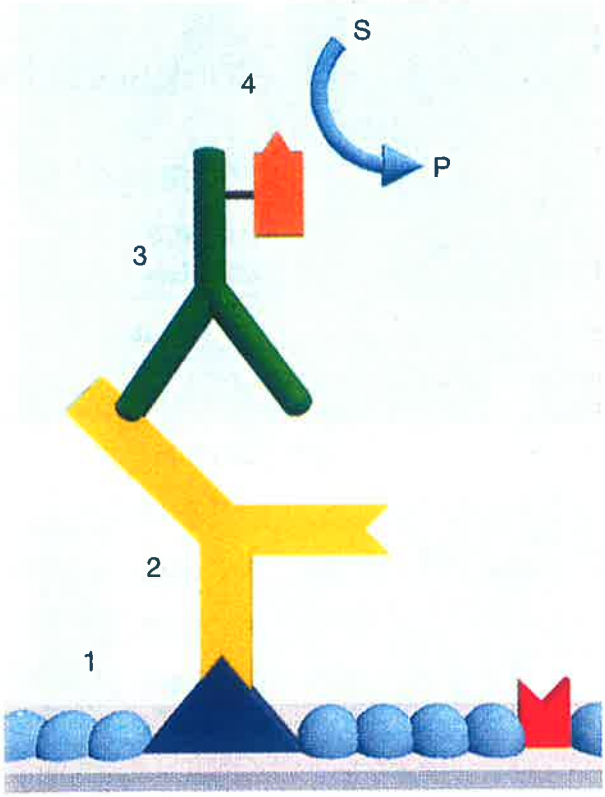


Figure 6.2 Description of the immunodetection of antigens bound to a nitrocellulose membrane (reproduced from BioRad, 1997).

1: the gelatine contained in the blocking solution occupies the empty sites on the membrane;

2: the primary antibodies (anti-HPF rabbit antibodies) recognise and bind the antigen (HPF);

3: the secondary conjugate antibodies (anti-rabbit goat antibodies linked to alkaline phosphatase) recognise and bind the primary rabbit antibodies;

4: the enzyme alkaline phosphatase catalyses the reaction in which the substrate (S) forms a coloured precipitate (P) at the site of the antigen-antibody complex.

Fresh Maurivin PDM yeast cells grown on glucose enriched medium to late exponential phase (refer to Section 4.2.3 for yeast growth) were harvested and washed (x 3) in phosphate-buffered saline (PBS ; 0.15 M NaCl, 0.15 M phosphate, pH 7.2). Cells [0.5% (v/v)] were resuspended in PBS.

The agglutination assay was carried out in a flat bottom microtitration plate according to the protocol in Table 6.1. The serum was added to rows A and B, and serially diluted by half along the row (50 µL, double serial dilution along the row from one well to the next one, 12 wells in total). The preimmune serum (serum before antigen injection) was serially diluted by half along the wells of row C (50 µL) and was referred to as the preimmune serum control. Row D contained only PBS (100 µL) and was referred to as the serum-free control row. 2-mercaptoethanol (2-ME, 15µL, 0.1 M) was added to row B, and a further volume of PBS (15 µL) was added to rows A, C and D. The yeast suspension (50 µL) was added to rows A, B, C and D. Each row was done in duplicate and contained a final volume of 165 µL. The microtitration plate was briefly mixed and left two hours at room temperature. The formation of macroscopic clumps was assessed with the naked eye over a white background.

6.2.8 IMMUNOELECTRON MICROSCOPY

6.2.8.1 Cell processing: fixation and embedding

Fresh Maurivin PDM yeast cells grown on glucose enriched medium to late exponential phase (refer to Section 4.2.3 for yeast growth) were harvested, washed with water (x 3) and fixed in 0.25% (v/v) glutaraldehyde in phosphate-buffered saline [PBS, 0.15 M NaCl, 6 mM phosphate (0.7 g NaH_2PO_4, 0.16 g KH_2PO_4), pH 7.2] containing 4% sucrose for 12 hours. Cells were washed in PBS containing 4% (w/v) sucrose (x 2, 30 min each time) and then dehydrated by successive washings in 70% (v/v) ethanol (x 2, 30 min each time), 90% (v/v) ethanol (x 2, 30 min each time), 95%(v/v) ethanol (x 2, 30 min each time), 100% ethanol (v/v) (x 2, 30 min each time and x 1, 60 min). Dehydrated cells were pre-embedded in 50% LR White Resin / 50% (v/v) absolute ethanol for 15 hours at 4°C and in 100% LR White Resin (x 3, 2 hours each time) at room temperature. After the third change of resin, cells were embedded in 100% LR White Resin and placed in an oven at 50°C for 24 hours, to allow the resin to polymerise.

Ultrathin resin sections (thickness around 50 nm) were cut with a Reichert Ultracut E (Reichert, Germany) at room temperature and collected on collodion coated nickel grids (diameter: 3 mm).

6.2.8.2 Immunogold labelling of ultrathin sections

During this procedure the grids were treated by floating them on top of drops (15-20 µl) of reagent dispensed onto sheets of parafilm.

The grids were placed on drops of 0.02 M glycine in PBS for 20 min, blotted onto filter paper and then floated on antibody buffer (consisting of PBS supplemented with 1% ovalbumin, 0.5% Tween-20 and 0.1% Triton-X-100) for 20 min. After blotting onto filter paper, the grids were placed on the primary antibody solution (IgG fraction diluted to 1:400, 1:600 with antibody buffer) for 15 hours at 4°C. At this stage, the negative controls were prepared as follows. To test for non-specific binding by the primary antibodies (IgG antibodies), the grids were placed onto a solution containing the preimmune serum (diluted to 1:400, 1:600 with antibody buffer) instead of IgG antibodies for 15 hours at 4°C. To test for non-specific binding by the gold probe, the grids were floated on antibody buffer in place of the primary antibody solution for 15 hours at 4°C. All sections were rinsed with 1% ovalbumin in PBS (x 6, each time 5 min) and blotted onto filter paper. The grids were then incubated on a solution of protein-A gold complex (gold probe) diluted with water to 1:50 for 60 min and rinsed with 1% ovalbumin in PBS (x 6, each time 5 min). Before staining, sections were washed with water (x 4).

For the staining procedure, sections were placed on 5% uranyl acetate (stabilised with glacial acetic acid and centrifuged before use) for 10 min and washed with water (x 4). The sections were then floated on lead citrate [1.3 g lead nitrate ($Pb(NO_3)_2$), 1.8 g sodium citrate, 8 mL 1N NaOH in 50mL water, centrifuged before use] for 5 min and washed with water (x 4).

The stained sections were examined with a Philips CM 100 Transmission Electron Microscope.

