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Mestrado em Biologia Molecular em Saúde

Purification of antimicrobial peptides secreted by *Saccharomyces cerevisiae* and proteomic analysis of cell membrane-associated proteins

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Resumo

Durante fermentações vínicas efectuadas com culturas mistas, a levedura *Saccharomyces cerevisiae* liberta péptidos antimicrobianos, que derivam da enzima gliceraldeído 3-fosfato desidrogenase (GAPDH) e provocam a morte prematura de leveduras não-*Saccharomyces*. Considerando o potencial destes péptidos antimicrobianos para serem usados como conservantes naturais do vinho, o primeiro objectivo deste trabalho consistiu na sua produção e purificação a uma escala preparativa, de forma a poderem ser aplicados em fermentações de adega. Primeiramente, produziram-se 3 L de sobrenadantes de fermentações efectuadas com *S. cerevisiae* que, em seguida, foram sujeitos a uma cromatografia de troca iónica utilizando um coluna preparativa DEAE-Sephadex. Foi obtida uma fracção bioactiva que exibia um perfil cromatográfico semelhante ao exibido pelos péptidos antimicrobianos previamente identificados. Embora tenha sido possível purificar os referidos péptidos com a coluna cromatográfica preparativa, os resultados deste trabalho mostraram que o sistema cromatográfico utilizado não foi suficientemente eficiente para isolar os péptidos de interesse num único passo. Assim propõe-se que de futuro se utilize um passo adicional de cromatografia de exclusão molecular que permita isolar a fração péptica (proteínas <10 kDa) antes da utilização da coluna de troca iónica.

Vários estudos têm demonstrado que a morte prematura de leveduras não-*Saccharomyces* durante fermentações vínicas é induzida por *S. cerevisiae* através de diferentes mecanismos: contacto célula-a-célula e secreção de péptidos bioactivos. Adicionalmente, um trabalho ainda não publicado mostrou que células de *S. cerevisiae* crescidas durante 48 h são capazes de induzir a morte de *Hanseniaspora guilliermondii* por contacto célula-a-célula, enquanto células crescidas durante 12 h não apresentaram o mesmo efeito. Tendo em consideração estes dados e sabendo que a GAPDH é uma proteína da parede celular da *S. cerevisiae*, formulou-se a hipótese destes péptidos bioactivos estarem presentes na parede celular de células crescidas durante 48 h e desta forma induzirem a morte de não-*Saccharomyces* por contacto célula-a-célula. Assim, o segundo objectivo deste trabalho consistiu na análise das proteínas de membrana de células de *S. cerevisiae* crescida durante 12 h e 48 h, respetivamente. A análise das proteínas de membrana por eletroforese bidimensional em géis de poliacrilamida revelou em ambos os proteomas a presença de dois *spots*,

exibindo pesos moleculares e pontos isoeléctricos muito semelhantes ao dos péptidos bioactivos previamente identificados. Para além disso, no proteoma das células crescidas durante 48 h os referidos *spots* mostraram estar sobre-expressos. Os referidos *spots* foram analisados por espectrometria de massa, o que confirmou a presença de péptidos derivados do GAPDH. Assim, este trabalho forneceu evidência experimental de que a morte de leveduras não-*Saccharomyces* por contacto célula-a-célula com *S. cerevisiae* pode resultar da presença dos péptidos antimicrobianos derivados do GAPDH. Porém, para confirmar definitivamente esta hipótese serão necessários ainda estudos futuros.

Palavras-chave: péptidos antimicrobianos; cromatografia preparativa; proteoma da membrana celular; morte celular

Abstract

Saccharomyces cerevisiae secretes antimicrobial peptides (AMPs) during mixed culture fermentations that are derived from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) enzyme and induce the early death of non-*Saccharomyces* yeasts. Considering the potential of AMPs to be used as natural preservatives in wine, the first aim of the present work was to produce and purify them in a preparative scale so that they could be applied in winery fermentations. Firstly, we produced 3 L of *S. cerevisiae* fermentation supernatants and subjected those to ion-exchange chromatography using a preparative DEAE-Sephadex column. One bioactive fraction, exhibiting a chromatographic profile similar to that exhibited by the previously found AMPs, was obtained. However, our work showed that the chromatographic system used was not fully efficient to purify the AMPs in a single step, requiring an additional size-exclusion chromatographic step.

Several studies have shown that early death of non-*Saccharomyces* yeasts during wine fermentations is induced by *S. cerevisiae* through cell-cell contact and secretion of AMPs. Moreover, unpublished work showed that *S. cerevisiae* cells pre-grown for 48 h are able to induce death of *Hanseniaspora guilliermondii* by cell-cell contact, while 12 h-grown cells are not. Considering these findings and knowing that GAPDH is a cell wall-associated protein in *S. cerevisiae*, we hypothesized that these AMPs could be present in the membranes of *S. cerevisiae* and in this way induce death of non-*Saccharomyces* by cell-cell contact. The second aim of our work was to analyze membrane proteins of *S. cerevisiae* cells grown for 12 h and 48 h, using two dimensional polyacrylamide gel electrophoresis. Proteomic analysis revealed the presence of two spots of molecular weight and isoelectric point similar to the previously found AMPs, which were differentially expressed in the two growth stages. The spots were analysed by mass spectrometry, confirming the presence of GAPDH-derived peptides. Thus, our work raised evidence that death of non-*Saccharomyces* yeasts by cell-cell contact might be due to the presence of GAPDH-derived AMPs in the membranes of *S. cerevisiae* cells. However, to definitively confirm this hypothesis further work is required.

Keywords: antimicrobial peptides; preparative chromatography; proteome of cell membrane; cell death

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Abbreviations

2D-PAGE - two dimensional polyacrylamide gel electrophoresis

2DE - two dimensional electrophoresis

AcAm - ammonium acetate

AMPs - antimicrobial peptides

AAMPs - anionic antimicrobial peptides

Cell/ml - cell per milliliter

CFU/ml - colony-forming unit per milliliter

CFU/g - colony-forming unit per gram

CHAPS - 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

DEAE - diethylaminoethyl

DTT - Dithiothreitol

EDTA - Ethylenediamine tetraacetic acid

ESI - electrospray ionization g/l - grams per liter

GAPDH - glyceraldehyde-3-phosphate dehydrogenase enzyme

GF - gel filtration or size exclusion chromatography

IEC - ion-exchange chromatography

IEF - isoelectric focusing

IPG - immobilized pH gradient

kDa - kilo Daltons

LAB - lactic acid bacteria

MALDI - matrix assisted laser desorption/ionization

MLF - malolactic fermentation

MS - mass spectrometry

MW - molecular weight

nm - nanometer

NMR - nuclear magnetic resonance

rpm - revolutions per minute

Phe - phenylalanine

p*I* - isoelectric point

RP-HPLC - reversed-phase high-performance liquid chromatography

SDS - sodium dodecyl sulfate

SGJ - synthetic Grape Juice

TCA - trichloroacetic

tdh3p - glyceraldehyde-3-phosphate dehydrogenase protein

Trp - tryptophan

TOF - time of flight

Tyr - tyrosine

YPD - yeast protein database

YEPD - yeast extract peptone dextrose

1 Introduction

1.1 Winemaking process and alcoholic fermentation

Winemaking process relies in some basic principles, mainly in the transformation of grape sugars into ethanol and carbon dioxide -alcoholic fermentation- by the yeasts belonging to the natural microflora of grape musts. After alcoholic fermentation, an additional fermentation process –malolactic fermentation– may take place in both red and white wines, which occurs by the intervention of lactic acid bacteria.

Depending on the process used, there are three types of wines: red, rosé and white wine. Following harvesting, grapes are destemmed and crushed and then the process diverges for white and red wines: white wine undergoes fermentation in the absence of grape skins and lees; while in red wine fermentation follows maceration and occurs in the presence of grape skins and lees. Since the red pigments of the red grape berries, the anthocyanins are located in the skin of grape berries, that difference on the winemaking process introduces a major distinction in the composition and taste of white and red wines. After vinification, fermented grape juice undergoes through several final operations: clarification, maceration, fining or stabilization, filtration and finally bottling (Pretorius, 2000, Bisson, 2004). The main steps of the production of red and white wines are represented in **Fig. 1.1**.

Wine is the result of the action of a diversity of microbiological interactions and, consequently, of a number of biochemical reactions, being the alcoholic fermentation the major and the most important biotransformation taking place during the process (Lambrechts and Pretorius, 2000, Fleet, 2003, Romano, 2003).

Yeasts have the ability of conducting alcoholic fermentation by fermenting grape sugars to ethanol and carbon dioxide in order to obtain the energy and biosynthetical material required for growth. This biotransformation was first studied and described by the well-known chemist Antoine Lavoisier in 1789. Lavoisier discovered that 100 parts by weight of sugar were converted to 60.17 parts of alcohol, 36.81 parts of carbon dioxide and 2.61 parts of acetic acid, being the first describing the chemical equation as “grape must = carbonic acid + alcohol”. In 1819, Joseph Gay-Lussac revised Lavoisier’s work, estimating that 100 parts of sugar were

converted in 51.34 parts of alcohol and 48.66 parts carbon dioxide. The general equation of alcoholic fermentation is:

$$C_6H_{12}0_6 \rightarrow 2C_2H_5OH + 2CO_2$$

Assuming this equation, 100 parts of sugar (glucose) are converted in 51.19 parts of alcohol (ethanol) and 48.90 parts of dioxide carbon, showing the great accuracy of the earlier studies of these researchers (Barnett, 1998, Barnett, 2003).

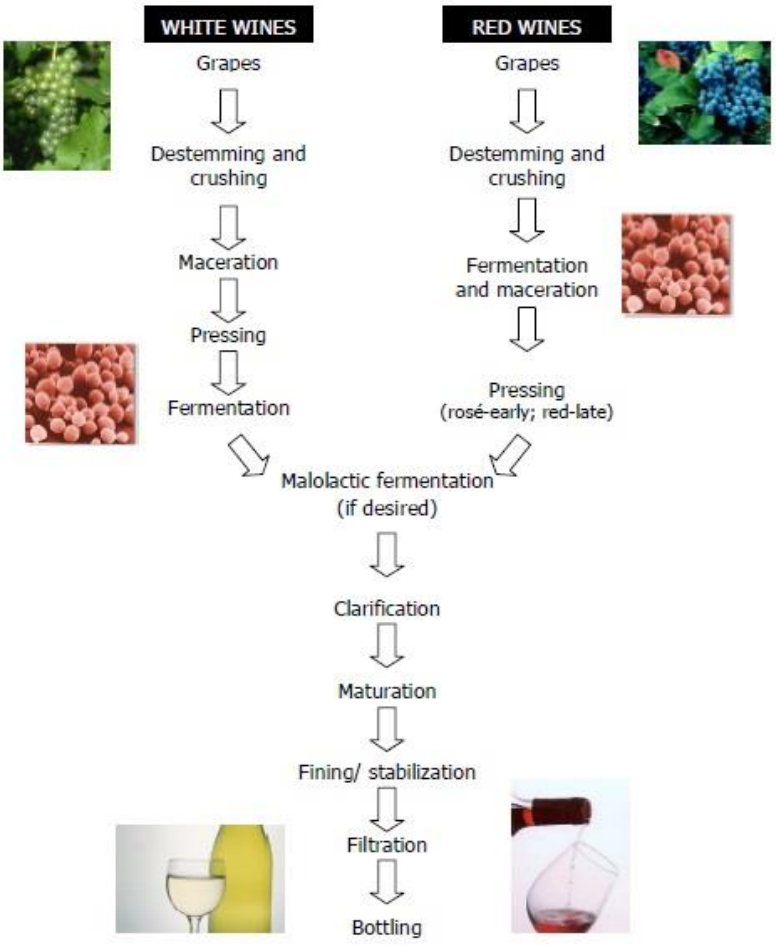

Figure 1.1 – The main steps of wine production (Pretorius, 2000)

1.1.1 Microorganisms associated with winemaking

Microorganisms associated with the winemaking process include yeasts, bacteria and filamentous fungi. Of the 100 yeast genera representing over 700 species, 16 are associated with winemaking: *Brettanomyces* (and its reproductive stage *Dekkera*) *Candida; Cryptococcus; Debaryomyces; Hanseniaspora* (and its

reproductive stage *Kloeckera*); *Kluyveromyces; Metschnikowia; Pichia; Rhodotorula; Saccharomyces; Saccharomycides; Schizosaccharomyces; Torulaspora* and *Zygosaccharomyces* (Heard and Fleet, 1985, Pretorius, 2000, Lambrechts and Pretorius, 2000, Esteves-Zarzoso *et al.*, 1998). The predominant species in the grape berries are apiculated yeasts, such as *Hanseniaspora uvarum* and its anamorphic form *Kloeckera apiculata*, and oxidative ones, such as *Candida*, *Pichia*, *Rhodotorula* and *Kluyveromyces* (Fleet, 2003). Contrary to popular belief, fermentative species of *Saccharomyces* (e.g. *S. cerevisiae*) occur at extremely low numbers on healthy, undamaged grapes and are rarely isolated from intact berries and vineyard soils (Frezier and Dubourdieu, 1992; Martini *et al*., 1996). In fact, the origin of *S. cerevisiae* is quite controversial. While some researchers believe that damaged grapes are an important source of *S. cerevisiae* (Mortimer and Polsinelli, 1999), others point to a direct association with artificial, man-made environments such as wineries and fermentation plants (Vaughan-Martini and Martini, 1995; Martini *et al*., 1996; Deak, 1998; Sabate *et al*., 2002).

1.1.2 Yeast population dynamics during wine fermentations

Yeast population on the surface of immature grape berries is low (*ca* $10\text{-}10^3$ CFU/g), increasing to about $10^4\text{-}10^6$ CFU/g as grapes mature to harvest. Apiculate yeasts of the genus *Hanseniaspora* are predominant on the surface of grape berries and in lower numbers appear other yeast genera such as *Candida*, *Brettanomyces*, *Cryptococcus*, *Kluyveromyces; Metschnikowia* and *Pichia* are also present. Fermentative species of *Saccharomyces* (e.g. *S. cerevisiae*) are frequently not isolated from intact grape berries being generally found at densities of *ca* $10\text{-}10^2$ CFU/g (Pretorius, 2000, Fleet, 2003).

The wine fermentation process may occur spontaneously, being conducted by the yeast belonging to the natural microflora of grape musts or it might start with the inoculation of a commercial strain. Spontaneous vinifications (without inoculation) are the result of the combined action of several yeast species, which grow more or less in succession throughout the fermentation process (Lambrechts and Pretorius, 2000, Esteves-Zarzoso *et al.*, 1998). In the early stages, wine fermentations are conducted by the low fermentative yeasts such as *Hanseniaspora, Candida* and *Metschnikowia*, reaching a density of about $10^6\text{-}10^7$ CFU/ml. At this stage, other

yeasts such as *Kluyveromyces* and *Pichia* and wine spoilage yeasts *Brettanomyces* and *Zygosaccharomyces* can also grow and ferment sugars. At mid-fermentation stages (with 3-4% of ethanol produced), non-*Saccharomyces* species begin to die-off giving way to the strongly fermentative *S. cerevisiae* strains which become predominant (10^7-10^8 CFU/ml) and complete the fermentation process (Pretorius, 2000, Fleet, 2003, Bisson, 2004).

Saccharomyces cerevisiae

S. cerevisiae is the most important and well-known yeast species due to its worldwide application in the production of wine, beer and bread. This species, also known as the "wine yeast", is the first choice in the formulation of yeast starters for winemaking due to its high fermentation capacity and high tolerance to the harsh environmental conditions of this process, i.e.: high levels of ethanol and other organic compounds, low pH values, low oxygen and scarce nutrient availability. Moreover, *S. cerevisiae* is the best physiologically and genetically characterized eukaryotic organism and a great biological model for multidisciplinary studies.

S. cerevisiae are dimorphic ascomycetous fungi that can occur in two forms: a unicellular oval-elliptic form and, under conditions of nitrogen deficiency, in a multicellular filamentous form, named pseudohyphae. *S. cerevisiae* can grow as diploid cells (where division is carried out through budding or fission) or as haploid cells forming ascopores (under starvation conditions) (Gimeno *et al*., 1992, Kurtzman and Piškur, 2005, Bergman, 2001, Bisson, 2004).

Fungal cell walls are rigid structures required for maintaining cellular shape and integrity by protecting cells against osmotic changes in the environment. They also act as selectively permeable membranes and play a role in processes such as flocculation, cell adhesion and pathogenicity. The cell wall exhibits a dynamic nature, undergoing profound changes during budding, apical extension of hypha, mating and dimorphic transition. (Klis, 2006; Pardo *et al*., 1999; Braconi *et al*., 2011).

S. cerevisiae has approximately 6000 functional genes, most of them encoding proteins of the plasmatic membrane (Goffeau *et al*., 1996). This membrane consists of a mixture of proteins and lipids which form an impermeable barrier to hydrophilic molecules. The major components of the cell wall are β-glucans, chitin (both are

responsible for cell wall strength) and mannoproteins that act as structural proteins or as enzymes involved in biogenesis. There are a variety of other specialized proteins that play a role in processes of solute transport, signal transduction, cytoskeleton anchoring and in glycolytic pathways such as the enzyme glyceraldehyde-3-phosphate (GAPDH; tdh3p) (Aliverdieva *et al.*, 2004, Bisson, 2004; Delom *et al*., 2006).

1.1.3 Lactic acid bacteria and the malolactic fermentation

Lactic acid bacteria (LAB) are Gram-positive, non-sporing and non-respiring bacteria that are able to transform malic acid into lactic acid – malolactic fermentation. LAB isolated from wine include species of the genera *Oenococcus*, *Lactobacillus* and *Pediococcus*. *Oenococcus oeni* is the preferred species used to conduct malolactic fermentation due to its tolerance to the extreme harsh conditions of the wine environment and the flavour profile they produce (Lerm, 2010).

Malolactic fermentation (MLF) is a secondary wine fermentation carried out by LAB that consists in an enzymatic-mediated reaction in which L-malic acid is decarboxylated into L-lactic acid and carbon dioxide. It promotes a desirable reduction of wine acidity an improvement in the microbial stability and in the organoleptic quality of the wine. MLF might occur spontaneously, concomitant with, or at the end of the alcoholic fermentation and its duration depends on the amount of malic acid in the medium. After the completion of MLF, the remaining LAB (*Lactobacillus* and *Pediococcus*) are still able to metabolize residual sugar, which could result in wine spoilage.