6.3 RESULTS AND DISCUSSION

HPF is a mannoprotein with an estimated M_r of 420 k. Because of its large size HPF was expected to be highly immunogenic and to induce a strong immune response when injected into the rabbit. As HPF consists of 70% of polysaccharides (97% mannose, 3% glucose) and 30% of protein (see Section 1.4.1), the antibodies generated would be directed against the mannan and/or the protein part of HPF.

6.3.1 SPECIFICITY AND CROSS-REACTIVITY OF THE POLYCLONAL ANTIBODIES

During immunisation, at the stage of pre- or final bleeding, the antiserum was tested by Ouchterlony's immunodiffusion assay to assess the specificity and cross-reactivity of the poyclonal antibodies produced. When placed into the wells of the gel (see Section 6.2.4), the antibodies of the antiserum and the antigens can diffuse freely from their wells into the gel and eventually meet each other. If the antibodies recognise and react with the antigen (reaction of identity), large immune complexes will form provided that the concentration of the antigen and antibodies are in the right ratio. As a result a continuous line of precipitation of these immune complexes will be visible between the wells. On the contrary, if the antibodies are not specific for the antigens no immune complexes are formed and thus no line of precipitation will appear between the wells.

Figure 6.3 shows the pattern obtained on the gel after immunodiffusion. Two continuous precipitation lines, typical for a reaction of identity, appeared in the gel between the well containing the antiserum and that of the antigen solution containing HPF. The presence of two lines indicated that the antibodies recognised two antigens of different M_r. If HPF was the sole component in the antigen solution, only antibodies against HPF would have been produced and only one line of precipitation would have been observed. Therefore the antigen solution injected into the rabbit contained two components.

6.3.1.1 Origin of the second component contained in the antigenic solution

Before being used for the immunisation the purified HPF had been stored as an aqueous solution at -20°C for over 12 months. The purity of this sample was analysed by size-exclusion chromatography (see Section 6.2.6). Only a single peak was detected with a M_r of 420 k as previously found by Waters *et al.* (1994; data not shown). Since no other peak was detected it was assumed that HPF had not decomposed into small M_r components during storage. Thus the second component present in the antigenic solution was not a breakdown product of HPF formed during the storage.

This second component might, however, have coeluted with HPF although after the successive steps of purification of HPF from red wine (Waters *et al.* 1994) (cation and anion-exchange chromatography, affinity chromatography and gel permeation chromatography) the final product obtained appeared to be homogeneous in size. To test this hypothesis, one sample showing satisfactory haze ability [SDS mannoprotein extract (see Chapter 3)] and hence containing HPF (due to a lack of purified HPF, it was not possible to test it by itself) were run in 1.4% agarose gel and blotted onto a nitrocellulose membrane (see Section 6.2.5). After immunostaining two fine bands of light intensity separated by only a few millimeters appeared in the first top quarter of the gel (very high M_r zone, membrane not shown). This result showed that two components of very close M_r reacted with the polyclonal antibodies and were most probably the same components as those detected with the Ouchterlony's immunodiffusion assay.

The two components could be either two different mannoproteins of similar M_r or two forms of the same mannoprotein. Due to the heterogeneity of their sugar moiety, it has been observed that mannoproteins such as invertase and acid phosphatase, migrate in a polydisperse way in an electrophoretic gel (Trimble and Maley 1977, Esmon *et al.* 1981) resulting in a pattern of several closely spaced fine bands. The two bands observed herein in agarose gels might represent two forms of HPF differing in the size of their sugar moiety. These two forms (or alternatively the two mannoproteins) could have been present in the HPF sample purified from red wine. The difference in size of the two components was presumably too small to be detected by gel permeation chromatography.

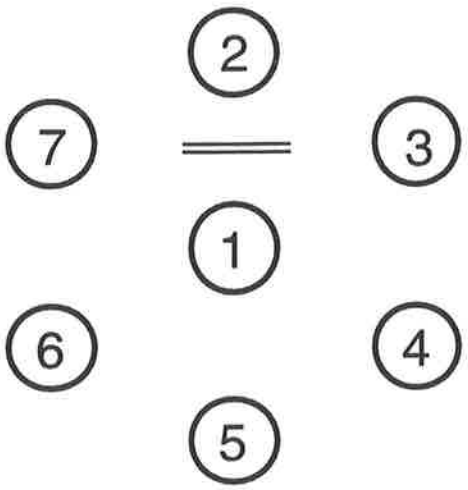


Figure 6.3 Description of the pattern of reactivity obtained between the antiserum (well 1) and HPF (well 2), yeast mannans (well 3), invertase (well 4) or BSA (well 5).
The immunodiffusion assay was carried out twice with either the antiserum diluted at 1:5 or 1:2. In both cases, the antigen concentration was around 1mg/mL and the same pattern of reactivity was observed.

The HPF purified sample from red wine had a high haze protective ability (Waters *et al.* 1994) suggesting that the two forms of HPF (or the two mannoproteins) were both active in reducing the haze. Therefore the use of the polyclonal antibody solution for further immunocytochemistry experimentation will enable the localisation of the two forms of HPF rather than HPF as a single component.

Alternatively, the introduction of a contaminant could have occured during the sample preparation for the immunisation. However, the chance that this contaminant would also be a yeast cell wall mannoprotein of similar size to HPF and present in the purified HPF sample is very low. Thus the presence of antibodies in the antiserum against a non yeast cell wall component would not distort the results for the localisation of HPF.

6.3.1.2 Cross-reactivity of the polyclonal antibodies

The cross-reactivity of the polyclonal antibodies produced was checked using the immunodiffusion technique. Antibodies raised against a specific mannoprotein can cross-react with other mannoproteins due to similar structural features of the sugar moieties (see Section 6.1 and Figure 6.1). Therefore antibodies against the mannan part of a given mannoprotein could also recognise the sugar moieties of other mannoproteins. Modena *et al.* (1986) experienced cross-reactions with antibodies raised against glucoamylase (a mannoprotein enzyme) since these antibodies also recognised invertase and mannans. Similarly, Marcilla *et al.* (1993) reported cross-reactivity between mannan and mannoproteins isolated from yeast.

Therefore potential cross-reacting components such as invertase (containing 50% mannose) or a commercial yeast cell wall mannan fraction and BSA (containing a variable but low amount of glucose) were tested with Ouchterlony's technique (Figure 6.3). No precipitation line appeared between the well containing the antiserum and those containing the different compounds mentioned above at two dilutions (1:5 and 1:2) of the antiserum. This observation showed that the polyclonal antibodies raised against HPF were not cross-reacting with invertase, mannan or BSA and that HPF did not share any antigenic determinant with those components.

Additionally, invertase and BSA were electrophoretically run in an 1.8% agarose gel and blotted onto a nitrocellulose membrane. During the immunostaining of the nitrocellulose membrane no colour development was observed either as a smeared spot or as a band for neither invertase nor BSA (membrane not shown). This result showed that even with a very sensitive method of detection the antibodies raised against HPF did not recognise potential cross-reacting components such as invertase or BSA.

By using immunodiffusion and immunoelectrophoretic techniques, no cross-reactivity was observed between anti-HPF antibodies and invertase, BSA or mannans. A partial deglycosylation of HPF in the course of the immunisation process could be responsible for the lack of cross-reactivity. Such a partial degradation of the outermost sugars of HPF [like α(1-3) or α(1-2) linked mannose units, see Figure 6.1] might have exposed the more internal mannose units such as α(1-6) linked and thus allowed the production of antibodies directed against this type of sugar. Accordingly, such antibodies would not be able to recognise the usually more exposed sugars like α(1-3) or α(1-2) linked mannose units of invertase or mannan and would explain the absence of any cross-reactivity. However, such antibodies would also not be able to recognise the sugar moiety of the non degraded HPF. Therefore the reaction of identity observed in the immnunoassay between HPF and the antiserum seems likely to be due to the antibodies raised against the protein moiety of HPF.

As no cross-reactivity was observed, it was not necessary to improve the specificity of the antibodies for the following immunochemical experiments.

6.3.2 Presence of HPF antigenic determinants on the Maurivin PDM cell surface. Interpretation of the direct agglutination assay

The agglutination assay performed was a modified version of the direct haemagglutination assay. Antibodies (for instance IgG and IgM) have multiple binding valency and are able to bind at the same time to several antigenic determinants. If the determinants recognised by the antibodies are located on different cells, the antibodies create bridges between the cells. As a result, provided that a sufficient amount of antibodies is present, the cells agglutinate and form clumps visible with the naked eye.

An agglutination assay was conducted using the antiserum obtained after the immunisation to examine the presence of HPF on Maurivin PDM yeast cells' surface.

An agglutination pattern (large circle of clumped cells in the well) was clearly visible in the first few wells of the antiserum test rows (see Section 6.2.7 and Table 6.1 for protocol). In the serum-free and preimmune serum control wells (see definitions in Section 6.2.7), the typical pattern of non-agglutination was observed (cells uniformly spread on the well bottom) at all dilutions of the antiserum. As the two type of controls did not show agglutination, the agglutination observed for the antiserum test wells was attributed to the antibodies present in the antiserum. This result implies that the polyclonal antibodies in the antiserum recognised antigenic determinants of HPF on the yeast cell surface and caused the agglutination. In the first wells where the antiserum concentration was high, the antibodies were in sufficient amounts to provide bridges between the cell surface antigenic determinants and to allow the cells to clump. In the wells further along the row where the antiserum was more diluted, too few antibodies were available to agglutinate the cells. The agglutination assay demonstrated that at least part of the HPF antigenic determinants were located on the yeast cell surface suggesting that HPF was present on the cell surface.

The row containing 2-mercaptoethanol (2-ME, row B) showed the same agglutination pattern as the row without 2-ME (antiserum test row). The reducing agent, 2-ME, can dissociate the IgM antibody pentamers so that they no longer give agglutination but since IgG antibodies are not multimeric, 2-ME has no effect on their agglutinating properties. Thus the agglutination observed in the wells was only due to the IgG antibodies. In the assay, the cells agglutinated to the same level with or without 2-ME present. This result suggested that IgG antibodies involved in the agglutination had a strong binding capacity to HPF determinants and indicated that the use of the IgG fraction for the immunolocalisation of HPF (see next Section) would give a good immunoresponse.

6.3.3 IMMUNOLOCALISATION OF HPF IN THE CELL WALL OF THE YEAST MAURIVIN PDM

The cells collected in late exponential phase were fixed and embedded in resin before being cut in ultrathin sections and examined by transmission electron microscopy (TEM).

During the fixation procedure the immunoreactivity between antibodies and the corresponding antigens might be altered because of the glutaraldehyde used as a fixing agent (Cailliez *et al.* 1992a). Some antigenic determinants are sensitive to the concentration and time of fixation in glutaraldehyde buffer and may loose their immunoreactivity. To determine whether such a loss occured in this study, the fixation was performed in 0.25% glutaraldehyde buffer for either 12 hours or 3 hours. As the same labelling was observed for both fixation times (data not shown) the 12 hour fixation was adopted for practical reasons.

For the immunogold labelling of the ultrathin sections (see Section 6.2.8.2) preliminary assays were carried out to determine the appropriate dilution range of primary antibody or gold probe in order to get minimum background on all sections and no labelling of the negative control sections (see definitions in Section 6.2.8.2, negative control sections not shown).

Figures 6.4 [Section A), B), C)] show the ultrathin sections after labelling with the anti-HPF IgG antibodies and staining. HPF was mainly detected on the cell wall and occasionally within the cytoplasm or in vacuoles. The percentage of gold particles in the cell wall outnumbered that in the vacuoles and in cytoplasm to such an extent that the cell wall could be considered as being the genuine site where HPF was located. The presence of labels in the yeast vacuoles during the immunodetection of cell envelope components is not uncommon. Similar observations were reported by Meyer and Matile (1975), Horisberger and Vonlanthen (1977), Linnemans *et al.* (1977) for the immunolabelling of acid phosphatase, mannans or invertase, respectively. No labelling was observed in the nucleus (data not shown) or the mitochondria [see Figures 6.4 A), B)].

6.3.3.1 Immunolabelling on the cell wall

The labelled material was not homogeneously distributed through the cell wall. Gold particles were more concentrated on the periphery (outer most layer) of the cell wall or near the cytoplasmic membrane (inner most layer of the cell wall) whereas sparse labelling was detected within the cell wall itself [Figures 6.4 A), B)]. Although this point will not be discussed in this thesis, it could also be noted that the labelling of the cell wall was also uniformly distributed at the closing septum of the daughter cell as for the mother cell (Figure 6.4 A).

Labelling was regularly found all over the outer most layer which means that HPF was present in significant amounts in this zone. This result is in agreement with that of the agglutination assay (see Section 6.3.2) which revealed the presence of HPF on the cell surface.

The labelling of the cell wall periphery occured in a zone where other mannoproteins have also been localised. Horisberger and Vonlanthen (1977) reported a similar strong labelling of the *Saccharomyces cerevisiae* cell wall surface in this zone with anti-mannan antibodies. This type of distribution was also observed for *Candida albicans* (Marcilla *et al.* 1993, Elorza *et al.* 1993). According to the molecular organisation of the cell wall as presented in Section 1.5.1.4, the surface of the cell wall is made up of a smooth amorphous layer of mannoproteins. Thus the intense labelling on the cell wall periphery strongly suggests that HPF was one of the mannoproteins forming the top layer of the cell wall.