Since LAB are very susceptible to various nutrient limitations, too acidic pH values, low temperatures, high levels of ethanol and sulfur dioxide, MLF are difficult to control. Thus nowadays, inoculation of wine fermentations with commercial malolactic starter cultures (*Oenococcus oeni*) is becoming a common oenological practice in wineries to better control MLF (Liu, 2002, Alexandre *et al*., 2003, Comitini *et al.*, 2005, Osborne and Edwards, 2007, Lerm, 2010, Izquierdo Cañas *et al.*, 2012).

1.1.4 Factors underlying dominance of *Saccharomyces cerevisiae*

As mentioned before, non-*Saccharomyces* yeasts grow during the early stages of wine fermentation but then begin to die-off, leaving way to *S. cerevisiae* strains to complete the fermentation process (Heard and Fleet, 1985). The ability of *S. cerevisiae* to displace other microbial species during wine fermentation has been always attributed to its higher fermentative power and capacity to withstand the increasingly adverse conditions established in the medium as the fermentation progresses, i.e.: high levels of ethanol and organic acids, low pH values, scarce oxygen availability and depletion of certain nutrients (Bisson 1999; Bauer and Pretorius 2000; Hansen *et al*., 2001).

The increasing levels of ethanol are known to inhibit cell growth. Ethanol modifies plasma membrane fluidity, stimulates the activity of plasma membrane H+-ATPase and inhibits glucose transport (Ansanay-Galeote *et al.*, 2001). Ethanol also triggers a stress response in *S. cerevisiae* cells consisting in the formation of heat shock proteins (Piper *et al*,. 1994).

The early death of non-*Saccharomyces* yeasts such as *Candida*, *Hanseniaspora*, *Kluyveromyces*, *Metschnikowia*, *Pichia* and *Torulaspora* has always been attributed to their incapacity to tolerate ethanol concentrations higher than 5-7% (v/v) (Fleet and Heard, 1993; Fleet, 2003). However, subsequent studies showed that some non-*Saccharomyces* species such as *Candida zemplinina* and *Kluyveromyces thermotolerans* exhibit similar ethanol tolerance to *S. cerevisiae* strains, especially under fermentations performed at temperatures lower than 20 ºC (Gao and Fleet, 1988). Other work on ethanol tolerance showed that the survival of *H. guilliermondii* at 25% (v/v) ethanol was strongly influenced by the conditions of cultivation prior to the ethanol challenge and a small increase in survival was observed for *H. uvarum* and *T. delbrueckii* in the cultures grown in aerobiosis (Pina *et al.*, 2004).

It is generally recognized that some *S. cerevisiae* strains are more ethanol-tolerant than others: cells of a given strain grown in the presence of ethanol are more ethanol-tolerant than the same cells grown in the absence of ethanol. The high ethanol tolerance of *S. cerevisiae* is based in the so-called survival factors (unsaturated long chain fatty acids and sterols) that play an essential role in the adaptive response of *S. cerevisiae* to ethanol: wine yeast strains usually contain higher levels of survival factors than non-wine *Saccharomyces* strains and their

physiological response to ethanol challenge is also greater than non-wine strains (Loureiro and van Uden, 1986; Lloyd *et al*., 1991; Ansanay-Galeote *et al*., 2001 Mishra and Kaur, 1991; Sajbidor, 1997).

During wine fermentations, oxygen and assimilable nitrogen can be rapidly depleted due to both semi-anaerobic growth conditions and poor initial nitrogen contents of grape musts. In the presence of oxygen, the increasing survival rate of yeasts is related to the enrichment of unsaturated long chain fatty acids and ergosterol in the phospholipid membrane (Alexandre and Charpentier, 1998; Chi and Arneborg, 1999).

Throughout the different stages of wine fermentation, several types of nutrients may become limited or exhausted which results in a decreasing in both the growth rate and the fermentation efficiency or even in a complete arrest of fermentation. Nutrient limitation and starvation are stress inducing conditions and result in a number of stress-associated responses (Bauer and Pretorius 2000). A study carried out with three strains of *H. uvarum*, *H. guilliermondii* and *C. stellata* (Albergaria *et al*., 2003) showed that the limited fermentation capacity exhibited by those yeast strains was, partially, due to nutritional limitations.

The killer phenomenon consists in the production of specific extracellular glycoproteins by certain killer yeast strains that are able to kill other sensitive yeast strains. The killer strains themselves are immune to their own toxin but remain susceptible to the toxins secreted by other killer yeasts (Schmitt and Breinig, 2002). Since first discovered in *S. cerevisiae*, killer strains have been isolated from several yeast genera, including *Candida*, *Cryptococcus, Hanseniaspora*, *Kluyveromyces*, *Pichia* and *Torulopsis* (Chen *et al*., 2000). *S. cerevisiae* produces three types of killer toxins K1, K2 and K28, but only K2 and K28 are functional during wine fermentations due to the low pH of grape must and wine. However, the killer toxins produced by *S. cerevisiae* strains have shown to be active only against strains of the same species. Thus, they cannot explain the antagonistic effect exerted by *S. cerevisiae* against non-*Saccharomyces* yeasts during wine fermentations.

Beside the above-mentioned factors usually reported to explain the yeast population dynamics of wine fermentations, more recent studies have shown that there are other causes for the early disappearance of non-*Saccharomyces* yeasts, namely: growth arrest mediated by a cell–cell contact mechanism (Nissen and Arneborg 2003; Nissen *et al*., 2003; Arneborg *et al*., 2005) and death mediated by

killer-like toxins (Chen *et al*., 2000; Comitini *et al*., 2005; Pérez-Nevado *et al*., 2006; Osborne and Edwards 2007; Albergaria *et al*., 2010).

Death mediated by cell–cell contact is another mechanism that has been reported to explain the early death of some non-*Saccharomyces* during wine fermentations. In a pioneer work, Nissen and Arneborg (2003) demonstrated that the early death of *K. thermotolerans* and *T. delbrueckii* in mixed culture fermentations performed with *S. cerevisiae* was not due to ethanol or any other toxic compound but rather to a cell-cell contact-mediated inhibition. More recent studies (Renauld *et al*., 2013, Kemsawasd *et al*., 2015) confirmed that *S. cerevisiae* cells at high cell density induce the early death of wine-related yeasts, although the exact death-inducing mechanism remains unclear.

Other studies (Comitini *et al*., 2005; Pérez-Nevado *et al*., 2006; Osborne and Edwards 2007; Albergaria *et al*., 2010) found evidences that certain *S. cerevisiae* strains produce killer-like toxins that are involved in the yeast–yeast and yeast–bacteria interactions established during wine fermentations. Pérez-Nevado *et al*. (2006) conducted a study with two non-*Saccharomyces* wine strains (*H. guilliermondii* and *H. uvarum*) performing enological fermentation both in single and in mixed culture with *S. cerevisiae*. Results showed that after the first 3 days of fermentation the non-*Saccharomyces* yeasts begun to die off in the mixed cultures, while in the single cultures the number of viable cells of non-*Saccharomyces* remained high (ranging 10^7–10^8 CFU/ml), regardless the increasing levels of ethanol. Furthermore, they also showed that death of the non-*Saccharomyces* strains was not induced by cell-cell contact but rather by one or more toxic compounds produced by *S. cerevisiae*. Subsequent studies (Albergaria *et al*., 2010) revealed the proteinaceous nature of the toxic compounds secreted by *S. cerevisiae* CCMI 885 and protein analysis demonstrated that the toxic compounds corresponded to antimicrobial peptides (<10 kDa) active against several wine-related non-*Saccharomyces* yeasts (i.e. *K. marxianus, K. thermotolerans*, *T. delbrueckii* and *H. guilliermondii*). Later, Branco *et al*. (2014) showed that *S. cerevisiae* CCMI 885 secretes antimicrobial peptides (AMPs) during alcoholic fermentation that are active against a wide variety of wine-related yeasts and bacteria (e.g. *Oenococcus oe*ni). Mass spectrometry analyses revealed that those AMPs correspond to fragments of the *S. cerevisiae* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein. Two main peptides with molecular weights of 1.638 and 1.622 kDa and the following amino acid

residues VSWYDNEYGYSTR and ISWYDNEYGYSAR were identified. The theoretical isoelectric point (p*I*) of these peptides (4.37) defines these peptides as anionic.

The involvement of GAPDH-derived peptides in wine microbial interactions was further sustained by results obtained in mixed cultures performed with *S. cerevisiae* single mutants deleted in each of the GAPDH codifying genes (*TDH1-3*).

Yeast-bacteria interactions in wine fermentations were also investigated by some authors. Comitini *et al*. (2005) found that certain *S. cerevisiae* strains produce proteinaceous compounds active against the malolactic bacteria. Also Osborne and Edwards (2007) found that a *S. cerevisiae* strain (Ruby.Ferm) secretes a peptide active against *Oenococcus oeni* bacteria that affected the malolactic fermentation process. However, neither of those AMPs were fully characterized.

1.2 Antimicrobial peptides: their nature, function and mode of action

AMPs are small biologically active molecules typically composed of fewer than 50 amino acid residues. They are evolutionary conserved components of the innate immune response and constitute the first line of antimicrobial defense for organisms across the eukaryotic kingdom. AMPs show a wide range of secondary structures such as α-helices and β-strands with one or more disulphide bridges, loop and extended structures, which are highly essential for the broad spectrum of antimicrobial activity they exhibit. Besides these properties, other factors such as size, charge, hydrophobicity, amphipathic stereo geometry and peptide self-association to the biological membrane of microbial cells are also important for their diverse mechanisms of action. In the vast majority of cases, AMPs are cationic and kill microorganisms through mechanisms that predominantly involve interactions between the peptide's positively charged residues and the anionic components of target membrane cells. There are also a number of cationic AMPs that appear to target internal anionic cell constituents such as DNA and RNA (Harris *et al*, 2009; Cézard *et al*, 2011; Pushpanathan *et al.*, 2013).

Although most AMPs are cationic in nature, anionic AMPs (AAMPs) have also been isolated from different organisms (Zasloff, 1987; López-García *et al*., 2006; Dang *et al*., 2006; Vera Pingitore *et al*., 2007; Kong *et al*., 2010.). AAMPs are

common to vertebrates and invertebrates, playing a role in their innate immune systems, which shows the ancient nature of these peptides and their important defense function. Structural characterization shows that AAMPs to generally range in net charge from -1 to -7 and in length from 5 residues to approx. 70 residues. Some AAMPs can be produced constitutively while others appear to be induced in response to microbial infection. A number of inducible AAMPs are encrypted within the primary structures of precursor proteins and require cleavage by proteolytic cleavage. The AAMPs have a broad spectrum of antimicrobial activity against bacteria, fungi, viruses, nematodes and insects. Their mechanism of action seems to rely mainly in membrane interactions. In general, AAMPs adopt amphiphilic conformations for membrane interaction; while some appear to interact *via* the use of receptors, others appear to use metal ions to form cationic salt bridges with negatively charged components of microbial membranes, thereby facilitating interaction with their target organisms (Harris *et al.*, 2009).

1.2.1 Purification methods of AMPs

Adequate purification of AMPs is necessary for their subsequent characterization.

For purification of AMPs it is often difficult to use methods similar to those applied in the purification of other organic compounds, mainly due to their complexity. The methods most commonly used in peptide purification usually utilize various principles of chromatography such as ion-exchange chromatography, gel filtration chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC) (Andersson and Persson, 2000). Chromatography is a technique in which solutes are resolved by their different elution rates as they pass through a chromatographic column. The separation is governed by their partitioning between the mobile phase and the stationary phase.

The common purification strategy consists in a sequence of procedures which include several steps: (1) sample preparation that consists in clarification before the first chromatographic separation step and may include extraction and/or concentration procedures; (2) capture of target molecules- initial purification in order to concentrate and isolate the target molecule; (3) intermediate purification - removal

of bulk contaminants; (4) polishing - removal of trace contaminants in order to obtain high level of purity.

Gel filtration or size exclusion chromatography (GF)

Gel filtration (GF) chromatography separates proteins according to differences in molecular size. The technique is highly efficient for separation of polymeric forms of peptides and for desalting of sample solutions. GF is a non-binding method, is independent of sample concentration and since buffer composition does not directly affect resolution, buffer conditions can be varied to suit the sample type or the requirements for the next purification or analysis step. Polyacrylamide matrices that separate molecules with a molecular weight lower than 10 kDa have been most useful in antimicrobial peptide purification. Disadvantages with gel filtration chromatography are the low capacity and the relatively slow flow-rates that can be applied for optimal separation on such columns (Cole and Ganz, 2000).

Ion-exchange chromatography (IEC)

Ion-exchange chromatography (IEC) technique is dependent on the ionic interaction between the support surface and charged groups of the peptide: the separation is based on the reversible interaction between a charged protein and an oppositely charged chromatography matrix. Target proteins are concentrated during binding and collected in a concentrated form. The mobile phase is typically an aqueous buffer solution and the stationary phase into which the mixture to be resolved is introduced is usually an inert organic matrix chemically derivatized with ionizable functional groups that carry a displaceable oppositely charged counter-ion. These counter-ions are in a state of equilibrium between the mobile and stationary phases, giving rise to two possible IEC formats, namely anion- and cation-exchange. Both cation and anion exchangers have been used with success for peptide purifications.

The net surface charge of proteins is dependent of the surrounding pH: if the net charge of a protein is above its p*I* it will bind to a positively charged anion exchanger; if it is below its p*I* the protein will bind to a negatively charged cation exchanger. Proteins bind as they are loaded onto a column at low ionic strength. The conditions are then altered so that bound substances are eluted differentially. Elution is usually performed by changing pH or altering the ionic strength. Changing the

mobile phase pH alters the net charge of the bound protein and its binding capacity to the matrix. Increasing salt concentration in the mobile phase alters affinity resulting in the displacement of the bound ionic species.

Exchangeable matrix counter-ions may include protons (H^+), hydroxide groups (OH^-), single charged monoatomic ions (Na^+, K^+, Cl^-), double charged monoatomic ions (Ca^{2+}, Mg^{2+}), and polyatomic inorganic ions (SO_4^{2-}, PO_4^{3-}), as well as organic bases (NR_2H^+) and acids (COO^-). A strong ion exchange medium has the same charge density on its surface over a broad pH range, whereas the charge density of a weak ion exchanger changes with pH. The selectivity and the capacity of a weak ion exchanger are different at different pH values (Cummins *et al*., 2011; Andersson and Persson).

Reversed-phase high-performance liquid chromatography (RP-HPLC)

The most powerful method for peptide purification is reversed-phase high performance liquid chromatography (RP-HPLC) that uses hydrophobic interactions as the main separation principle. Separation depends on the hydrophobic binding-capacity of the solute molecule towards the mobile phase or towards the immobilized hydrophobic ligands attached to the stationary phase. It is characterized by the use of a stationary phase (sorbent) and an aqueous mobile phase containing an organic solvent. The solute mixture is initially applied to the sorbent in the presence of aqueous buffers, and the solutes are eluted by the addition of organic solvent to the mobile phase. Elution is usually performed by an increasing organic solvent concentration, in order to increase molecular hydrophobicity. Acetonitrile, methanol, ethanol and propanol are common used organic solvents. RP-HPLC exhibits an excellent resolution that can be achieved under a wide range of chromatographic conditions for very closely related molecules as well as structurally quite distinct molecules; its chromatographic selectivity can be manipulated through changes in mobile phase characteristics; gives the generally high recoveries, shows high productivity and reproducibility. However, RP-HPLC can cause the irreversible denaturation of protein samples resulting in the loss of protein activity (Marie-Isabel Aguilar, 2004).

In the purification of AMPs from biological samples such chromatographic techniques are commonly used to isolate the peptides of interest to further

characterization. In order to isolate and characterize a class of AMPs, magainins from *Xenopus* skin, Zasloff (1987) performed a series of purification steps that included: a sample clarification process by centrifugation to prepare sample for an initial ion-exchange chromatography in a carboxymethyl-cellulose matrix (CM52column); the fraction recovered was further concentrated and fractionated by gel filtration (Gel P-30); finally, active fractions were purified in a Vydac C4 HPLC column, eluted with a gradient of organic solvent acetonitrile. The two active forms purified showed growth inhibition activity against bacteria and fungi. In a 2006 study performed on oriental fruit fly, *Bactrocera dorsalis* (Hendel), one antibacterial fraction was isolated and purified by a pre-purification step, followed by cation-exchange chromatography, gel filtration chromatography and RP-HPLC. Results of physical and biological analysis revealed that this AMP is heat stable and showed strong activities against Gram-positive bacterial growth. Pingitore and colleagues (2007) described different techniques applied to purify some bacteriocins from LAB. Bacteriocins are natural peptides secreted by several bacteria that exert bactericidal activity against other bacterial species. The applied techniques involved salt precipitation followed by various combinations of ion-exchange and reverse phase C18 solid phase extraction, absorption-desorption (AD) and RP-HPLC. The authors emphasized the importance of knowing the characteristics of the different bacteriocins to apply the best purification strategy possible.

The identification of a novel GAPDH-derived AMPs secreted by *S. cerevisiae* during wine fermentation was achieved following two main purification steps: fractionation by GF and then purification using a ion-exchange chromatography and by mass spectrometry analysis. The fermentation supernatants containing the secreted peptides were first ultrafiltered with 10 kDa membranes and then concentrated with 2 kDa membranes. The concentrated fraction was first fractionated by gel filtration chromatography, using a Superdex-Peptide column (10/300 GL) coupled to a High-Performance Liquid Chromatography (HPLC) system equipped with an UV detector. The fractions were eluted with ammonium acetate 0.1 M. All fractions were collected, freeze-dried and screened for antimicrobial activity and an active fraction was then further purified using a strong anion-exchange column (QResource). Peptides were eluted at neutral pH using a gradient of ammonium acetate of 5–500 mM. All the collected fractions showed antimicrobial activity. Active fractions followed sequencing by liquid chromatography electrospray

ionization-tandem mass spectrometry (LC-ESI-MS/MS). Sequence analysis revealed that all peptides present in each anionic fraction correspond to fragments of the *S. cerevisiae* GAPDH isoenzymes, GAPDH2/3 and GAPDH1which are encoded by the *TDH*2, *TDH*3 and *TDH*1 genes, respectively. This work demonstrated that these AMPs identified are anionic (at neutral pH) and are active against several wine-related yeasts (e.g. *D. bruxellensis*) and bacteria (e.g. *O. oeni*) (Branco *et al.*, 2014).

1.2.2 Characterization of AMPs

Peptide characterization is the following step in the process of analyzing AMPs. Peptide mass fingerprinting is the identification of a protein (or a peptide) sequence after its cleavage into small fragments by tryptic digestion followed by mass spectrometry (MS). Mass spectrometry relies on the formation of gas-phase ions (positively or negatively charged) that can be isolated electrically (or magnetically) based on their mass-to-charge ratio (*m/z*). Two main ionization techniques, electrospray ionization (ESI) and matrix assisted laser desorption/ionization (MALDI), are commonly used in MS (Henzel and Watanabe, 2003; Thiede *et al.*, 2005; El-Aneed *et al.*, 2012). Regardless of the ionization source, the sensitivity of a mass spectrometer is related to the mass analyzer where ion separation occurs. Both quadrupole and time of flight (TOF) mass analyzers are commonly used and they can be configured together as QToF tandem mass spectrometric instruments. Tandem mass spectrometry (MS/MS), as the name indicates, is the result of performing two or more sequential separations of ions usually coupling two or more mass analyzers (El-Aneed *et al.*, 2012).

The structural and dynamical characterization of peptides can be performed using a variety of standard techniques as X-ray crystallography, electron diffraction, nuclear magnetic resonance (NMR) and circular dichroism.

1.3 Proteomic analysis of *Saccharomyces cerevisiae* during wine fermentations

Proteomic analysis aims the characterization of all proteins present within a given biological sample. The standard methodology that has been used is the combination of 2 procedures: protein separation by two–dimensional polyacrylamide gel electrophoresis (2D-PAGE/ 2DE) and mass spectrometry (MS) based methods for identification of the resolved proteins. Due to its high resolution and sensitivity, 2D-PAGE is a powerful tool for the analysis and detection of proteins from complex biological sources with the ability to separate thousands of proteins at once. Applications include: whole proteome analysis, post- and co-translational modifications, which cannot be predicted from the genomic sequence; cell differentiation, detection of biomarkers and disease markers; bacterial pathogenesis, purity checks, microscale protein purification and product characterization (O'Farrell, 1975, Bond and Blomberg, 2006, Rabilloud *et al.*, 2009, Sameh Magdeldin *et al.*, 2014).

1.3.1 2D-PAGE

In 1975, O'Farrell and Klose (1975) established a technology for the global study of protein expression: by the two–dimensional polyacrylamide gel electrophoresis (2DE). They applied this method to the analysis of proteins of *Escherichia coli* and to complex protein mixtures of animal extracts. The method consists of two steps of protein separation according with two independent properties: in the first dimension (isoelectric focusing) protein molecules are resolved according to the charge of proteins (i.e. their isoelectric point), while in the second dimension the focused proteins are fractionated according to their molecular weight (O'Farrell, 1975, Klose, 1975).

The principle of isoelectric focusing (IEF) is that electrophoresis is carried out in a pH gradient, allowing each protein to migrate to its isoelectric point (p*I*). The second dimension begins with the equilibration of the isoelectric-focusing gel in a solution of sodium dodecylsulfate (SDS), which is an amphipathic detergent that binds non-specifically to all proteins and confers a uniform negative charge, thereby allowing proteins to be separated only by their molecular mass, under an electric field (O'Farrell, 1975, Klose, 1975).

The 2D-PAGE principle has remained basically the same throughout time, although new improves have been introduced to overcome reproducibility problems with this methodology. Initially, the first dimension was performed in glass tubes and the process of taking out the gels and transferring them onto the slab gel for the second dimension was very difficult, sometimes damaging the gels. Moreover, IEF with carrier ampholytes had reproducibility problems because ampholytes are mobile synthetic molecules with tendency to drift towards the cathode (causing the progressive loss of the basic portions) and distorting the gels. Later on, two main events improved the 2D-PAGE technology such as the replacement of glass tube gels by strip gels (i.e. easier to handle) and the introduction of immobilized pH gradient gels (IPG) in which buffering groups are attached to the gel matrix. There was also developments in sample preparation that helped achieving reproducibility and decreasing variability such the different extraction buffers for specific samples and protein precipitation to clean interfering substances and concentrate samples (Primrose and Twyman, 2008; Rabilloud *et al.*, 2009; Sameh Magdeldin *et al.*, 2014).

For protein visualization, there is a variety of staining methods such as the Coomassie brilliant blue, silver nitrate and fluorescent stains and specific methods as immunodetection and glycoprotein detection (Primrose and Twyman, 2008; Rabilloud *et al.*, 2009).

Gel image analysis is performed with 2D gel image *softwares*, like ImageMaster™ 2D Platinum, which allows us to know the total number of spots in a given gel; the corresponding p*I* and molecular weight (MW) and analyze different protein expression levels between two or more gels.

1.3.2 *Saccharomyces cerevisiae* proteome and surfome analysis

Proteomics is recognized as one of the most important tools in numerous areas of research, namely in the study of *S. cerevisiae*, since this is one of the best physiologically and genetically characterized eukaryotic organism. Thus, proteomic analysis allows an understanding of the global protein expression, as well as the identification and quantitation of proteins. Besides it also gives information regarding proteins function and localization, the biological systems in which a protein is involved and also allows the comparison of different developing stages and

of the physiological responses towards changes in environmental conditions (Pham and Wright, 2007).

Proteome

In the 1990s and early 2000s, numerous studies were conducted in order to construct the whole proteome of *S. cerevisiae*, using 2DE gel-based techniques. In an attempt to construct a gene-protein map, Boucherie *et al.* (1995) were able to correlate proteins of *S. cerevisiae* resolved on 2DE gels with their corresponding genes. They used the 2DE protein map of *S. cerevisiae* strain S288C as a reference map and 200 polypeptides spots were detected after the experiment. The p*I* of the separated proteins ranged between 4.2 and 6.8 and their relative MW between 15 kDa and 150 kDa. They reported the identification of 36 novel polypeptides on the yeast protein map, which corresponded to the products of 26 genes. The proteins identified concerned with four major areas of yeast cellular physiology: carbon metabolism, heat shock proteins, amino acid biosynthesis and purine biosynthesis. One year later, another project with the aim of linking genome-proteome, with the same yeast strain S288C, used a combination of 2DE gels and MALDI and nano-ESI-MS/MS to analyze the proteins. Although a substantial number of proteins were found in the 2DE gel coordinates different from the ones expected based on their sequence, they stated that a total of 150 gel spots were successfully analysed, greatly enlarging the yeast 2DE gel data base. Besides, more than 32 proteins were novel and matched to previously uncharacterized open reading frames in the yeast genome (Shevchenko *et al.*, 1996). In the same year, Sanchez and coworkers (1996) performed 2DE of *S. cerevisiae* proteins with the intention of describing the yeast SWISS-2D-PAGE database. Therefore, they used *S. cerevisiae* X2180-1A and X2180-2B strains as 2D-PAGE reference map. The innovative aspect of that work consisted in the use of IPGs for the IEF separation, since several yeast 2D-PAGE databases already established by that time were based in first dimension separation using carrier ampholyte pH gradients. They reported the identification of more than 100 polypeptides that were identified by gel comparison, amino acid composition analysis and/or microsequencing; several of those proteins were newly mapped. The identified spots included among others: alchohol dehydrogenase, enolases, fructose-biphosphate aldolase, phosphoglycerate mutase, pyruvate decarboxylase isoenzyme, heat shock proteins and glyceraldehyde 3-phosphate dehydrogenases 1,2,3 (Sanchez

et al., 1996). Proteome studies in *S. cerevisiae* conducted by Garrels and colleagues (1997) aimed the identification and characterization of abundant proteins in order to complete the yeast 2DE map of abundant proteins. The results obtained extended the yeast 2DE protein map to 169 identified spots based on the yeast genome sequence and showed that methods of spot identification based on predicted p*I*, predicted MW and determination of partial amino acid composition from radiolabeled gels were powerful enough for the identification of at least 80% of the spots representing abundant proteins. Comparison of proteins predicted by the Yeast Protein Database (YPD) to be detectable on 2DE gels based on calculated MW, p*I* and codon bias (a predictor of abundance) with proteins identified in this study, suggested that many glycoproteins and integral membrane proteins were missing from the 2DE gel patterns. The authors also performed 2DE gel experiments to analyse and characterize the yeast proteins associated with:(i) an environmental change (heat shock), (ii) a temperature-sensitive mutation, (iii) a mutation affecting post-translational modification (N-terminal acetylation) and (iv) a purified subcellular fraction (the ribosomal proteins), using the 2DE gel map and the information available in the YPD. Another work extended the yeast 2DE protein map with 92 novel protein spots, which were identified by three methods: gene overexpression, amino acid composition and mass spectrometry. The results were recorded in the Yeast Protein Map server (Perrot *et al*., 1999). In 2002, a 2DE reference map of very alkaline yeast cell proteins was established by using IPGs up to pH 12 (IPG 6–12, IPG 9–12 and IPG 10–12) for 2DE and by using MALDI-TOF peptide mass fingerprinting for spot identification. 106 proteins with theoretical p*I* up to pH 11.15 and MW between 7.5 and 115 kDa were localized and identified. Most of the proteins (66 spots) were identified from the IPG 6–12 gel due to the improved resolution of narrow IPGs. In addition 37 spots were analysed using IPG 9–12 and 29 spots using the IPG 10–12. 49 of the 106 spots identified were ribosomal proteins. The remaining 57 were eight of unknown function, 17 oxidoreductases, 7 lyases, 6 transferases, 4 hydrolases, 3 isomerases (including 2 chaperones), 3 ligases, 2 heat shock proteins/chaperones, 3 DNA binding proteins including histones H2B and H2A-1, 2 transcription factors, 1 protein conjugation factor and 1 transporter channel protein (Wildgruber *et al.*, 2002).

Several comparative proteomic analysis studies have been carried out in an attempt to study the yeast response to different environmental conditions such as:

induced fermentation stress conditions in a wild-type wine strain of *S. cerevisiae* (Trabalzini *et al*., 2003); studies in yeast growth in chemostat cultures limited by glucose and ethanol (Kolkman *et al*., 2005); comparative proteomic analysis of transition of *S. cerevisiae* from glucose-deficient medium to glucose-rich medium (Giardina *et al*., 2012); proteome analysis of recombinant xylose-fermenting yeast strain, comparing conditions in which glucose or xylose was the carbon source (Salusjarvi *et al*., 2003); proteomic response to amino acid starvation in *S. cerevisiae* (Yin *et al*., 2004) and several other studies on changes in the yeast proteome as a function of the stimuli in the environment, such as cadmium, lithium, hydrogen peroxide, sorbic acid, amongst others.

Surfome

Yeast's cells are delimited by a membrane structure that is composed by a cytoplasmatic membrane and a cell wall that are separated by the periplasmic space. The rigid cell wall is an essential structure required for maintaining cellular shape and integrity by protecting cells against osmotic changes in the environment. Cell membranes act as filters, permitting the passage of some molecules while excluding others. The cell membrane is not an inert structure and undergoes profound changes as a consequence of cellular processes such as budding, apical extension of hypha, dimorphic transitions and mating. Membranes also play an important role in cellular processes such as flocculation, adhesion and pathogenicity (Pardo *et al*., 1999; Pardo *et al*., 2000; Braconi *et al*., 2011). The cell wall of *S. cerevisiae* is composed by an internal layer consisting of a flexible network of β-1,3-glucan molecules with covalently attached β-1,6-glucan and chitin, and an external fibrillar layer of mannoproteins, which are mannose-containing glycoproteins (Braconi *et al*., 2011). Synthesis of *S. cerevisiae* cell wall components could take place in two steps. First, chitin and β-1,3-glucan are synthesized by the cytoplasmatic membrane bound enzyme complexes through a vectorial process in which the formed chains are extruded through the cytoplasma membrane. Mannoproteins are synthesized and secreted through the secretory pathway. Part of β-1,6-glucan synthesis may take place in the endoplasmic reticulum or Golgi. Following this, all components will then interact and assemble to form a functional cell wall (Pardo *et al*., 1999). β-1,3-glucan and chitin are responsible for the cell wall strength while mannoproteins determine its porosity, act as structural proteins and as enzymes involved in cell wall

biogenesis. The complex nature of the cell wall is emphasized by the fact that approximately 20% of the *S. cerevisiae* genome is required for the biogenesis of this structure (Braconi *et al*., 2011).

Cytoplasmatic membrane is composed mainly by an amphoteric bilayer of phospholipids that form an impermeable barrier to hydrophilic molecules and within which are enclosed proteins. Membrane proteins are highly specialized and mediate a variety of crucial cellular functions such as sensing/signaling external environmental conditions, transport of nutrients, endocytosis/exocytosis, cytoskeleton anchoring, generation of membrane potential and cell wall synthesis and maintenance. Membrane phospholipids are, in their majority, composed by phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (Aliverdieva *et al.*, 2004, Bisson, 2004; Delom *et al*., 2006).

Several studies have been performed in order to analyze the protein composition of the cytoplasmatic membrane and of the cell wall using different protein separation and identification strategies such as 2DE and mass spectrometry. Two main works conducted by Pardo *et al.* (1999; 2000) analysed the proteins secreted by regenerating protoplasts as a way to understand the cell wall biogenesis and identify cell wall proteins. They reported the identification of several proteins with different functions: proteins involved in cell wall construction (β-1,3-glucanosyl transferase; GPI cell wall protein; exoglucanase); enzymes involved in glycolysis or fermentation (alcohol dehydrogenase, enolases, fructose biphosphate aldolase, pyruvate decarboxylase, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase (*TDH* 1,2,3)); heat shock proteins (PIR proteins and Hsp70 family); amongst others. Aliverdieva *et al.*, (2004) designed a simplified method for the isolation of cell membranes fractions for the evaluation of their protein composition and they were able to identify several spots in 2DE gels that in their majority corresponded to protein transporters (high-affinity hexose transporter, cation transporter, phosphate transporter, lactate and pyruvate transporter, sodium/phosphate symporter, low-affinity iron transporter, ammonium ion transporter, general amino acid transporter and others).

The surfome corresponds to the cell surface proteome i.e. proteins that are exposed at the surface of the cell. A relevant study (for the aim of the present work) on the analysis of the surfome of a wild-type wine *S. cerevisiae* strain during wine fermentation was performed by Braconi *et al*. (2011). In that work cell surface-

exposed proteins were extracted by trypsin “shaving” of intact cells and the 2DE resolved proteins were identified by nLC-ESI-LIT-MS/MS. Those authors reported a total of 42 identified proteins, out of which 16 were specifically expressed at the beginning of the fermentation and 14 at the end of the process. Carbon metabolism-related proteins (particularly involved in glycolysis and fermentation) accounted for 21% of the 42 identified and the following information can be summarized:

- GAPDH was detected both at the beginning and at the end of fermentation. GAPDH (an enzyme involved in the glycolysis pathway) incorporation into the cell wall is considered responsive to environmental factors and not requiring de novo protein synthesis, i.e. stress conditions cause the incorporation of pre-existing GAPDH into the yeast cell wall.
- Hexokinase 2 (Hxk2p, catalyzing glucose phosphorylation in the cytosol) was detected as surface-expressed proteins at the beginning of fermentation.
- Phosphogluconate dehydrogenase catalyzes the second oxidative reduction of $NADP^+$ to NADPH. It is also important for protecting yeast from oxidative stress, since NADPH is an essential cofactor for several enzymes involved in the cell protection against oxidative damages
- Enolase is among the most abundant enzymes in S. cerevisiae cytosol. It catalyzes the conversion of 2-phosphoglycerate to phosphoenolpyruvate in the fourth glycolytic step.
- Pyruvate decarboxylase isozyme, Pdc1p is a key enzyme in alcoholic fermentation, decarboxylates pyruvate to acetaldehyde; subjected to glucose-, ethanol-, and autoregulation, it is involved in amino acid catabolism.

Stress response proteins (14%) were also detected: heat shock proteins (Hsp), SSA1 and SSA2 that are ATPases involved in protein folding and nuclear localization signal and members of the 70 heat shock proteins family. SSA1 protein was found at the end and SSA2 at the beginning of fermentation. Two other stress responsive proteins were collected at the end of fermentation: Hsp82p, belonging to the Hsp90 family, and Sod1p (cytosolic superoxide dismutase). Related to protein biosynthesis (48% of the total protein), the authors reported several ribosomal and elongation factors in both stages of fermentation. 10% of the proteins identified were involved in the control of cellular organization: protein BMH1, exo-1,3-bglucanase (Exg1p), and cell wall mannoprotein PST1 exclusively at the end of fermentation.

Bmh1p is involved in yeast physiology regulation through a variety of cell signaling pathways, including chitin synthesis at the cell wall and Pst1p is known to be involved in a repair mechanism activated upon cell damage (Braconi *et al*., 2011).

2 Materials and methods

2.1 Microorganisms

In this study two yeast species were used: *Saccharomyces cerevisiae* strain CCMI 885 (Culture Collection of Industrial Microorganisms, ex-INETI, Lisbon Portugal) originally isolated form the indigenous microflora of grape musts of Alentejo region and *Hanseniaspora guilliermondii* strain NCYC 2380 (National Collection of Yeasts Cultures, Norwich, United Kingdom) originally isolated from grapes of Douro region. All strains were maintained in YEPD-agar slants, stored at 4°C and reactivated periodically.

2.2 Culture media and inocula

Alcoholic fermentations were performed in a Synthetic Grape Juice (SGJ), which has a chemical composition similar to the natural grape musts. The SGJ was prepared by mixing three solutions (A, B and C), as described in Pérez-Nevado *et al*. (2006). The final composition of the SGJ was (per litre): (from solution A) D-glucose, 110 g; D-fructose, 110 g; (from solution B) L-(1)-tartaric acid, 6.0 g; L-(2)-malic acid, 3.0 g; citric acid, 0.5 g; (from solution C) YNB (yeast nitrogen base), 1.7 g; CAA (vitamin-free Casamino Acids), 2.0 g; CaCl2, 0.2 g; arginine-HCl, 0.8 g; L-(2)-proline, 1.0 g; L-(2)-tryptophan, 0.1 g. Solutions B and C were buffered at pH 3.5 with NH_4OH and H_3PO_4, respectively.