The labelling in the inner most layers of the cell wall was rather homogeneous and intense [arrow, Figure 6.4 A)]. This suggested that after being extruded into the extracytoplasmic medium, HPF mainly accumulated in the inner most layers of the cell wall. The periplasmic space (space between the cytoplasmic membrane and the glucan-mannoprotein network of the cell wall) is located in the inner most layers of the cell wall but could not be visualised under the staining and magnification conditions used in this study. However, the location of HPF in the inner most layers of the cell wall most probably corresponded to an accumulation of HPF in the periplasmic space. Similar accumulation of cell wall mannoproteins and secreted glycoproteins in the inner most layers of the cell wall was observed using immunolocalisation techniques by Elorza *et al.* (1993) and Cailliez *et al.* (1992a), respectively. In addition Lu *et al.* (1994) showed that α-agglutinins (cell wall surface mannoprotein) accumulated in

significant amounts in the periplasmic space of cells in exponential phase before migrating to the wall. This observation confirmed the finding of Pastor *et al.* (1984) who observed cell wall mannoproteins in transit through the periplasmic space before reaching their destination on the outer surface. Therefore to localise HPF in large amounts in the most inner part of the cell wall was not in contradiction with HPF being a cell wall surface mannoprotein. In addition, the quantity of surface mannoproteins in transit through the cell wall of growing cells which show a high metabolic activity (the cells used herein were in late exponential phase and thus were metabolically very active), would be expected to be high. More experiments, such as the determination of HPF turnover, will be necessary however to confirm this hypothesis.

The labelling in the central part of the cell wall was sparse and more or less randomly spread. No preferential secretion pathways, as reported by Cailliez *et al.* (1992b) for the secretion of killer toxins or by Poulain *et al.* (1989) for the secretion of glycoproteins, could be observed. HPF was therefore probably freely diffusing from the inner most part of the cell wall to its destination on the cell wall surface .

6.3.3.2 Immunolabelling of the cytoplasm

The cytoplasmic labelling was not uniform but mainly concentrated on the periphery near the cytoplasmic membrane [Figures 6.4 A), B), C) ; the section shown in Figure 6.4 C) was atypical in that the peripheral labelling of the cell wall was not as strong as for other sections]. The pattern of gold particles lined up on the external side of the cytoplasmic membrane visible in section C) was often observed at high magnification in other sections.

During the cell wall mannoprotein biosynthesis the protein and mannan moieties are synthesised intracellularly and modified within the endoplasmic reticulum in the course of their migration to the cell wall (see Section 1.5.2). Thus the anti-HPF antibodies might have recognised a precursor form of HPF [or alternatively the mature form accumulated intracellularly as suggested by Cailliez *et al.* (1994) for other cell wall glycoproteins] before its extrusion in the extracytoplasmic medium. Immunological cytoplasmic detections of cell wall mannoproteins have already been reported for acid phosphatase and secreted glycoproteins by Linnemans *et al.* (1977) and Cailliez *et al.* (1992a), respectively.

In some sections (not shown herein), gold particles were also detected in small vesicles which could correspond to secretory vesicles. However, the number of them was too small to consider this observation as significant. Detailed studies would be needed to determine the secretion pathway of HPF and thus to know if the cytoplasmic form observed was either a precursor or mature form.

6.4 CONCLUSION

By producing anti-HPF antibodies and applying different immunological approaches, HPF could be detected and localised in the cell wall of cells in late exponential phase. Using the agglutination assay and the immunolabelling of sections examined by transmission electron microscopy, the presence of HPF on the cell wall surface was confirmed. This result correlated well with the observation of a specific release of HPF from the yeast cells by treatment with non-destructive agents such as EDTA or SDS [HPF was released with EDTA or SDS treatment or simply by boiling the cells. All these methods implied the destruction of non-covalent bonds only and therefore could not deeply disorganise the cell wall (see results Section 4.4)]. Furthermore, the location of HPF on the cell surface could also account for the ease of HPF release into the fermentation culture (see results Section 5.4).

HPF was also localised in the inner layers of the cell wall and more sparsely in the central layers. The distribution of HPF in the wall was in agreement with that of other cell wall mannoproteins like α-agglutinins or secreted glycoproteins which also accumulated in the inner most parts of the wall before further migration to the outer surface (Pastor *et al.* 1984, Cailliez *et al.* 1992a, Lu *et al.* 1994). Therefore HPF might also temporarily accumulated in the inner most part of the wall of growing cell before migrating to the wall surface. Further studies on the distribution of HPF at the different stage of cell growth (for cells newly budded or in stationary phase) could provide more information about the transitional presence of HPF in the inner most part of the cell wall.

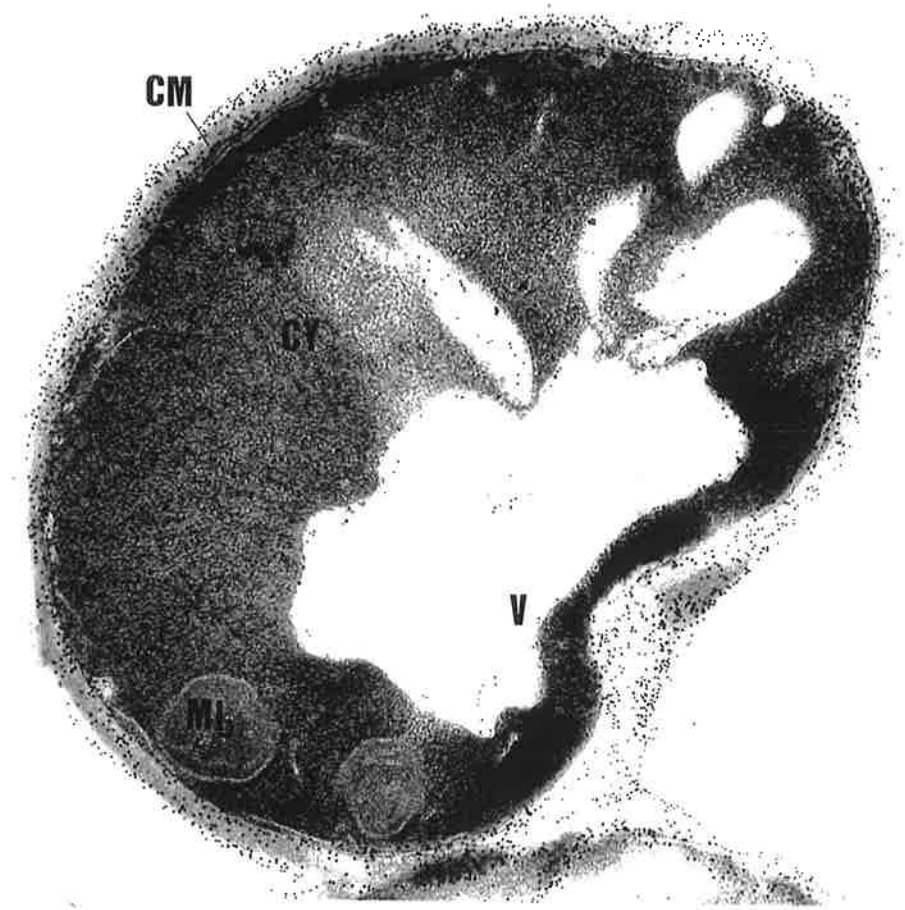
Figures 6.4 Sections of *Saccharomyces cerevisiae* Maurivin PDM in late exponential phase labelled with anti-HPF antibodies [dilution 1:400 for A), dilution 1:600 for B) and C)] and gold probe (size gold particle: 10 nm, dilution 1:50), and stained with lead citrate and uranyl acetate.

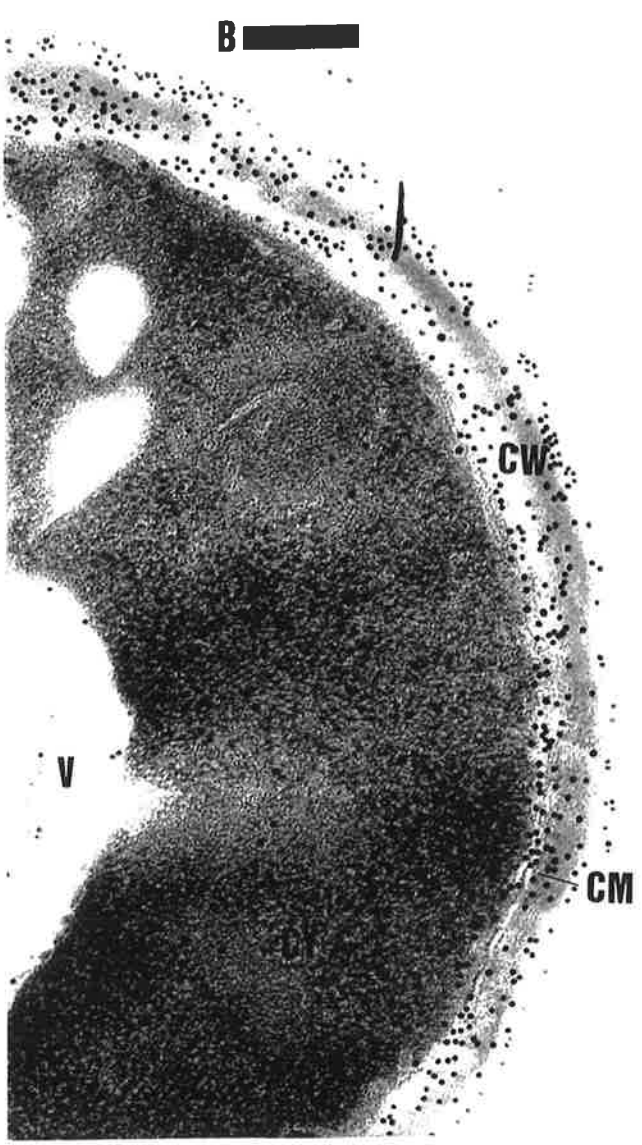
CM: cytoplasmic membrane; **CW**: cell wall; **CY**: cytoplasm; **MI**: mitochondria; **V**: vacuoles. The nucleus is not shown in these sections.

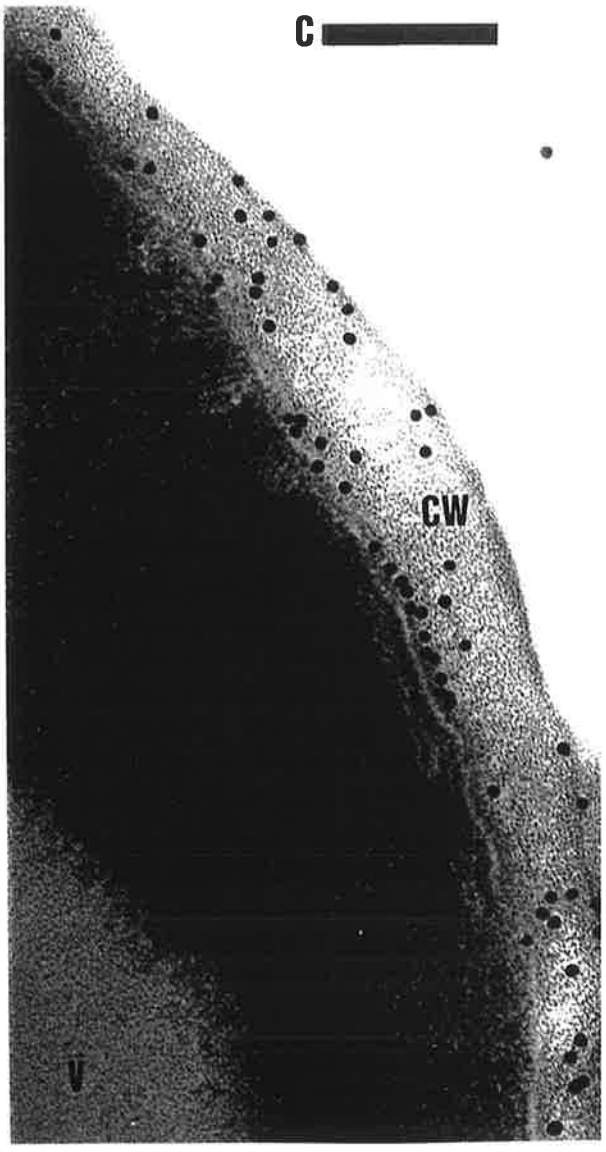
A) This section of a whole cell showed the specific labelling of the cell wall compared to that of the cytoplasm or vacuoles. The cell wall was intensely marked with a preferential labelling of either the inner most or peripheral zones. The bar represents 500 nm.

B) This section showed the labelling of the cell wall, cytoplasm and vacuoles. The cytoplasmic membrane was partly visible (arrow). The bar represents 200 nm.

C) This section of the cell clearly showed the cell wall limits. Both cell wall and cytoplasmic labellings were observed. The bar represents 200 nm.


A
CM
CY
V
M1


B
CW
V
CM
CY


C
CW
V

Chapter 7

CONCLUDING REMARKS

Production and localisation of haze protective material from *Saccharomyces cerevisiae.* Special reference to Maurivin Prise de mousse strain.