Inocula of *S. cerevisiae* and *H. guilliermondii* were prepared by transferring biomass from one YEPD-agar slant (pre-grown with each strain at 30ºC for 48 h) into 250 ml Erlenmeyer-flasks containing 100 ml of YEPD medium (10 g/l yeast extract, 20 g/l peptone and 20 g/l glucose). The respective single cultures were incubated in an orbital shaker (G25 Incubator Shaker, New Jersey, USA) at 30º C and 150 rpm of agitation for 16 h.

2.3 Purification of antimicrobial peptides (AMPs) secreted by *S. cerevisiae* during alcoholic fermentation

2.3.1 Production of alcoholic fermentations supernatants

To obtain large amounts of AMPs secreted by *S. cerevisiae* during alcoholic fermentation, we performed three microvinifications in 2-L Erlenmeyer flasks containing each 1000 ml of SGJ. Each flask was inoculated with 10^5 cells/ml of *S. cerevisiae* (inoculum prepared as described in section 2.2.) and incubated in an orbital shaker (G25 Incubator Shaker, New Jersey, USA) at 30ºC and 150 rpm of agitation, for 7 days. Microvinifications were controlled by measuring cell growth, as well as sugars consumption and ethanol production.

Daily samples were taken from each microvinification and cell growth was evaluated by the number of colony forming units (CFU), determined by the classical plating method. Briefly, 100 µl of culture sample were diluted in deionized water and after appropriated dilutions (decimal serial dilution method) inoculated in YEPD-agar plates. The plates were incubated at 30 ºC in a vertical incubator (Infors, Canada) and the number of CFU enumerated after 2-3 days.

Sugars (glucose and fructose) and ethanol concentrations were determined by High Performance Liquid Chromatography (HPLC) in cell-free samples obtained by filtration with 0.45 um Millipore membranes (Merck Millipore, Algés, Portugal). The HPLC system (Merck Hitachi, Darmstadt, Germany) was coupled with a refractive index detector (HPLC, Merck Hitachi, Darmstadt, Germany). Samples were injected (20 µl) into a Sugar-Pak™ column (Waters, Milford, USA) and eluted with a degassed mobile phase (50 mg/ml of CaEDTA) at 90ºC with a flow rate of 0.5 ml/min. Glucose, fructose and ethanol standards at concentrations of 15, 7.5 and 3.75 g/l were used for calibration curves.

At the end of the alcoholic fermentation process (residual sugars <2 g/l), the whole culture media (1000 ml for each fermentation) was collected and filtrated twice by 0.45 µm Millipore membranes (Merck Millipore, Algés, Portugal). The cell-free supernatants were then sterilized by filtration through 0.22 µm membranes (Merck Millipore, Algés, Portugal). Total protein concentration of the supernatants was quantified by spectrometry using a NanoDrop™ 2000 spectrophotometer (Thermo Scientific, Delaware, USA) and measuring absorbance at 280 nm. Sterile

supernatants (3000 ml in total) were stored at 4 ºC until utilization in the preparative anion-exchange chromatographic column (DEAE-Sephadex).

In order to confirm the presence of the bioactive peptides (2-10 kDa) previously found in alcoholic fermentation supernatants (Branco *et al*, 2014). 45 ml of the cell-free supernatants were ultrafiltered through centrifugal filter units (Vivaspin 15R, Sartorius, Göttingen, Germany) equipped with 10 kDa membranes and 2 kDa membranes. First, the supernatant was ultrafiltered through the 10 kDa membranes and then the permeate (<10 kDa) was concentrated (10-fold) by passing through the 2 kDa membranes. Ultrafiltration was performed at 20 ºC and 6000 x *g* in a bench centrifuge (Sigma 2-16 K, Sartorius, Germany) and the concentrated fraction (2-10 kDa) was frozen and kept at -80ºC.

2.3.2 Purification of the AMPs from the fermentation supernatants using a preparative anion-exchange column

Proteins of the alcoholic fermentation supernatants (approx. 3000 ml) were fractionated in a preparative chromatographic system using an anion-exchange resin diethylaminoethyl (DEAE) Sephadex A-25 (GE Healthcare, Uppsala, Sweden). This gravity-flow chromatographic system was composed of a preparative glass column (34 cm long x1.5 cm inner diameter) filled with the 588 ml of DEAE-Sephadex resin. A gradient elution system composed of two 2-L Erlenmeyer flasks (of 2-L each), one containing 2000 ml of 2 M ammonium acetate and the other 2000 ml of 2 mM ammonium acetate, was coupled to the column. Both Erlenmeyer flasks were connected by a plastic tube, with the low salt concentration flask being agitated to ensure that the gradient was reached. Elution was carried out by gravimetric force.

Each purification procedure was performed by loading the preparative column with 500 ml of alcoholic fermentation supernatant (diluted 1:2 with deionized water and pH adjusted to 8) and proteins eluted with the ammonium acetate salt gradient above-mentioned. Protein fractions collected into glass tubes, (approx. 25 ml in each tube), until the elution gradient was finished. Subsequently, all protein fractions collected were analysed by spectrophotometry and the spectrum of absorbance range from 250 to 800 nm (UV-1800, Shimadzu UV-Vis Spectrophotometer). Samples (25 ml each) exhibiting similar absorbance spectra were grouped in major fractions.

These major DEAE-Sephadex-protein fractions were frozen and kept at -80ºC for further analysis.

2.3.3 Antimicrobial activity of the protein fractions obtained in the preparative anion-exchange DEAE-Sephadex column

In order to search for the previously found AMPs (Albergaria *et al*., 2010; Branco *et al*., 2014), the antimicrobial effect of each DEAE-Sephadex-protein fraction was evaluated in growth assays performed with a sensitive yeast strain. Antimicrobial active fractions have 2-10 kDa AMPs (Albergaria *et al*., 2010; Branco *et al*., 2014) and yellow color (data not published), therefore 100 ml of only yellow-colored fractions (obtained from DEAE-Sephadex column) were ultrafiltered using centrifugal filter units (Vivaspin 15R, Sartorius, Göttingen, Germany) equipped with 10 kDa membranes. The <10 kDa fractions were then concentrated 10-fold by passing through the 2 kDa membranes. Ultrafiltration was performed at 20 ºC and 6000 x *g* in a bench centrifuge (Sigma 2-16 K, Sartorius, Germany). The protein concentration of the 2-10 kDa fractions was determined by spectrometry using a NanoDrop^{TM} 2000 spectrophotometer (Thermo Scientific, Delaware, USA) and measuring absorbance at 280 nm and concentrated fractions were then vacuum–dried until further use.

Antimicrobial activity of the 2-10 kDa fractions was tested against the sensitive strain *H. guilliermondii* and performed in 96-well microplates. Lyophilized fractions were resuspended in a total volume of 100 µl of YEPD with 30 g/l of ethanol and a pH 3.5, to a final protein concentration of 1 mg/ml. Control assay was performed without addition of any fraction. Each well was inoculated with 10^5 cells/ml of *H. guilliermondii* (inocula prepared as described in section 2.2) and the microplates were incubated in a thermo-shaker (Infors HT, Bottmingen, Switzerland) at 30 °C and 700 rpm of agitation. The inhibitory activity was evaluated by means of growth measurements using a microplate reader (Dinex Technologies Inc., Chantilly, USA). Growth was followed by absorbance measurements at 590 nm and by CFU counts in YEPD-agar plates using the classical plating method. All antimicrobial tests were performed in triplicates.

2.3.4 Analysis of the bioactive fractions obtained in the preparative DEAE-Sephadex column by gel filtration and ion-exchange chromatography

In order to compare analytic profiles of bioactive fractions against the sensitive strain *H. guilliermondii* (Branco *et al*., 2014), the protein fractions were purified and analysed by filtration followed by ion-exchange chromatography using the same procedure as Branco *et al*. (2013). First, protein fractions were subjected to gel filtration chromatography, using an HPLC system (Merck Hitachi, Darmstadt, Germany) equipped with an UV detector (Merck Hitachi, Darmstadt, Germany). 200 µm of each fraction were loaded into a Superdex Peptide column (10/300 GL, GE Healthcare, London, UK) and eluted with 0.1 M ammonium acetate at a flow rate of 0.7 ml/min. The fraction of interest, which exhibits an apparent molecular weight (MW) of 8 kDa, was collected after approx. 25 min of sample running. This 8 kDa gel filtration fraction was first lyophilized and then further analysed in a strong anion-exchange column (QResource 6 ml, GE Healthcare, London, UK). Samples were eluted at neutral pH using a gradient of ammonium acetate (ranging from 5–500 mM) between 10 and 40 min at a flow rate of 1 ml/min.

2.4 Proteomic analysis of the cell wall-associated proteins (surfome) of *S. cerevisiae* cells during alcoholic fermentation

2.4.1 Extraction of S. cerevisiae cell-wall associated proteins

In order to extract the proteins associated to the plasma membrane of *S. cerevisiae* cells, four cultures were prepared in 2-L Erlenmeyer-flasks containing 1000 ml of SGJ medium. Each flask inoculated with 10^5 cells/ml of S. cerevisiae inoculum and incubated in an orbital shaker (G25 Incubator Shaker, New Jersey, USA) at 30º C and 150. Two flasks were incubated for 12 h and the other two for 48 h, respectively, and daily samples were taken from each culture to determine the number of viable cells (CFU/ml). After 12 h and 48 h of incubation, respectively, the entire culture broth was centrifuged for 5 min at 4 ºC at 10000 *g* in a bench centrifuge and the cell pellets separated were collected while the supernatants were discarded.

Isolation of plasma membranes from 12 h and 48 h grown cells for extraction of proteins was performed as described by Van Leeuwen *et al*. (1991) with slight modifications. Grown *S. cerevisiae* cells were centrifuge to separate supernatants from the cell pellet. The cell pellet was washed twice with ice-cold distilled water and once with buffer A (0.1 M glycine, 0.3 mM KC1 at pH 7.0) in a Sigma centrifuge (Sigma 2-16K, Sartorius, Germany) for 20 min at 4˚C and 9000 rpm of agitation. *S. cerevisiae* cells were resuspended in a proportion of 15 g cells per 15 ml of buffer A containing a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail Tablets, Roche, USA). The cell lysis was carried out in a French press (Thermo Scientific, Delaware, USA) at 20000 psi.

Then, 50 ml of buffer A was added to the lysate and centrifuged for 10 min at 2100 x *g*, 4˚C, in a Sigma centrifuge (Sigma 2-16K, Sartorius, Germany). The supernatant was filtered with a 0.22 µm membrane (Merck Millipore, Algés, Portugal) and centrifuged with the conditions mentioned above. By slowly addition of 1 M HCl and at constant stirring, the supernatant (kept on ice) was adjusted to pH 4.9 to aggregate mitochondrial membranes and centrifuged straightaway. The supernatant was adjusted to pH 7.0 with 1 M KOH, immediately after, and the pellet was resuspended in 10 ml of buffer A, repeating the last procedure (pH adjustment to 4.9 followed by centrifugation). The supernatants with the plasma membranes combined supernatants with the plasma membranes were adjusted to pH 5.0 and centrifuged for 3 min at 7700 x *g* and 4˚C. The final supernatant was again adjusted to pH 7 and kept at -80˚C.

The purified plasma membranes were defrosted at room temperature and centrifuged for 20 min at 100000 x *g*, 4˚C, in an ultracentrifuge (Beckman Coulter, OptimaTM LE-80K, California, USA). The supernatant was discarded and the pellet resuspended in 30 ml of buffer B (1 mM EDTA, 10 mM Tris, pH 7.4) and centrifuged once again, with the same conditions. The final pellet was resuspended and in 0.5 ml sample buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer pH 3-10), aliquoted and stored at -80˚C.

2.4.2 Two dimensional polyacrylamide gel electrophoresis

Prior to 2D electrophoresis, the samples were cleaned from contaminants by a precipitation method using a 2D Clean-Up Kit (GE, Healthcare, London, UK). The obtained pellet was air-dried for 5 minutes and the proteins were resuspended in the rehydration solution (7 M urea, 2 M thiourea, 2% (m/V) CHAPS, 0.5% (V/V) IPG buffer (pH 3-10), 0.002% (m/V) bromophenol blue and 0.28% (m/V) dithiothreitol (DTT)). The protein concentration was determined by the Bradford method (BIO-RAD Protein assay, California, USA).

Rehydration of Immobiline DryStrip gel strips with linear 4-7 pH gradient, 7 cm in length, (GE Healthcare, London, UK) was performed with 125 µl of rehydration solution (containing 35 µg of sample proteins), for 16 h.

The first dimension was run in the Ettan IPGphor III system (GE Healthcare, London, UK) with in the following conditions: 200 V for 1 h; 500 V for 30 min; voltage gradient up until 1000 V for 30 min; voltage gradient up until 5000 V for 1h30; 5000 V for 1h30; for a total of 12825 Vh at 20 ˚C.

After isoelectric focusing, equilibration of the strips was performed in two steps. In the first one, strips were equilibrated in equilibration buffer (6 M urea, 50 mM tris-HCl pH 8.8, 30% (V/V) glycerol, 2% (m/V) SDS and bromophenol blue) with 10 mg/ml of DTT for 20 min. In the second step, the procedure was repeated with 25 mg/ml of iodoacetamide instead of DTT. Both equilibration steps were performed in a rocking platform shaker (VWR International, USA).

In the second dimension, the strips were placed onto gradient 4-12% Bis-Tris SDS-PAGE gels (NuPAGE® NOVEX® Zoom® Protein Gels, 1.0 mm, IPG well, Life Technologies, Thermo Fisher Scientific, USA). The gel was also loaded with weight molecular markers (diluted 1:10) (Mark12™ Unstained Standard, Life Technologies, Thermo Fisher Scientific, USA). The electrophoresis was run at 150 V for 1h25 in a XCell SureLock™ Mini-Cell Electrophoresis System (Life Technologies, Thermo Fisher Scientific, USA), using MES running buffer (1 M MES, 1 M Tris Base, 69.3 mM SDS, 20.5 mM EDTA, pH 7.3 – stock solution).

After SDS-PAGE electrophoresis, gels were silver-stained using the following protocol.

Table 1 Silver Staining Protocol

Steps	Reagents	Time
Fixing 1st step 2nd step	40% methanol, 10% trichloroacetic acid (TCA), 120 ml deionized water	Overnight 30 min
Sensitization	30% methanol, 0.632 g thiosulfate sodium pentahydrate, 6.8 g sodium acetate, 70 ml deionized water	30 min
Washing	100 ml deionized water	5 x 5 min
Silver staining	100 mg silver nitrate, 100 ml deionized water	20 min
Washing	100 ml deionized water	2 x 1 min
Development	5 g sodium carbonate, 200 µl formaldehyde 37%, 100 ml deionized water	~ 5 min
Stopping	3.7 g Titriplex III; 100 ml deionized water	10 min
Washing	100 ml deionized water	3 x 5 min

All steps were performed in a rocking platform shaker (VWR International, USA) at room temperature.

Image and statistical analysis of proteins was performed using ImageMaster 7.0 software (GE Healthcare). The intensity level of the spots was determined by the relative spot volume of each protein compared to the normalized volume of proteins. The relative abundance of each protein spot was compared between the cell wall-associated protein at 12 h and 48 h; the spots with an intensity ratio higher than 5 were considered significantly different.

3 Results and discussion

3.1 Purification of antimicrobial peptides from wine fermentation supernatants

Albergaria *et al*. (2010) found that *S. cerevisiae* secretes AMPs during mixed culture fermentation which cause the early death of non-*Saccharomyces* yeasts. Afterwards, Branco *et al*. (2014) purified those AMPs by gel filtration and ion-exchange chromatography. The isolated peptides were then sequenced by mass spectrometry showing that these peptides correspond to fragments of the *S. cerevisiae* GAPDH protein. The AMPs revealed to possess a great potential as natural preservatives against spoilage microorganisms in several industrial food processes, such as in winemaking.

Considering the potential of application of the above-mentioned AMPs, the aim of the present work was to produce and isolate them in a preparative scale so that sufficient amounts of purified AMPs could be obtained and applied in winery fermentations.

3.1.1 Production of supernatants from alcoholic fermentations

To obtain large amounts of AMPs, we performed three microvinifications in 2-L Erlenmeyer flasks containing each 1000 ml of SGJ. Alcoholic fermentations with *S. cerevisiae* were performed under enological growth conditions (slow agitation) during a seven-day period. Culturability as well as sugars consumption (glucose and fructose) and ethanol production were determined during the whole fermentation period (**Fig. 3.1-a,b**). Analysis of yeast growth (**Fig. 3.1a**) shows that there was an initial exponential growth phase during the first day of fermentation, where cell density reached a maximum of about 10^7 cells/ml. The apparent absence of a lag phase was most likely due to lack of sampling analysis in the initial hours after inoculation and to the pre-inoculum status that allowed a rapid initiation of cell division. In the following days a stationary growth phase was observed up to the 3rd day of fermentation. After the 3rd day of fermentation no CFU values were obtained due to technical problems with the plating method. Although there was no data related to cell viability for the last days of fermentation, analysis of sugars

consumption and ethanol production profiles (**Fig. 3.1b**) shows that fermentation was almost complete at the 3rd day of fermentation.

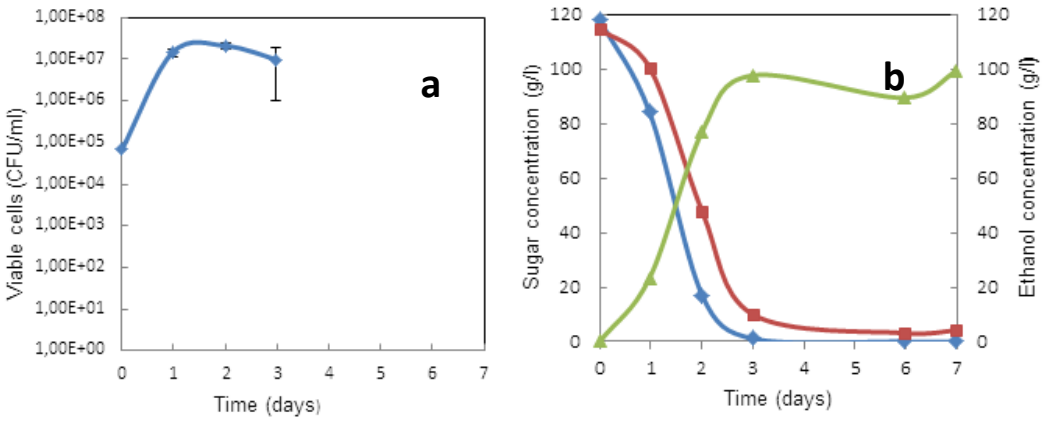

Figure 3.1 - Viable cells of *S. cerevisiae* **(a)** and sugars consumption (*blue filled diamonds –glucose; red filled squares-fructose*) and ethanol production (*green filled triangles)* **(b)** during alcoholic fermentation. Data presented are mean values of three independent experiments.

Glucose and fructose initial concentrations were 118 g/l and 114 g/l, respectively. *S. cerevisiae* sugars consumption profiles demonstrates that after 3 days of fermentation yeasts consumed nearly 117 g/l of glucose and 104 g/l of fructose, with glucose being completely consumed by the 4th day, while fructose was almost completely consumed at the 6th day of fermentation (with 110 g/l consumed). This difference in the sugars consumption profile is due to the glucophilic behavior of *S. cerevisiae* that has higher affinity towards glucose than fructose. Ethanol concentration reached 97 g/l by the 3rd day of fermentation, followed by a slight increase up to 99 g/l at the 7th day of fermentation. These results show that cells kept their viability throughout the entire stationary growth phase (i.e. from the 4th to the 7th day) and also that the fermentation proceeded as expected, i.e.: glucose was fully depleted by the 7th day and conversion of sugars to ethanol reached 89% of the maximal theoretical yield.

3.1.2 Purification of the AMPs from the fermentation supernatants using a preparative anion-exchange column

Branco *et al*. (2014) found that the AMPs produced by *S. cerevisiae* during alcoholic fermentation are anionic (at neutral pH), with a theoretical p*I* of 4.37. Therefore, to isolate and purify these AMPs we used a preparative scale ion-exchange chromatographic column.

Cell-free supernatants obtained from the previously described alcoholic fermentations were fractionated using an anion-exchange resin constituted by a cross-linked dextran matrix (Sephadex A-25), with positively charged groups of diethylaminoethy (DEAE). Since the pH of the fermentation supernatants was 3.5, it was necessary to raise the pH to a value of 8.0 before loading the chromatographic column in order to retain the anionic peptides (p*I* of 4.37) in the ion-exchange resin. Then, 500 ml of a 1:2 diluted supernatant (pH=8.0) were loading into the chromatographic system and proteins eluted with a mobile phase of ammonium acetate using a salt gradient of 2 mM-2 M. First, the column was washed with 2 mM ammonium acetate and the respective liquid fraction recovered. That fraction, front solvent (FS), contained all the supernatant proteins that did not bind to the resin. In the end of the salt gradient elution, a final fraction eluted with 2 M of ammonium acetate was also recovered for further analysis (F85).

Fractions (25 ml each) collected from the chromatographic column were analysed by spectrophotometry in the absorbance spectra of 250-800 nm. It is well-known that proteins absorb light at 280 nm but also between 200-220 nm due to strong absorption of peptidic bonds at those wavelengths. However, salts can also absorb light between 200-220 nm, and thus we found high absorbance in all samples at these wavelengths due to the presence of ammonium acetate in the samples. Thus, after analyzing the absorbance spectra of all the 85 protein fractions (25 ml each) we grouped those fractions exhibiting similar absorbance spectra and strong absorbance at 280 nm.

In **Fig. 3.2** are presented the absorbance spectra of the following fractions:

- FI- V=0-250 ml;
- FII- V=275-875 ml;
- FIII- V=900-1625 ml;
- FIV- V=1650-2000 ml;
- FV- V=2025-2125 ml.

Since we know from unpublished data of the LNEG's research group that the AMPs fractions from alcoholic fermentations exhibit a light-yellow color, we included visible light in the absorbance spectra (400-750 nm) to help to detect fractions containing the AMPs we were interested on.

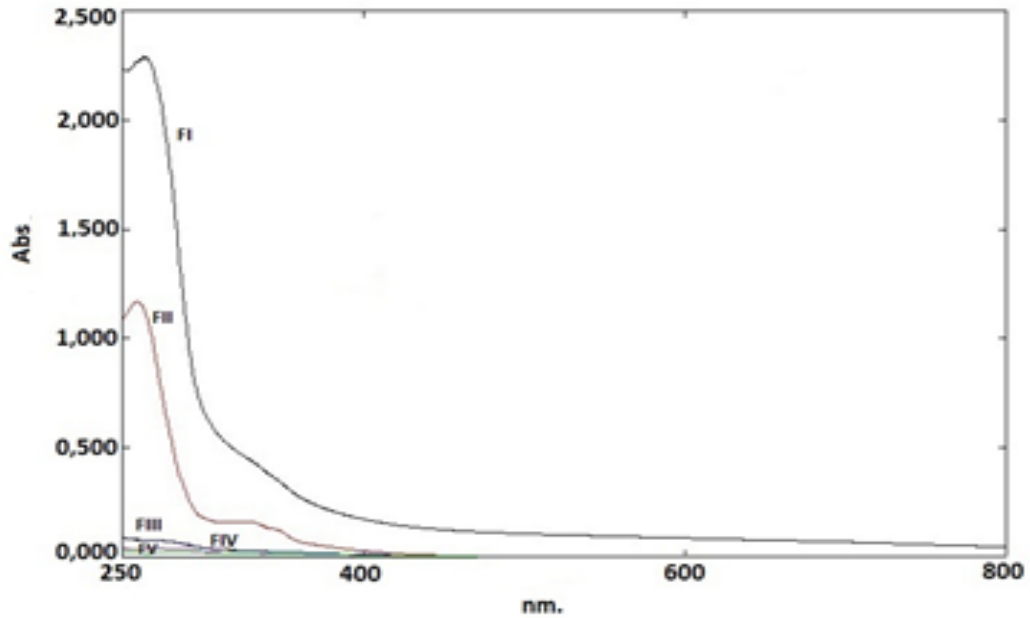

Figure 3.2 - Absorbance spectra (UV-visible) of the supernatant protein fractions FI, FII, FIII, FIV and FV collected from the DEAE-Sephadex ion-exchange chromatographic column.

Fig. 3.2 shows that only fractions FI and FII exhibit high values of absorbance at 280 nm, indicating they are the only ones with high protein content; the remaining fractions show values of absorbance close to zero over the entire spectrum; and, finally, there are no peaks in the visible zone corresponding to the yellow color (565-590 nm) in any of the fractions.

Since this analysis showed there was no significant absorbance in the visible zone, the wavelength of 280 nm was selected to detect the peptidic compounds.

Thus, we plotted the absorbance values of samples at 280 nm in function of the volume collected from the chromatographic column (**Fig. 3.3**). Besides, peptides containing aromatic amino acid residues such as tryptophan (W), Tyrosine (Y) and Phenylalanine (F) are known to strongly absorb light at 275-280 nm. And, we know from Branco *et al*. (2014) that the AMPs we are searching for contain these amino acids in their sequences (VSWYDNEYGYSTR/ ISWYDNEYGYSAR).

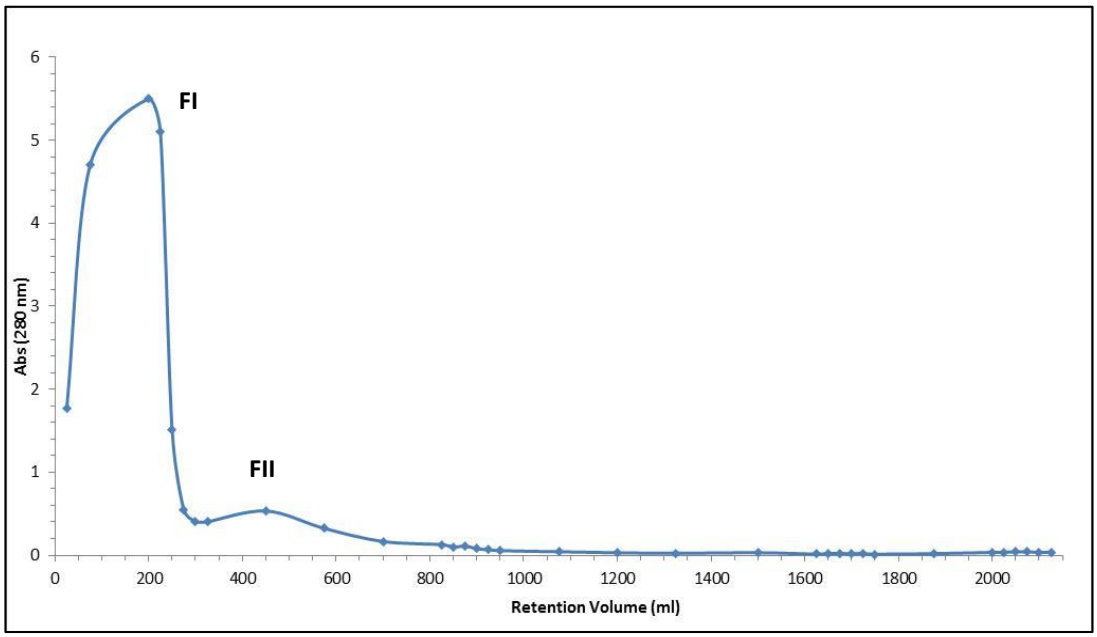

Figure 3.3 - Chromatogram of the protein fractions collected from the DEAE-Sephadex chromatographic column. Sample: 500 ml of alcoholic fermentation supernatant (diluted 1:2; pH=8.0).Gradient elution of 2 mM-2 M ammonium acetate. Absorbance at 280 nm

Fig. 3.3 shows that the first band eluted corresponds to FI (V=25-250 ml) and the second one to fraction FII (V=275-875 ml). At 280 nm, fraction FI shows a high absorbance peak (of approx. 6 AU), whereas fraction FII has a maximum absorbance of approx. 0.5 AU. The following fractions show no significant absorbance at 280 nm, which means they do not contain proteic compounds.

Since there are only two visible peaks along the entire chromatogram, we can conclude that the anionic proteins present in the supernatants were not properly resolved by this anion-exchange chromatographic system. Therefore, purification with this DEAE-Sephadex A-25 column does not seem to be completely efficient since a single large peak with abundant protein content was obtained, indicating that fraction FI probably contains more than one protein in it.

Taking this into consideration, we further analysed these 2 fractions (FI and FII) using chromatographic techniques; first we fractionated proteins according with their MW using a gel filtration column (Superdex-peptides) and then we purified small peptides (< 8.0 kDa) by anion-exchange chromatography (Q-resource column).

3.1.3 Antimicrobial activity of the protein fractions obtained in the preparative anion-exchange DEAE-Sephadex column

In order to search for the previously found AMPs (Albergaria *et al*., 2010; Branco *et al*., 2014), fractions exhibiting light-yellow color (FI, FII and FS) obtained from the DEAE-Sephadex column were tested for antimicrobial activity using growth assays performed with a sensitive yeast strain (*Hanseniaspora. guilliermondii*).

To obtain the 2-10 kDa peptidic fraction containing the AMPs reported by Albergaria *et al*. (2010) and Branco *et al*.(2014) we first purified fractions FI, FII and FS, using the procedure reported by those authors. First, the fractions were ultrafiltrated using centrifugal filter units equipped with 10 kDa membranes. Then, permeates (fraction<10 kDa) were concentrated 10-fold by passing them through centrifugal filter units equipped with 2 kDa membranes and finally the 10-fold concentrated fractions were vacuum–dried. For desalting control purposes, the inhibitory effect of fraction F85 (eluted from the DEAE-Sephadex column with 2 M ammonium acetate) was tested and used as a control of proper desalting of samples. Growth inhibitory assays were performed as described by Branco *et al*. (2014) in 96-well microplates. Lyophilized fractions were resuspended in YEPD medium with 30 g/l of ethanol and a pH of 3.5, which simulates enological conditions, and a final protein concentration of 1 mg/ml (maximum concentration of AMPs in alcoholic fermentation supernatants). Control assays were performed using the same medium but without any addition of protein fraction. Each well was inoculated with 10^5 cells/ml of *H. guilliermondii*. Growth was followed by absorbance measurements at 590 nm and by CFU counts that were obtained in YEPD-agar plates using the classical plating method.

Analysis of the inhibitory activity of the fractions by optical density (**Fig. 3.4**) shows that fractions FS, FI and FII inhibited the growth of *H. guilliermondii* throughout cultivation (approx. 46 h). Both the control assay and the F85 assay,

which was used as a control of proper desalting of samples, showed exponential growth until approx. 23 h, followed by a slight decrease of absorbance in the control till the end of the assay, while fraction F85 remained at high density values, confirming that a proper desalting of samples was achieved and salts had no influence in the inhibition of *H. guilliermondii* growth.

CFU counts (**Fig. 3.5**) shows a decreasing of *H. guilliermondii* cell density from 10^5 cells/ml at 0 h to 10^4 cells/ml 24 h later, for fractions FS and FII, while fraction FI reached these CFU values within 15 h after inoculation. After 24 h, *H. guilliermondii* in the FS assay recovered its viability to values close to 10^5 cells/ml after 46 h. In the FI assay *H guilliermondii* lost its culturability up to final values lower than 10^2 cells/ml at the end of the growth assay. It was not possible to evaluate the growth of *H. guilliermondii* in the FII assay after 24 h, since the sample dried making difficult the execution of plating method. Control assay shows a cell growth up to e 10^8 cells/ml at 15 h, after which cell density decreased to 10^7 cells/ml and remained around these values until 46 h.

The sharp decline of *H. guilliermondii* viability subject to FI activity shown by CFU counts and the lack of growth in DO measurements, since the beginning of the assay and throughout the 46 h, confirms the strong fungicide effect of this fraction. Fungistatic effect is observed in the assays with F1 and FII, since there was only an arrestment of the cellular growth and no cellular death, as it is seen in CFU counts.

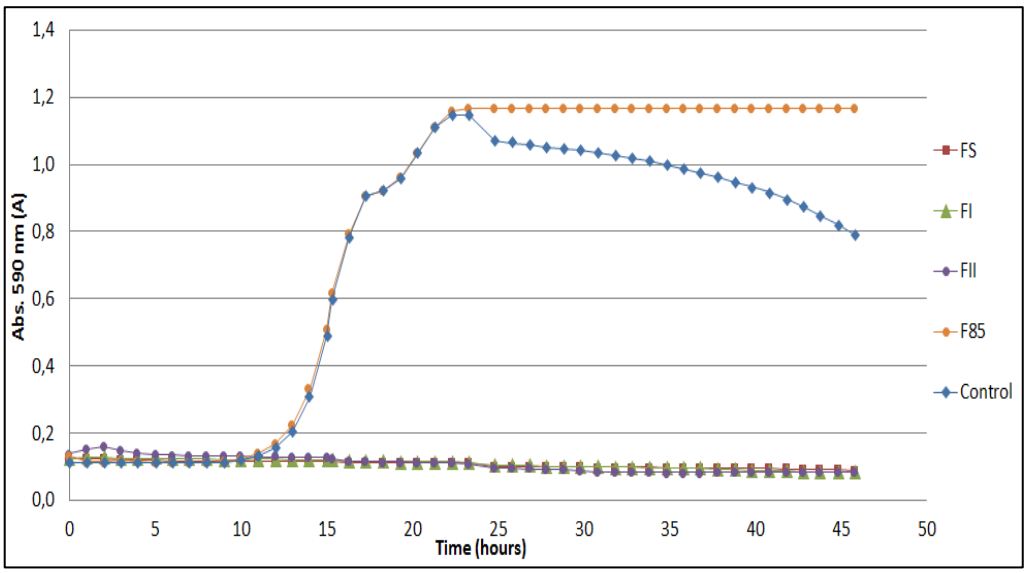

Figure 3.4 - Optical density of *H. guilliermondii* cultures in the antimicrobial tests performed using fractions FS (*red filled squares*), FI (*green filled triangles*), FII (*purple filled circles*), F85 (*yellow filled dots*) and control (*blue filled diamonds*) in YEPD medium at pH 3.5. Absorbance measurements ate 590 nm. Data presented are mean values of three independent assays.

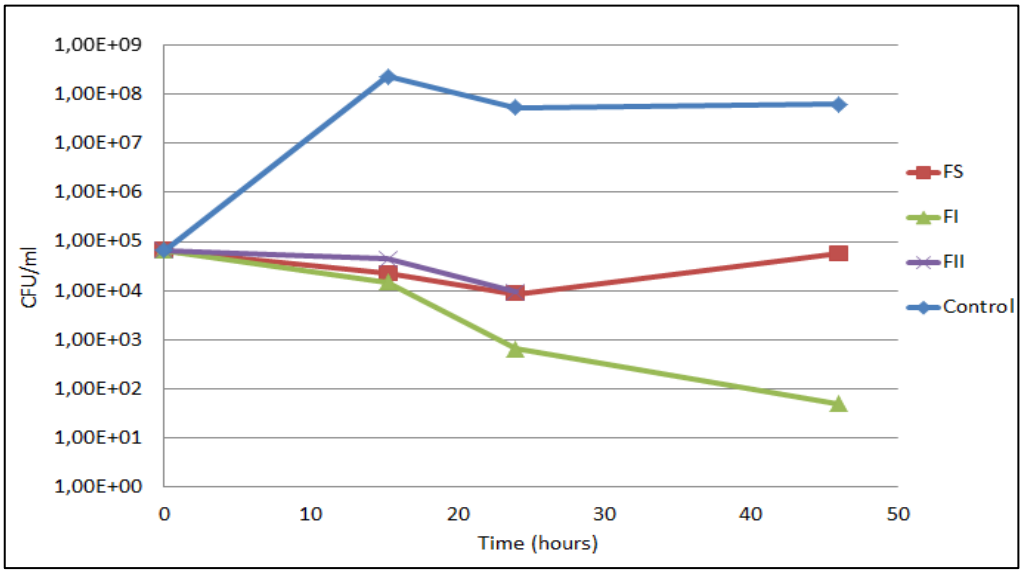

Figure 3.5 - Viable cell profiles of *H. guilliermondii* during the antimicrobial tests performed with fractions FS (red, *filled squares*), FI (green, *filled triangles*), FII (purple, *crosses*) and control (blue, *filled diamonds*) in YEPD medium at pH 3.5.