In this thesis the extraction and release profiles of yeast haze protective material (HPM) were investigated. Destructive methods (disruption of the covalent bonds) involving chemical (DTE treatment), enzymatic (zymolyase treatment) or physical action (French press, autoclave methods) did not release mannoprotein material showing good haze protective ability. Extraction of HPM was specific for methods having a mild impact (disruption of the non-covalent bonds) on the cell envelope such as EDTA or SDS treatments (extraction from whole cells or cell walls) or boiling whole cells in Tris buffer. Accordingly, HPM was thought to be non-covalently linked to other cell wall components and loosely associated with the cell wall.

HPM was found to be naturally released by the yeasts into the medium during fermentation and storage on yeast lees. Collection by ultrafiltration or ethanol precipitation of the extracellular material released by the yeast, in particular from yeasts grown to stationary phase in a mannose enriched medium, gave satisfactory yields of material active in reducing the haze. In the future, this natural release could be seen as a viable way to produce HPM on a large scale under a continuous cultivation process.

The localisation of HPF (using antibodies to HPF purified from red wine) in the yeast Maurivin Prise de mousse cell led to a better understanding of the position of HPM in the yeast cell (Figure 7.1). This material was distributed in the inner most and peripheral layers of the cell wall. This distribution was common to other cell wall mannoproteins (Horsiberger *et al.* 1976, Horisberger and Vonlanthen 1977).

High M_r mannoproteins are often reported to be covalently linked to the glucan network and thus contribute to the cell wall structural architecture (Sentandreu *et al.* 1984, Ruiz-Herrera 1992, Cid *et al.* 1994). On the basis of the results obtained in this research, the 420 k mannoprotein(s) (contained in the HPF sample purified from red wine) could represent a new type of non structural high M_r mannoproteins which are located in the amorphous mannoprotein surface layer of the cell wall instead of being linked to the glucan network. As far as we know no mannoprotein with a given function was ever reported to be similar to the 420 k mannoprotein(s) by the size, sugar or amino acid content, or by the extraction or release profile.

Further studies on the function of mannoprotein(s) with haze reducing ability would be very valuable to understand their role in the cell wall and the reason for their release into the medium. With regard to an optimised production, this information would be valuable to eventually oblige the cell to overproduce HPM. Additionally, such production could be enhanced by working with a genetically modified yeast overproducing HPM. Further genetic or molecular biology studies could also lead to the HPM natural release property being used as a means for the secretion of heterologeous proteins.

Extraction with SDS or EDTA treatments or by boiling cells

Natural release into the culture medium

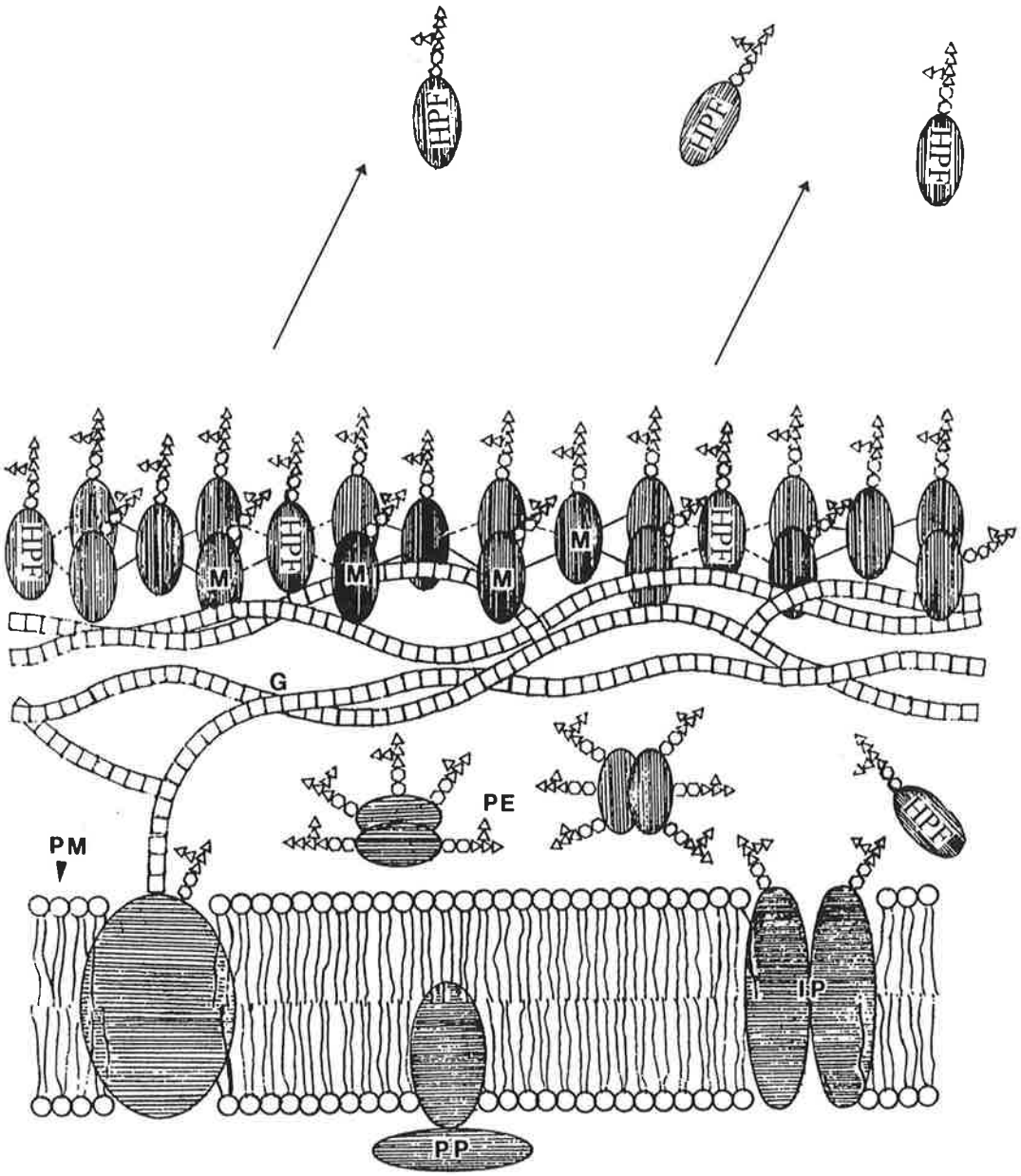
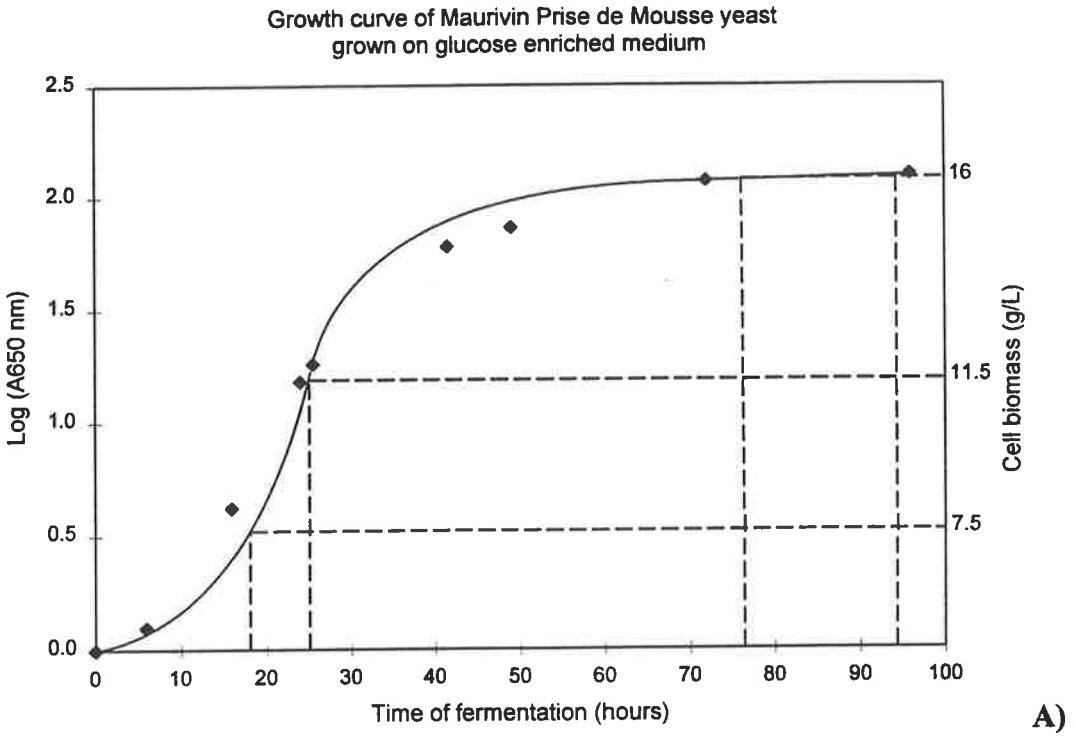


Figure 7.1 Localisation of HPM in the simplified molecular organisation of the yeast cell wall surface [adapted from Schekman and Novick (1982) and modified for this thesis].

G, glucan; **M**, mannoprotein; **PE** periplasmic enzyme; **IP**, integral membrane protein; **PP**, peripheral membrane protein, **HPF**, haze protective factor .

- - - - : no covalent bonds

APPENDIX A



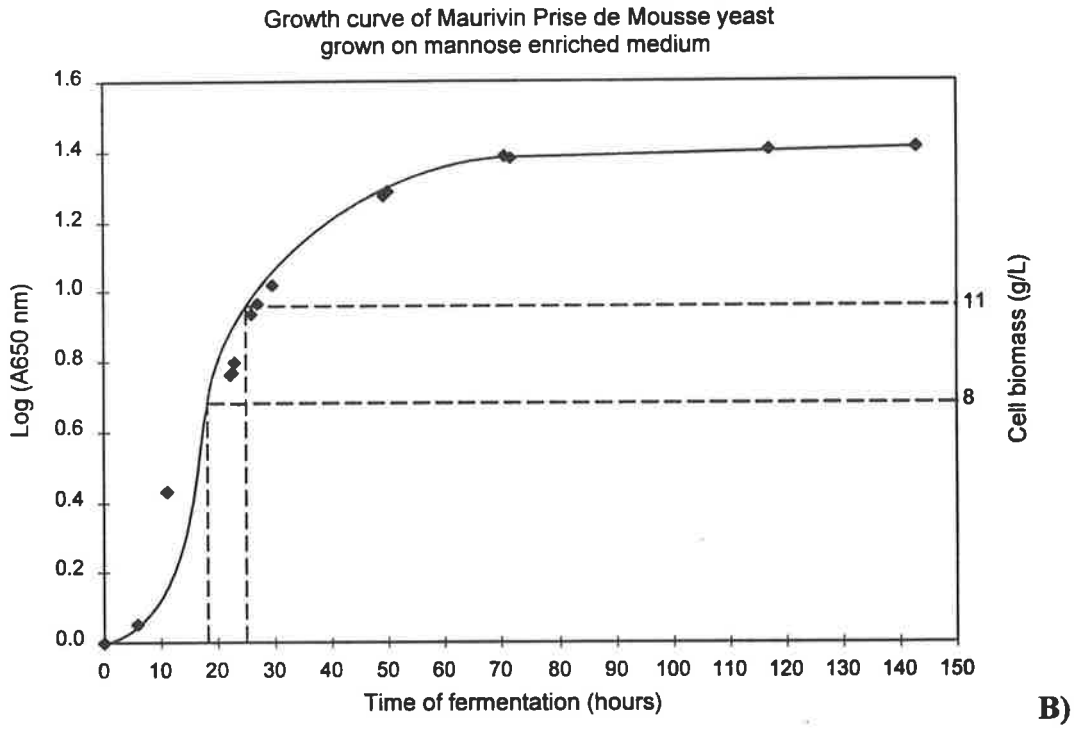


Figure 1 Growth curves of Maurivin prise de mousse grown on glucose (A) or on mannose (B) at the conditions mentioned in Section 3.2.3 or 3.2.4.

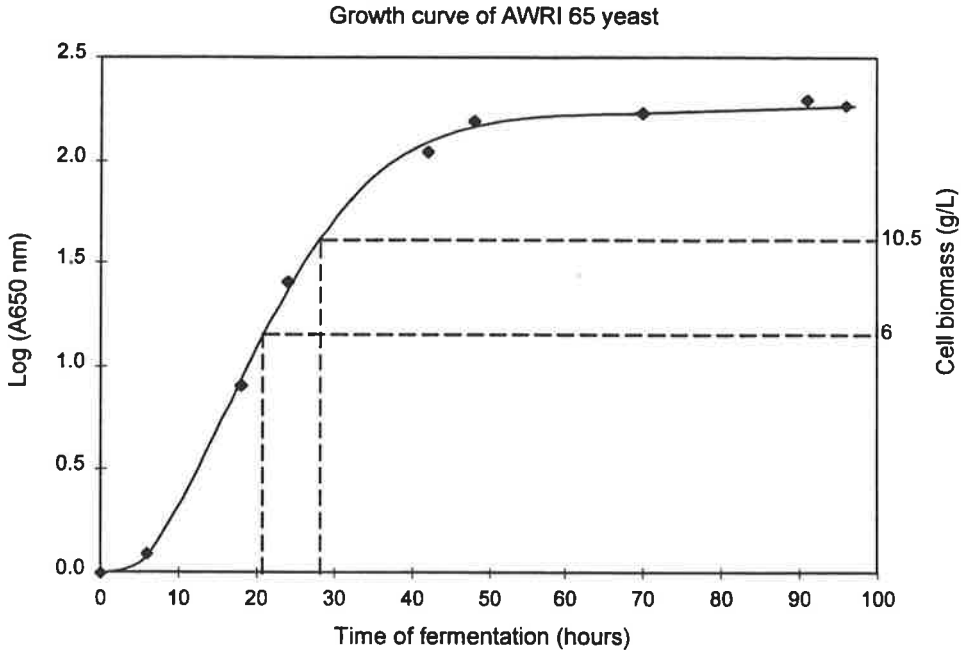


Figure 2 Growth curves of AWRI 65 yeast grown on glucose at the conditions mentioned in Section 3.2.3.