3.1.4 Analysis of the bioactive fractions obtained in the preparative DEAE-Sephadex column by gel filtration and ion-exchange chromatography

In order to compare the chromatographic profiles of the bioactive fractions (i.e. fractions FI and FS) obtained in the DEAE-Sephadex column with the ones previously identified by Branco *et al*. (2014), we purified those fractions using exactly the same chromatographic procedure as used by Branco *et al*. (2014). It was not possible to analyse fraction FII due to unavailability of the sample. For comparative purposes, we also analysed the chromatographic profiles of the 2-10 kDa fraction from the original alcoholic fermentation supernatants. First, protein fractions were subjected to gel filtration chromatography coupled to a HPLC system, using a Superdex Peptide column and eluted with 0.1 M ammonium acetate.

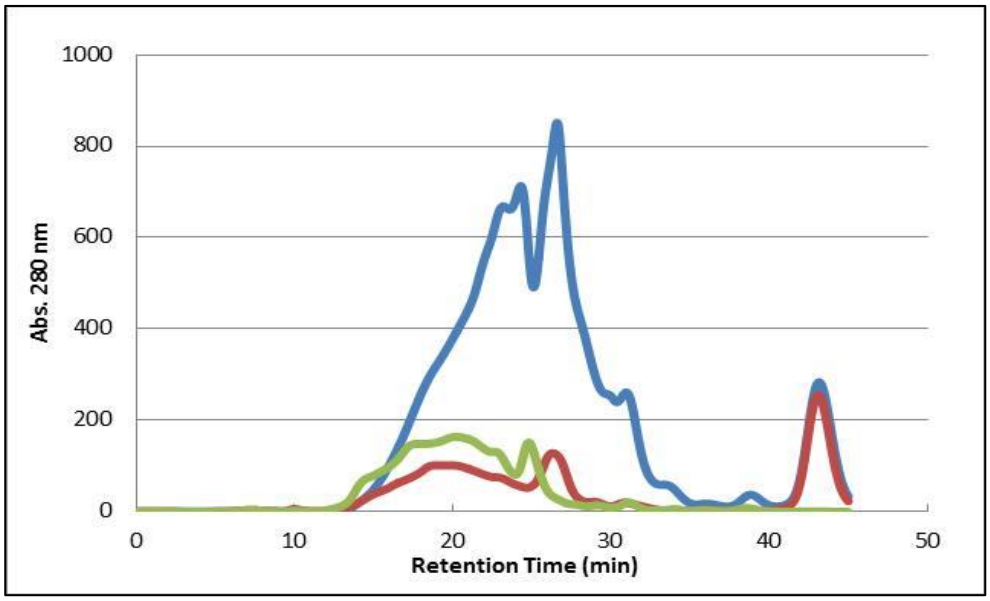

Figure 3.6 - Chromatographic profiles of FS (*red*), FI (*green*) and 2-10 kDa fractions (*blue*) fractionated by HPLC gel filtration using a Superdex Peptide column. Elution of the fractions with 0.1 M ammonium acetate at a flow rate of 0.7 ml/min. Absorbance measured at 280 nm.

Gel filtration chromatographic profiles (**Fig. 3.6**) show that 2-10 kDa fraction exhibits much higher protein content than DEAE-Sephadex-column fractions, i.e. FS and FI. This difference in protein concentrations results from the treatment of the samples obtained in the preparative DEAE-Sephadex column for injection in the SuperdexPeptide column. This fraction, and also fraction FS, show a peak at about 45 min that is not seen in fraction FI. This probably indicates the presence of cationic

peptides with positive charge that are not present in fraction FI because this fraction is the only one that was selectively retained in the anionic DEAE-Sephadex column.

Fractions FS and FI show similar elution profiles, except between 42-45 min of sample running, exhibiting the typical 25-28 min peak that contains the AMPs that were identified by Branco *et al.* (2014) in the 2-10 kDa fraction from *S. cerevisiae* fermentation supernatants.

The peak of interest (i.e. the peak between approx. 25-28 min), which contains peptides with an apparent molecular weight (MW) of 8.0 kDa, was collected for each fraction (i.e. for fractions FS, FI and 2-10 kDa). The 8.0 kDa gel-filtration fractions were first lyophilized and then further fractionated in a strong anion-exchange column (Q-Resource). Samples were eluted at neutral pH using a gradient of ammonium acetate (ranging from 5–500 mM).

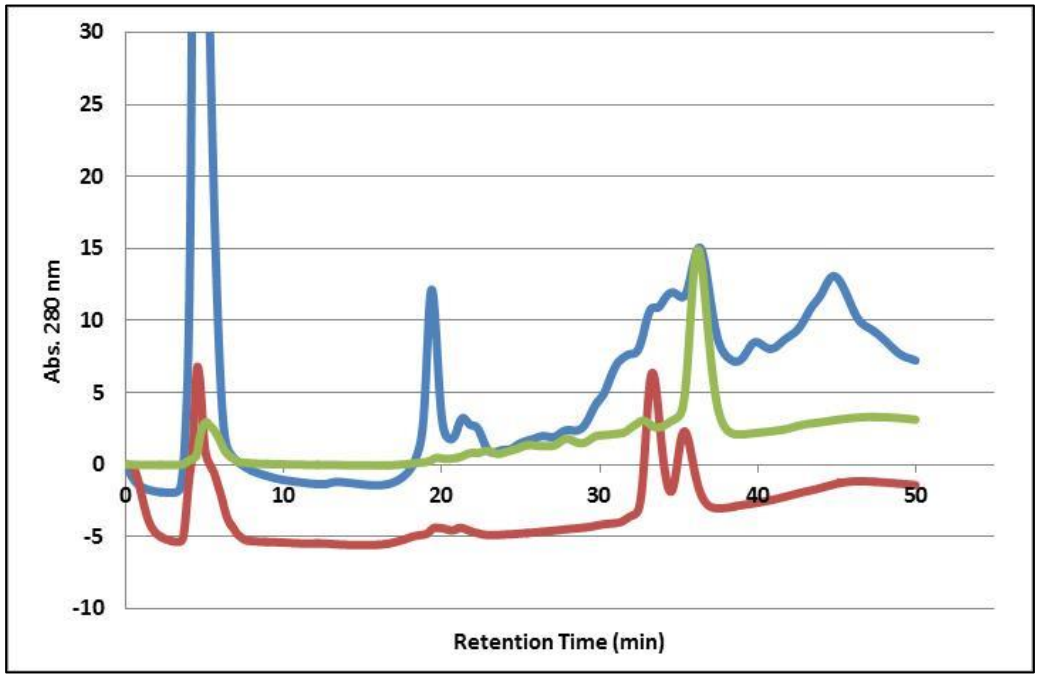

Figure 3.7 - Chromatographic ion-exchange (Q-Resource column) profiles of the 8.0 kDa fractions (F8) obtained from gel filtration of the three bioactive fractions: F8 [FS] (*red*); F8 [FI] (*green*) and F8 [2-10 kDa] (*blue*). Elution of the fractions using a gradient of ammonium acetate (ranging from 5–500 mM) at a flow rate of 1 ml/min. Absorbance measured at 280 nm.

The Q-Resource profile (**Fig. 3.7**) of the F8 [2-10] kDa fraction shows a separation into several peaks, with a maximum peak at about 5 min corresponding to cationic and neutral proteins and minor peaks at approx. 20 min, 35 min and 45 min, respectively, corresponding to negatively charged proteins. This profile is very

similar to the one reported by Branco *et al*. (2014) where the GAPDH-derived AMPs were identified. The profile of the F8 [FS] fraction shows two peaks between 30 and 40 min, beyond an initial peak (at about 5 min) containing the positively charged proteins. Meanwhile the Q-resource profile of the F8 [FI] fraction shows only one intense peak at about 35-36 min, which means that this sample is highly purified.

This analysis allows us to conclude that the purification procedure of the AMPs secreted by *S. cerevisiae* during alcoholic fermentation can be successfully achieved with the DEAE-Sephadex ion-exchanger if a first and additional gel filtration step is used to retain the small peptides (< 8.0 kDa) from the supernatants.

3.2 Proteomic analysis of membrane-proteins of *S. cerevisiae* cells grown for 12 and 48 h

Several studies have shown that the early death of non-*Saccharomyces* during wine fermentations are due to yeast-yeast interactions induced by *S. cerevisiae* that are mediated by different mechanisms: cell-cell contact (Nissen *et al*., 2003) and secretion of AMPs (Pérez-Nevado *et al.,* 2006; Albergaria *et al*., 2010). Recently, those AMPs were identified by Branco *et al*. (2014) as peptides derived from the GAPDH protein. Previously, Delgado *et al*. (2001) had also reported that GAPDH is a cell wall-associated protein in *S. cerevisiae*. Besides, in an unpublished work carried out by LNEG's research group it was found that *S. cerevisiae* cells pre-grown for 48 h were able to induce death of *H. guilliermondii* cells by cell-cell contact, while 12 h-grown cells were not. Taken together these findings strongly suggest that GAPDH-derived AMPs might be present in the cell wall of *S. cerevisiae* 48 h grown cells and for this reason they induced death of the sensitive *H. guilliermondii* yeast.

In the present work we investigated the presence of GAPDH-derived AMPs in the membranes of *S. cerevisiae* cells pre-grown for 48 h and 12 h, respectively. With this purpose, *S. cerevisiae* cells were grown for 12 h and 48 h and the proteins of cell membranes resolved in 2D-PAGE gels and the respective proteomes analysed.

The *S. cerevisiae* cells were grown for 12 and 48 h, and then followed by separation of the cell membrane fractions to extract the membrane cell-associated proteins. The protocol used for extraction of cell membranes did not separate cytoplasmic membrane fractions from cell wall fractions.

After 2D electrophoresis, the 2DE gels were silver-stained, digitalized and then analysed by ImageMaster 2D software. Silver-stained 2DE gels images of membrane-associated proteins of *S. cerevisiae* cells pre-grown for 12 h and 48 h are shown in **Figs. 3.8 and 3.9**, respectively.

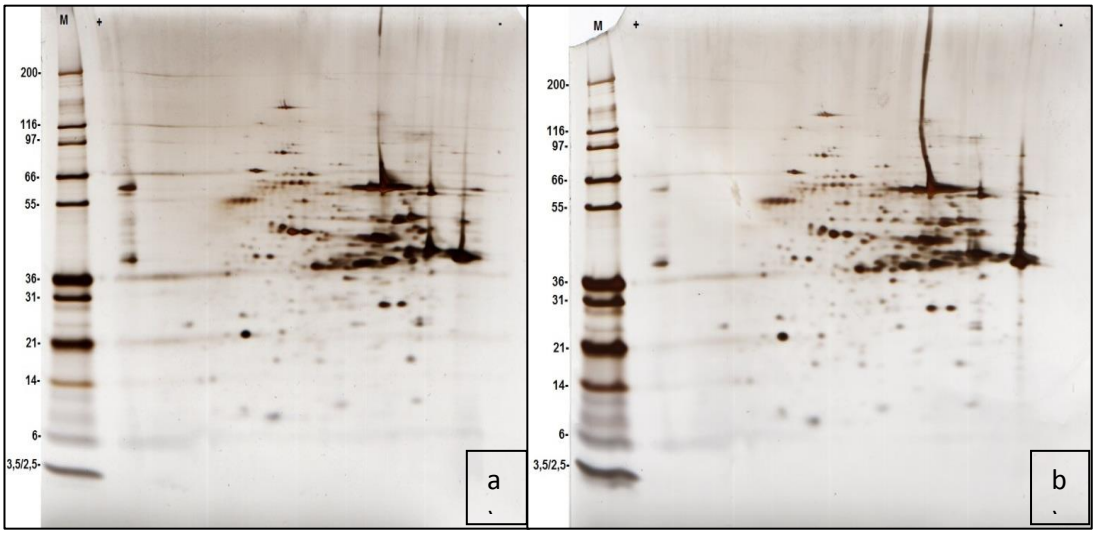

Figure 3.8 - 2D-PAGE map of *S. cerevisiae* membrane-associated proteins extracted from cells grown for 12 h (*a*, *b* are replicates). 35 µg of protein sample. IPG strip 4-7 pH gradient, 7 cm. 4-12% Bis-Tris gel. Gels were silver stained. 1x MES running buffer. *M* – molecular weight marker (kDa). + acidic end; - basic end

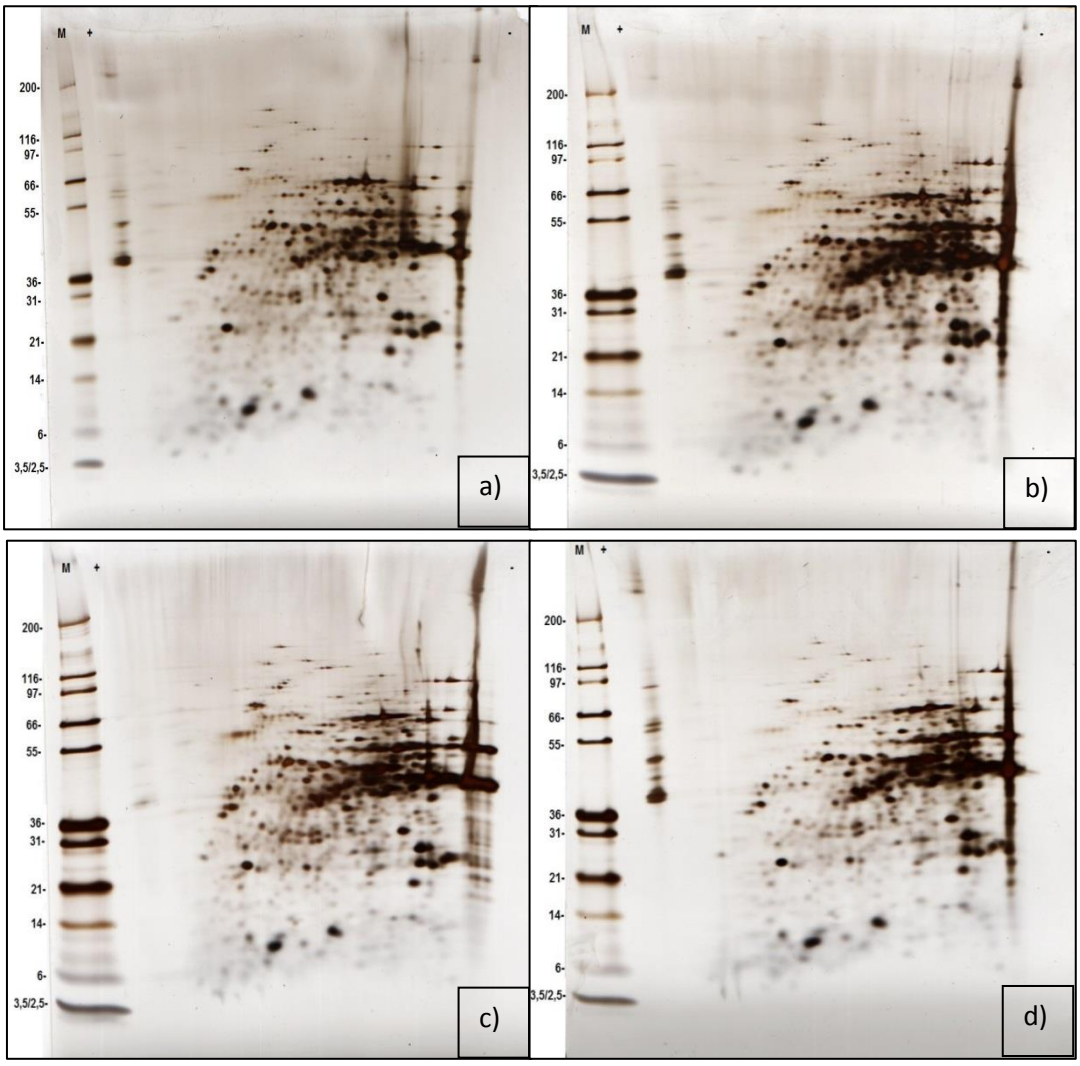

Figure 3.9 - 2D-PAGE map of *S. cerevisiae* membrane-associated proteins extracted from cells grown for 48 h (*a*, *b* are replicates; *c* is a replicate with addition of GAPDH and *d* is a replicate with addition of GAPDH and GAPDH-derived AMPs). 70 µg of protein sample. IPG strip 4-7 pH gradient, 7 cm. 4-12% Bis-Tris gel. The gels were silver stained. 1x MES running buffer. *M* – molecular weight marker (kDa). + acidic end; - basic end

The 2DE gels of *S. cerevisiae* membrane-associated proteins of cells grown for 12 h and 48 h show some similarities although there is a much higher number of proteins in the 2DE gel of cells grown for 48 h. Although the increased number of spots seem to be the result of cell growth (from 12 h to 48 h), we cannot discard the possibility of this increase being due to the higher protein concentration in the gels representing membrane proteins of cells grown for 48 h. As expected, 2DE gels (of both growth stages) express higher abundance of proteins in the 4.8-6.6 pH range (approximately) with relative MW between 66 and 31 kDa. Results of statistical analysis of the 2DE gels performed with the ImageMaster software (**Table 2**) indicate a total of 160 spots at 12 h and of 334 spots at 48 h (these results correspond to mean values of replicates).

To determine if a protein is differentially expressed it is common to use a fixed fold change (i.e. ratio of spot intensities between two different 2DE gels) threshold of 2. However, in this situation this fold change could not be used since the amount of total protein loaded in the 2DE gels was not identical. In normal conditions, the slope from the scatter plot is 1, since the amount of total protein loaded in the 2DE gels is the same. In this case, the mean of the slopes from the scatter plots was 2.5 (**Fig. 3.10**). To overcome this problem, we decided to multiply the fold change for the value of the slope from the scatter plots. In this way, we used a threshold of 5. Spots showing a volume intensity ratio higher than 5 and a *p*-value (ANOVA) less than 0.05 were considered differentially expressed (**Appendices 1 and 2**).