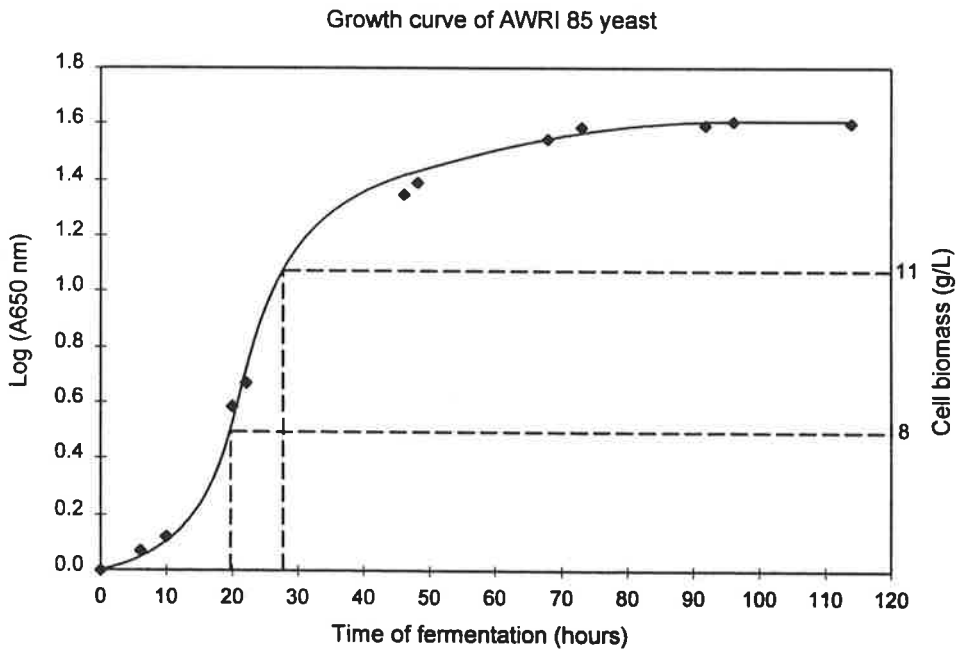


Figure 3 Growth curves of AWRI 85 yeast grown on glucose at the conditions mentioned in Section 3.2.3.

APPENDIX B

Table 1 Values of the haze obtained for mannoprotein materials extracted with SDS, full zymolyase, autoclave, French press treatments on thawed late exponential phase Maurivin DPM cells or with SDS treatment on cells previously French pressed. Haze was measured in the heat test as described in Section 3.2.7.

Concentration of the mannoprotein extract (mg/mL wine)	Treatments				
	SDS	Full zymolyase	Autoclave	French press	SDS on cells previously French pressed
0	100 *	100	100	100	100
0.5	26	34	57	72	53
1	26	33	52	96	43
1.5	34	37	55	97	50
2	42	38	56	127	53

* Values are expressed in % of haze obtained compared to the initial haze value (100%)

Table 2 Values of the haze obtained for mannoprotein materials extracted with SDS, full zymolyase, autoclave, French press treatments on thawed late exponential phase AWRI 85 cells. Haze was measured in the heat test as described in Section 3.2.7.

Concentration of the mannoprotein extract (mg/mL wine)	Treatments			
	SDS	Full zymolyase	Autoclave	French press
0	100 *	100	100	100
0.5	32	36	70	94
1	43	43	53	117
1.5	30	41	61	219
2	39	57	62	248

* Values are expressed in % of haze obtained compared to the initial haze value (100%)

Table 3 Values of the haze obtained for mannoprotein materials extracted with SDS, full zymolyase, autoclave, French press treatments on thawed late exponential phase AWRI 65 cells. Haze was measured in the heat test as described in Section 3.2.7.

Concentration of the mannoprotein extract (mg/mL wine)	Treatments			
	SDS	Full zymolyase	Autoclave	French press
0	100 *	100	100	100
0.5	85	69	82	117
1	76	76	77	117
1.5	75	77	93	133
2	85	76	88	145

* Values are expressed in % of haze obtained compared to the initial haze value (100%)

Table 4 Values of the haze obtained for mannoprotein materials extracted with the full zymolyase treatment on thawed late exponential or stationary phase Maurivin PDM cells grown either on glucose or mannose enriched medium. Haze was measured in the heat test as described in Section 3.2.7.

Concentration of the mannoprotein extract (mg/mL wine)	Full zymolyase treatment			
	Cells grown on glucose		Cells grown on mannose	
	Late exponential phase	Stationary phase	Late exponential phase	Stationary phase
0	100 *	100	100	nd
0.5	34	56	36	nd
1	33	30	35	nd
1.5	37	35	40	nd
2	38	40	55	nd

* Values are expressed in % of haze obtained compared to the initial haze value (100%)
nd not determined

APPENDIX C

Table 1 Values of the haze obtained for mannoprotein materials extracted with EDTA treatment (see Section 4.2.5.2) at pH 3, 5.5 or 8 on fresh late exponential phase Maurivin PDM cells. Haze was measured in the heat test as described in Section 4.2.7.

Concentration of the mannoprotein extract (mg/mL wine)	EDTA treatment		
	pH 3	pH 5.5	pH 8
0	100 *	100	100
0.5	59	56	44
1	83	75	47
1.5	97	91	50
2	133	114	62

* Values are expressed in % of haze obtained compared to the initial haze value (100%)

Table 2 Values of the haze obtained for the mannoprotein material extracted with the SDS treatment on Lallemand yeast hulls. Haze was measured in the heat test as described in Section 4.2.7.

Concentration of the mannoprotein extract (mg/mL wine)	SDS treatment on Lallemand yeast hulls
0	100 *
0.5	75
1	85
1.5	116
2	117

* Values are expressed in % of haze obtained compared to the initial haze value (100%)

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