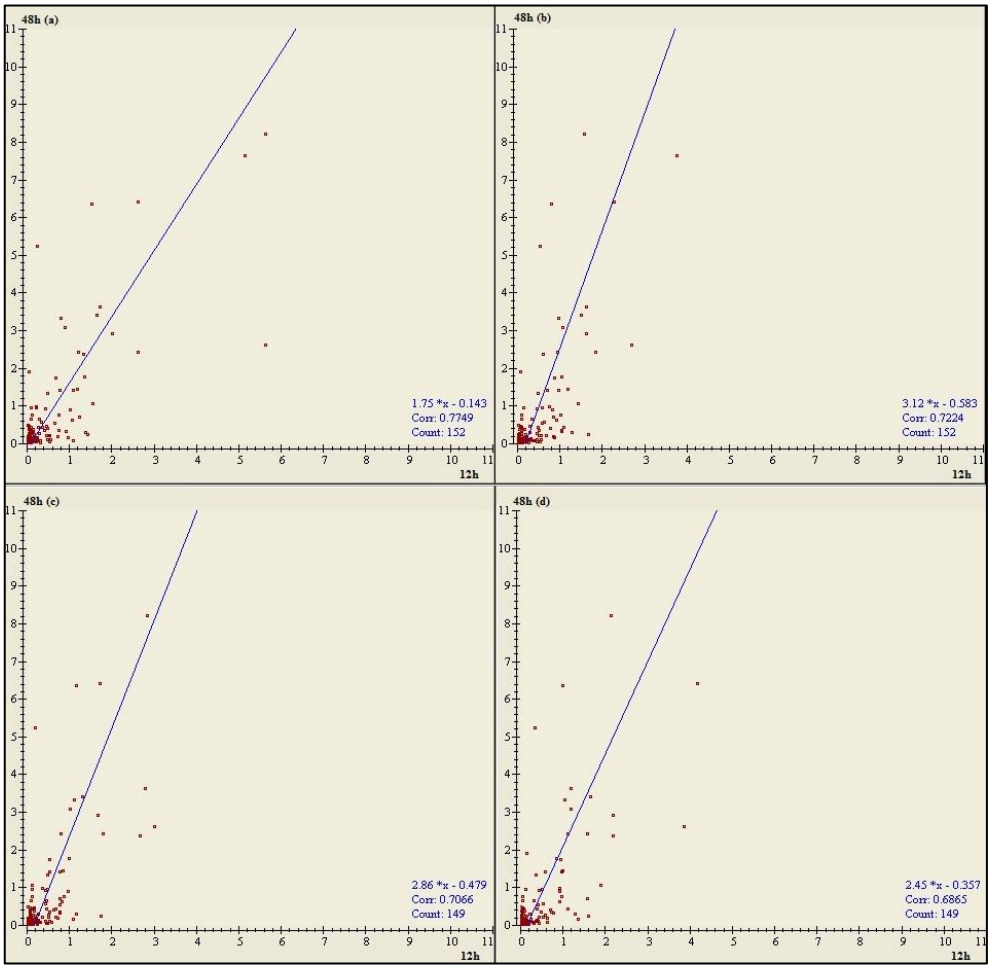

Figure 3.10 - Scatter plots of 2DEgels.

Using these criteria, 13 spots at 12 h and 5 spots at 48 h were considered overexpressed. At 12 h there were found only 8 exclusive spots; however at 48 h, 184 spots were identified as new proteins (**Table 2**).

Table 2 Statistical analysis of the *S. cerevisiae* membrane-associated proteins extracted from cells grown for 12 h and 48 h, respectively (these are mean values of 2 replicates of cells grown for 12 h and 4 replicates of cells grown for 48 h).

S. cerevisiae cell membranes	**Growth time**	
	12 h	**48 h**
Total number of spots	160	336
Exclusive	8	184
Overexpressed	13	5

The GAPDH protein has a MW of approx. 36 kDa and the p*I*'s of its isoforms are between 6.59-6.98. Through calculations of the relative MW of proteins (**Appendix 3**) it was deduced that spot 1 (**Fig. 3.11**) could represent the GAPDH protein. As the image of the 2DE gels shows, this spot is present in both growth stages; however the spot is saturated which makes impractical its quantification and intensity comparison. Therefore, spot 1 was not considered in the statistical analysis (**Appendices 1 and 2**).

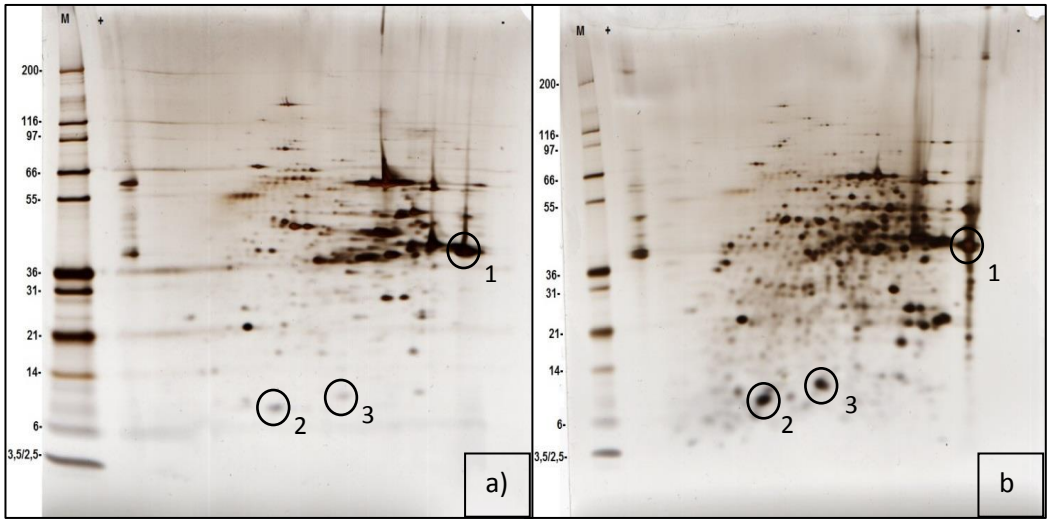

Figure 3.11 *a, b* – 2DE gels of *S. cerevisiae* membrane-associated proteins extracted from cells grown for 12 h and at 48 h, respectively. Spots *1*- identified as GAPDH and spots *2, 3* identified by MALDI-TOF/TOF as GAPDH-derived AMPs.

To confirm the presence of the GAPDH enzyme we performed a 2D-PAGE in three different conditions: a) protein fraction collected from cells grown for 12 h aside a commercial molecular weight marker (described in section 2.3.4.2); b) protein fraction collected from cells grown for 12 h supplemented with the commercial GAPDH protein aside the commercial molecular weight marker; c) protein fraction collected from cells grown for 12 h supplemented with the commercial GAPDH protein, aside the same commercial GAPDH used as molecular weight marker. **Fig. 3.12** shows an increase of intensity of the putative GAPDH spot in gel *b)*; it also shows a strong smear (with the same molecular weight of GAPDH) in gel *c)* – indicating an excessive load of commercial GAPDH. These results confirm the spot 1 as the GAPDH protein, proving this protein is present in both samples from the *S. cerevisiae* cell membranes.

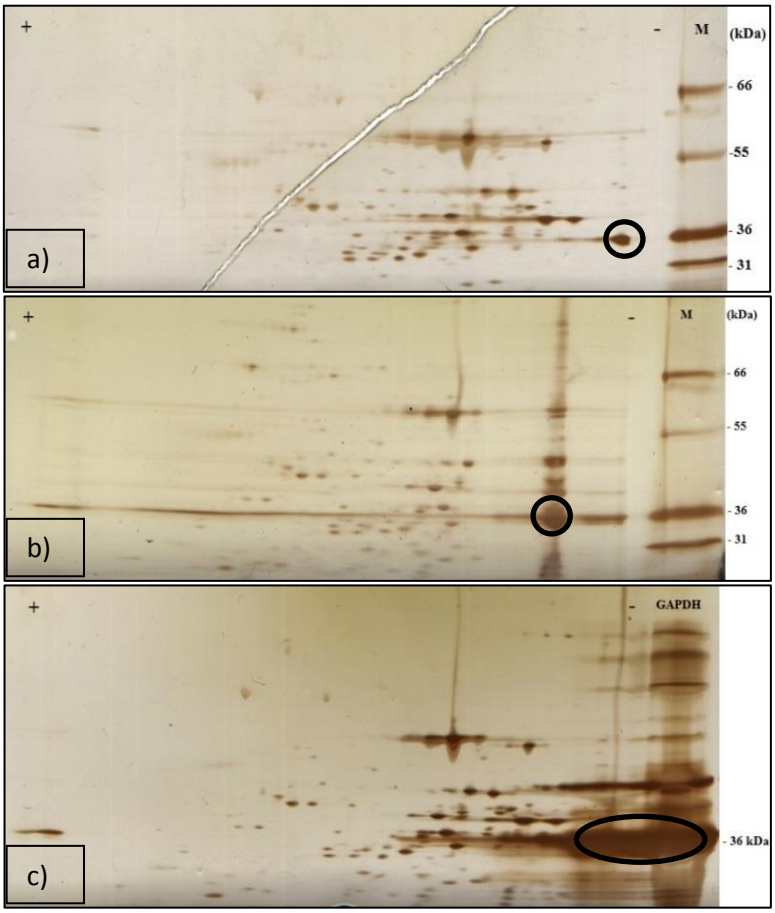

Figure 3.12 - 2D-PAGE map of *S. cerevisiae* membrane-associated proteins extracted from cells grown for 12 h. IPG strip 4-7 pH gradient, 7 cm. Silver stained 4-12% Bis-Tris gel *a)* without addition of GAPDH; *b)* with GAPDH and *c)* with GAPDH and GAPDH as molecular marker. *M* – molecular weight marker. + acidic end; - basic end.

According to Branco *et al.* (2014), the AMPs identified as GAPDH-derived peptides have a theoretical p*I* of 4.37 and are present in a bioactive fraction of 8 kDa. Regarding these data and the relative MW of the peptides (**Appendix 3**), spots 2 and 3 (**Fig. 3.11**) were pointed out as possible GAPDH-derived peptides. Statistical results indicated that spot 2 is not differentially expressed (it shows a volume intensity ratio of 0.5); in its turn, spot 3 is overexpressed with a 48 h/12 h ratio of 6 (> 5) and a *p*-value of 0.0041 (< 0.05). To confirm the identity of these spots as GAPDH-derived peptides the gel plugs were excised and examined by mass spectrometry using the method MALDI-TOF/TOF. The results of the analysis by mass spectrometry are listed as follows:

Table 3 Results from mass spectrometry analysis. Method used MALDI-TOF/TOF. Protein score is -10*Log(P), where P is the probability that the observed match is a random event. Protein scores greater than 52 are significant ($p<0.05$). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Gel spots	Protein Hits	Nominal Mass (M_r)	Protein Score	Sequence Coverage (%)	Identified peptides
Spot 2	Glyceraldehyde-3-phosphate dehydrogenase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	35728	43	17	-.MIRIAINGFGR.I, R.VPTVDVSVVDLTVK.L, K.IVSNASCTTNCLAPLAK.V, K.LISWYDNEYGYSAR.V, K.KIVSNASCTTNCLAPLAK.V
Spot 3	Pyruvate decarboxylase isozyme 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	61457	92	11	K.LTAATNAKQ.-, K.LIDLTQFPAFVTPMGK.G, R.WAGNANELNAAYAADGYAR.I, K.NPVILADACCSRHDVKAETK.K
Spot 3	Pyruvate decarboxylase isozyme 2 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	61873	92	10	K.NPVILADACASRHDVK.A, R.WAGNANELNAAYAADGYAR.I, K.STPANTPMKQEWMWNHLGNFLR.E
Spot 3	Pyruvate decarboxylase isozyme 3 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	61542	92	9	K.FALQNLLK.V, K.NPVILSDACASRHNVK.K, R.WAGNANELNAAYAADGYAR.I, K.VKNATFLGVQMKFALQNLLK.V
Spot 3	Glyceraldehyde-3-phosphate dehydrogenase 1 OS=Saccharomyces cerevisiae (strain ATCC 204508 / S288c)	35728	68	17	R.VPTVDVSVVDLTVK.L, K.ELDTAQKHIDAGAK.K, K.LISWYDNEYGYSAR.V, K.IVSNASCTTNCLAPLAK.V

Indeed, the peptides extracted from spot 2 were identified as GAPDH fragments. However, the protein score was 43 only, which is not statistically significant. Spot 3, however, matched with two proteins, the pyruvate decarboxylase isozyme and the GAPDH, with a protein score of 68 for the latter, which confirmed its GAPDH origin.

In summary, we confirmed the presence of the GAPDH protein in the 2DE gels supplemented with GAPDH (**Fig. 3.12**) and in the 2DE gels *c)* and *d)* with extra addition of GAPDH (**Fig. 3.9**). In both experiments it was observed an increase of the GAPDH spot intensity; in the 2DE gel *c)* (**Fig. 3.12**) the GAPDH spot shows a great excess of protein. However, it was not possible to conclude about expression levels of GAPDH at 12 h and 48 h due to the saturation of the GAPDH spots. This spot saturation can be a result of various factors such as incorrect protein quantification that led to an increase of protein sample in the gel; the high sensitivity of silver nitrate as a staining method and its susceptibility for spot saturation; a larger development time during the staining process; among other factors. Although it was not possible to assess it correctly, the GAPDH seems to have a tendency to increase its expression in the cell membranes of 48 h-grown cells by comparison with 12 h-grown cells. In fact, Delgado *et al*. (2001) found that each of the three GAPDH polypeptides encoded by the *TDH1-3* genes is associated with the cell wall of *S.*

cerevisiae. The same authors also demonstrated that GAPDH accumulates in the cell wall of *S. cerevisiae* in response to starvation and temperature upshift (Delgado *et al*. 2003). This could explain the increase of the GAPDH expression since at 48 h of fermentation the environmental stress is much higher than at 12 h. Further work must be carried out to prove that the expression of GAPDH is actually increased in the membranes of cells at 48 h of fermentation.

It was shown that AMPs are differentially expressed in the membranes of *S. cerevisiae* cells at 48 h by comparison with cells at 12 h of growth. It was also confirmed that the AMPs are derived from GAPDH proteolysis. These results are in line with a previous work published by Albergaria *et al.* (2010) that showed that *S. cerevisiae* begins to secrete AMPs to the extracellular medium at the end of the exponential growth phase (1–2 days) in alcoholic fermentations, as well as with the new findings by Branco *et al*. (2014) revealing that these AMPs correspond to fragments of the *S. cerevisiae* GPADH protein. Moreover, Silva *et al*. (2011) identified GAPDH as a specific target of metacaspases in *S. cerevisiae*, thus proving that GAPDH is associated with apoptosis, which is sustained by the results obtained by Branco *et al*. (2014) showing that a mutant strain of *S. cerevisiae* deleted in the metacaspase *YCA1* gene significantly prevents death of *H. guilliermondii* during alcoholic fermentation.

Taken together, these findings suggest that the presence of GAPDH-derived peptides in the cell membranes of *S. cerevisiae* at the end of the exponential growth phase might be due to apoptotic cells inducing the cleavage of GAPDH by metacaspases. However, to definitively establish the connection between apoptosis and secretion of AMPs further investigation must be carried out.

4 Final remarks and future work

The present work is comprised of two parts: the first part consisted in the production and purification in a preparative scale of AMPs, so that they can be used in winery fermentations in a purified form; the second part aimed to analyze the membrane proteome of *S. cerevisiae* cells pre-grown for 12 and 48 h, respectively, and to verify the presence of the previously identified GAPDH-derived AMPs in this cell structure.

In the first part we produced 3000 ml of *S. cerevisiae* fermentation supernatants and purified the cell-free supernatants in a preparative chromatographic system using an anion-exchange resin (DEAE-Sephadex A-25). Proteins were eluted using a 2-2000 mM gradient of ammonium acetate and protein fractions were collected and analyzed by spectrophotometry. Two fractions (fraction FI and FII) exhibiting significant protein content were tested for antimicrobial activity against the sensitive yeast *H. guilliermondii*. Fraction FI exhibited highest antimicrobial activity and thus was further analysed by chromatographic methods (i.e. gel filtration and anion exchange chromatography) and the respective profiles were compared with those exhibited by the 2-10 kDa peptidic fraction of *S. cerevisiae* supernatants where Branco *et al*. (2014) found the GAPDH-derived AMPs.

From all the work performed we can conclude that purification of the AMPs secreted by *S. cerevisiae* during alcoholic fermentation can be successfully achieved using the preparative DEAE-Sephadex ion-exchanger if a first and additional gel filtration step is used to retain small peptides (< 8.0 kDa) from the supernatants.

To improve the efficiency of the purification procedure of AMPs, the chromatographic system should be coupled to an absorbance detector, in order to monitorize the whole process and obtain elution profiles for each fraction, and a pumping system should be used to control not only the flow rate of the eluent but also to obtain a consistent elution gradient of the mobile phase.

In the second part of this work, *S. cerevisiae* cells were grown for 12 and 48 h, respectively, the cell membranes were isolated and membrane-associated proteins extracted. The proteomes of these cell membranes were analysed by 2D-PAGE. We detected a total of 160 spots in the membrane-proteome of *S. cerevisiae* cells grown for 12 h and 336 spots in the membrane-proteome of *S. cerevisiae* cells grown for 48

h; Statistical analysis of the membrane-proteomes revealed that 13 spots were overexpressed in cells grown for 12 h by comparison with cells grown for 48 h and 8 spots were exclusively found in 12 h-grown cell membranes. In the membrane-proteome of *S. cerevisiae* cells grown for 48 h 5 proteins were overexpressed and 184 were identified as new proteins. Proteomic analysis also allowed detecting the presence of two spots of low MW (ca 10 kDa) and p*I* (4-5) that were overexpressed in the membranes of 48 h-grown cells. These spots were excised from the 2DE gel and identified by mass spectrometry to be GAPDH-derived peptides.

Concluding, the proteomic analysis demonstrated that the GAPDH-derived AMPs previously identified by Branco *et al*. (2014) are present in cell membranes of *S. cerevisiae* and overexpressed in 48 h-grown cells by comparison 12 h-grown cells. These findings suggest that death mediated by cell-cell contact reported by Nissen *et al*. (2003) could be the result of the presence of AMPs in cell membranes of *S. cerevisiae*. Moreover, overexpression of these AMPs in membranes of *S. cerevisiae* cells grown for 48 h is in agreement with previous work carried out by LNEG's research group that found that *S. cerevisiae* cells pre-grown for 48 h are able to induce death of *H. guilliermondii* cells by cell-cell contact, while 12 h-grown cells are not. It is possible that these two different death-inducing mechanisms (i.e. cell-cell contact and AMPs) are connected and that one mechanism (cell-to-cell mechanism) could rely in the other (AMPs present in cell membranes) mechanism. Further work must be carried out in order to understand the mechanisms underlying cell-cell contact death and the role of AMPs in this phenomenon.

It was also demonstrated that the AMPs are derived from GAPDH proteolysis. Moreover, Silva *et al*. (2011) identified GAPDH as a specific target of metacaspases in *S. cerevisiae*, thus proving GAPDH is associated with apoptosis in *S. cerevisiae*. Albergaria *et al*. (2010) showed that *S. cerevisiae* begins to secrete AMPs to the extracellular medium at the end of the exponential growth phase (1–2 days) in alcoholic fermentations. In addition, Branco *et al*. (2014) also showed that a mutant strain of *S. cerevisiae* deleted in the metacaspase *YCA1* gene significantly prevents death of *H. guilliermondii* during alcoholic fermentation. Taken together, these findings suggest that the presence of GAPDH-derived peptides in the cell membranes of *S. cerevisiae* at the end of the exponential growth phase might be due to apoptotic cells inducing the cleavage of GAPDH by metacaspases.

We also confirmed the presence of GAPDH in the membrane-proteome of *S. cerevisiae* cells by performing 2D-PAGE gels with membrane proteins to which a commercial GAPDH protein was added to verify the exact position of this protein in the 2DE gel. However, it was not possible to include the GAPDH in the statistical analysis since the spots were too saturated. Although it was not possible to determine statistically, it seems that GAPDH is overexpressed in the membranes of *S. cerevisiae* cells grown for 48 h by comparison with 12 h-grown cells. Delgado *et al*. (2003) demonstrated that GAPDH accumulates in the cell-wall of *S. cerevisiae* cells in response to starvation and temperature upshift. Thus, the increase of GAPDH in the membranes of *S. cerevisiae* cells grown for 48 h could explain why GAPDH-derived peptides are also increased in the membranes of those cells.

Future work of these AMPs secreted by *S. cerevisiae* during alcoholic fermentations should include:

- Further 2D-PAGE analysis to confirm the overexpression of the GAPDH-derived AMPs and of GAPDH protein.
- Structural characterization of these AMPs using a variety of techniques such as X-ray crystallography or nuclear magnetic resonance, in order to understand its structure and mode of action.
- Cell-cell contact assays involving *S. cerevisiae* mutant strains deleted in the *TDH1-3* genes (GAPDH) or with mutant strains deleted in the *YCA1* gene (metacaspase) that prevent the production of GAPDH-derived peptides in order to verify if there are still any death of non-*Saccharomyces* yeasts.
- Further identification of proteins associated with cell membranes of *S. cerevisiae* that showed to be new proteins and differentially expressed in 2DE gels in both 12 h and 48 h-growth cells.

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Appendix 1 – Relative volumes of intensity of spots

Table A.1 Relative volumes of the spots, means of relative volumes and 48/12h ratio

Match ID	12h A1	12h A2	48h A1	48h A2	8h+GAPDI	+GAPDH+AN	Mean 12h	Mean 48h	48h/12h
107	0,493074	0,383215	0,020838	0,013708	0,015377	0,009213	0,438145	0,014784	0,033742
59	5,24302	4,92493	0,251708	0,536799	0,204214	0,338997	5,083975	0,33293	0,065486
105	1,91347	0,273444	0,057394	0,07467		0,144349	1,093457	0,092138	0,084263
10	0,375208	0,614056	0,036846	0,0403	0,081442	0,020637	0,494632	0,044806	0,090585
8	0,110725	0,279905	0,018707	0,014148	0,029397	0,010869	0,195315	0,01828	0,093593
9	0,257397	0,442267	0,039388	0,022665	0,065421	0,018482	0,349832	0,036489	0,104304
85	0,181557	0,221601	0,021955	0,022584	0,030359	0,018477	0,201579	0,023344	0,115805
11	0,175664	0,643447	0,035495	0,024609	0,129059	0,013878	0,409556	0,05076	0,12394
139	0,077667	0,049427	0,003289	0,008465	0,007	0,012757	0,063547	0,007878	0,123968
84	0,771104	0,744476	0,11184	0,098493	0,130432	0,055991	0,75779	0,099189	0,130892
40	0,958987	0,801431	0,098544	0,136917	0,125984	0,12785	0,880209	0,122324	0,138971
118	0,656505	0,613076	0,096491	0,105843	0,103965	0,05195	0,634791	0,089562	0,141089
119	0,181905	0,173853	0,022926	0,033876	0,040243	0,008689	0,177879	0,026434	0,148605
126	0,041082	0,037586	0,007727	0,007008	0,006078	0,003164	0,039334	0,005994	0,152396
125	0,453872	0,303792	0,053762	0,062768	0,084024	0,053297	0,378832	0,063463	0,167522
113	0,035314	0,0255	0,005703	0,009715	0,002465	0,003027	0,030407	0,005227	0,171913
4	6,36237	5,05053	1,53448	0,791471	1,15627	1,00657	5,70645	1,122198	0,196654
124	0,367298	0,29642	0,068189	0,088042	0,067066	0,039854	0,331859	0,065788	0,19824
123	0,266418	0,161581	0,048297	0,038813	0,070846	0,029591	0,214	0,046887	0,219097
3	3,31856	5,18634	0,80915	0,975368	1,11363	1,03315	4,25245	0,982825	0,23112
138	0,070347	0,051619	0,009247	0,020008	0,017591	0,011869	0,060983	0,014679	0,2407
108	0,125355	0,114685	0,037974	0,035652	0,028669	0,017203	0,12002	0,029874	0,248912
92	0,086914	0,07287	0,020331	0,027747	0,018878	0,012899	0,079892	0,019964	0,249884
17	0,050601	0,036485	0,014889	0,01153	0,0146	0,00519	0,043543	0,011552	0,265305
112	0,114044	0,111888	0,030911	0,027488	0,045663	0,019515	0,112966	0,030894	0,273483
114	0,431472	0,331607	0,108039	0,098027	0,141006	0,074643	0,38154	0,105429	0,276325
96	0,067554	0,138264	0,035354	0,033365	0,027751	0,017504	0,102909	0,028493	0,276881
122	0,073399	0,08448	0,015623	0,020357	0,041017	0,011665	0,07894	0,022166	0,280794
110	0,029652	0,05577	0,012303	0,011474	0,019763	0,005348	0,042711	0,012222	0,286162
28	0,148538	0,10607	0,092243	0,014158	0,020047	0,029584	0,127304	0,039008	0,306414
100	0,194954	0,319413	0,075717	0,099974	0,088082	0,058108	0,257184	0,08047	0,312891
120	0,039729	0,039125	0,025198	0,009197	0,009851	0,01345	0,039427	0,014424	0,365843
79	1,34859	1,14923	0,480418	0,491031	0,496418	0,365224	1,24891	0,458273	0,366938
88	0,124024	0,246283	0,08023	0,077553	0,080754	0,03646	0,185154	0,068749	0,371309
62	0,388882	0,396799	0,152876	0,162265	0,172293	0,099043	0,392841	0,146619	0,373229
128	6,40893	7,9001	2,63173	2,27359	1,73236	4,17757	7,154515	2,703813	0,377917
0	3,08191	2,42647	0,893081	1,05835	1,02511	1,19493	2,75419	1,042868	0,378648
29	0,076252	0,114893	0,050819	0,043553	0,028478	0,026304	0,095572	0,037289	0,390162
24	8,20724	7,3393	5,62639	1,57855	2,84495	2,14067	7,77327	3,04764	0,392067
63	0,286189	0,28132	0,13073	0,103328	0,14722	0,081079	0,283755	0,115589	0,407356
86	0,022111	0,040132	0,011747	0,010004	0,022536	0,00689	0,031121	0,012794	0,411102
61	3,40289	4,03098	1,6517	1,50103	1,32293	1,64223	3,716935	1,529473	0,411488
54	1,7541	1,74974	0,670648	0,865308	0,536207	0,936346	1,75192	0,752127	0,429316
146	0,081009	0,089474	0,048958	0,039648	0,029446	0,028404	0,085242	0,036614	0,429529
98	0,077293	0,093648	0,046917	0,042901	0,043936	0,022204	0,085471	0,038989	0,456171
52	0,943171	0,9183	0,220283	0,590215	0,483762	0,510724	0,930736	0,451246	0,484827
6	2,43223	1,71769	1,21631	0,944503	0,806259	1,12082	2,07496	1,021973	0,492527
44	0,059011	0,054386	0,033397	0,027344	0,027203	0,025237	0,056699	0,028295	0,499045
58	0,930335	0,837809	0,435965	0,462436	0,429414	0,439066	0,884072	0,44172	0,499643

Appendix 1 (cont.) – Intensity relative volumes of spots

Match ID	12h A1	12h A2	48h A1	48h A2	8h+GAPDI	+GAPDH+AN	Mean 12h	Mean 48h	48h/12h
20	0,652237	0,74766	0,301646	0,333546	0,458946	0,308228	0,699949	0,350592	0,500882
151	7,64714	10,0181	5,14879	3,75631			8,83262	4,45255	0,504103
106	0,128288	0,140959	0,064011	0,076269	0,08814	0,048727	0,134624	0,069287	0,51467
5	1,76963	2,32521	1,36038	1,0486	0,990557	0,844771	2,04742	1,061077	0,518251
68	3,62257	3,37827	1,72993	1,622	2,78223	1,20189	3,50042	1,834013	0,523941
32	0,110128	0,11361	0,061355	0,082562	0,053067	0,038767	0,111869	0,058938	0,526847
80	1,42407	0,946984	0,77616	0,698393	0,540554	0,583357	1,185527	0,649616	0,547955
109	0,069505	0,120389	0,049933	0,060548	0,068796	0,03136	0,094947	0,052659	0,554615
121	0,127915	0,088032	0,075566	0,042773	0,099576	0,023433	0,107974	0,060337	0,558812
23	0,451522	0,607932	0,454148	0,280118	0,176632		0,529727	0,303633	0,573187
117	0,028568	0,025591	0,020306	0,02648	0,012726	0,005746	0,027079	0,016315	0,602472
12	0,143433	0,207421	0,113017	0,108287	0,128418	0,074022	0,175427	0,105936	0,603874
137	0,069748	0,073843	0,040902	0,047865	0,063972	0,024362	0,071796	0,044275	0,616685
15	0,975417	0,871312	0,222555	0,757534	0,374822	0,92986	0,923365	0,571193	0,618599
83	1,41588	1,57602	1,09181	0,96684	0,776796	0,980595	1,49595	0,95401	0,637729
89	0,058294	0,083747	0,076579	0,057199	0,030269	0,018337	0,071021	0,045596	0,642009
34	0,123993	0,160618	0,087656	0,09972	0,107772	0,07065	0,142306	0,091449	0,642627
103	0,11333	0,21111	0,101068	0,094724	0,128407	0,096418	0,16222	0,105154	0,64822
99	0,253148	0,274596	0,159093	0,197429	0,206392	0,154279	0,263872	0,179298	0,679489
136	0,102842	0,149581	0,076802	0,080384	0,119862	0,067861	0,126212	0,086227	0,683196
90	2,92269	2,42427	2,0275	1,62961	1,68213	2,17745	2,67348	1,879173	0,702894
33	1,44339	1,39713	1,1873	1,18865	0,845199	0,999595	1,42026	1,055186	0,742953
132	0,089102	0,063115	0,070843	0,053343	0,068173	0,040299	0,076108	0,058165	0,764233
147	2,3661	2,04626	1,32375	0,61168	2,66118	2,17424	2,20618	1,692713	0,767259
116	0,058154	0,074491	0,043476	0,054456	0,072708	0,033428	0,066322	0,051017	0,769229
127	0,302981	0,30965	0,157658	0,296777	0,275219	0,221767	0,306316	0,237855	0,776504
104	2,42014	2,60502	2,61674	1,83537	1,80356	1,57228	2,51258	1,956988	0,778876
46	0,041954	0,034053	0,044994	0,025533	0,031214	0,018103	0,038004	0,029961	0,788364
111	0,028577	0,012842	0,004084	0,020653	0,025686	0,016593	0,020709	0,016754	0,809014
53	0,121873	0,147917	0,064442	0,164966	0,13376	0,104114	0,134895	0,116821	0,866011
39	0,160192	0,16967	0,162871	0,150445	0,1658	0,107979	0,164931	0,146774	0,88991
16	0,030422	0,037401	0,02437	0,038978	0,032377	0,025188	0,033911	0,030228	0,891392
45	0,159076	0,131944	0,097595	0,170267	0,146991	0,113896	0,14551	0,132187	0,908441
95	0,139528	0,202958	0,182631	0,162451	0,165058	0,114797	0,171243	0,156234	0,912354
74	0,050023	0,070568	0,056315	0,05	0,087948	0,040867	0,060295	0,058783	0,974911
56	0,060601	0,081563	0,053802	0,117555	0,072112	0,037826	0,071082	0,070324	0,989337
93	0,165981	0,158248	0,161396	0,173146	0,179574	0,128449	0,162115	0,160641	0,990912
101	0,573404	0,415705	0,350935	0,629685	0,437014	0,544514	0,494555	0,490537	0,991877
102	0,088189	0,124293	0,082928	0,115149	0,125025	0,108678	0,106241	0,107945	1,016037
51	0,121558	0,068923	0,073818	0,153039	0,092533	0,073161	0,095241	0,098137	1,030415
75	0,425661	0,385696	0,358422	0,491644	0,451776	0,378581	0,405679	0,420106	1,035563
36	0,050176	0,039813	0,048437	0,050438	0,057742	0,029992	0,044994	0,046652	1,036844
70	0,053078	0,053901	0,053842	0,047391	0,079017	0,042663	0,05349	0,055728	1,041856
2	0,541597	0,61052	0,665546	0,579663	0,768357	0,412803	0,576059	0,606592	1,053005
78	0,085081	0,089608	0,090036	0,103175	0,128603	0,063538	0,087344	0,096338	1,102968
143	0,045945	0,068879	0,070477	0,070542	0,06481	0,05576	0,057412	0,065397	1,139086
87	0,912244	0,609732	1,0104	0,835841	0,961954	0,911327	0,760988	0,929881	1,221938
26	0,158218	0,212367	0,252689	0,19024	0,221631	0,249103	0,185293	0,228416	1,232731
130	0,077271	0,126927	0,123861	0,162567	0,10231	0,120087	0,102099	0,127206	1,245909
38	0,048954	0,054454	0,072836	0,058751	0,067961	0,059347	0,051704	0,064724	1,251811

Appendix 1 (cont.) – Intensity relative volumes of spots

Match ID	12h A1	12h A2	48h A1	48h A2	8h+GAPDI	+GAPDH+AN	Mean 12h	Mean 48h	48h/12h
97	0,341437	0,40527	0,414063	0,520748	0,52753	0,477752	0,373354	0,485023	1,299099
145	0,049789	0,042977	0,075571	0,070209	0,052107	0,046093	0,046383	0,060995	1,315016
149	1,05359	0,833768	1,55974	1,42407	0,126961	1,90435	0,943679	1,25378	1,328609
65	0,75092	0,573797	0,763862	1,00882	0,880796	0,982426	0,662359	0,908976	1,372332
35	0,132074	0,136261	0,23064	0,209656	0,15304	0,149725	0,134168	0,185765	1,384577
27	0,105798	0,212191	0,222523	0,174621	0,267766	0,220058	0,158995	0,221242	1,391507
21	2,62195	2,64078	5,62968	2,69808	3,00442	3,84907	2,631365	3,795313	1,442336
22	0,052833	0,016571	0,025533	0,083824	0,041727		0,034702	0,050361	1,451251
141	0,069471	0,051495	0,08581	0,103596	0,097518	0,078363	0,060483	0,091322	1,50988
133	0,104953	0,078626	0,16634	0,15876	0,100911	0,128589	0,091789	0,13865	1,510522
41	0,626379	0,584454	1,06148	0,923996	0,831422	0,912825	0,605417	0,932431	1,540148
81	0,274535	0,290716	0,284095	0,456795	0,500038	0,5856	0,282626	0,456632	1,615679
14	0,013285	0,051706	0,048682	0,074432	0,04612	0,041495	0,032496	0,052682	1,621208
13	0,025688	0,01925	0,055827	0,029006	0,03144	0,032373	0,022469	0,037161	1,653924
66	0,706079	0,6132	1,23335	0,854825	0,765751	1,58636	0,65964	1,110072	1,682846
48	0,410518	0,304928	0,488659	0,787391	0,633681	0,692062	0,357723	0,650448	1,818301
73	0,448859	0,355059	0,276121	1,08502	0,676541	1,12419	0,401959	0,790468	1,966539
142	0,06037	0,048822	0,095961	0,136254	0,127543	0,091436	0,054596	0,112798	2,066053
91	0,354912	0,429797	0,74889	1,05224	0,779946	0,722064	0,392355	0,825785	2,104691
76	0,025499	0,037062	0,059045	0,053518	0,102078	0,050723	0,03128	0,066341	2,120849
148	0,053867	0,06026	0,130942	0,12237	0,15231	0,086351	0,057063	0,122993	2,155386
55	0,024193	0,044879	0,084314	0,08972	0,090919	0,041242	0,034536	0,076549	2,216486
60	0,231162	0,210064	0,465683	0,691996	0,371432	0,431333	0,220613	0,490111	2,221587
19	0,138208	0,080513	0,028487	0,305156	0,260122	0,378572	0,109361	0,243084	2,222775
64	0,049215	0,05555	0,135587	0,113632	0,121555	0,096781	0,052383	0,116889	2,231432
57	0,052395	0,047861	0,123778	0,106723	0,134326	0,097397	0,050128	0,115556	2,305203
135	0,031331	0,020864	0,047358	0,048428	0,056963	0,091362	0,026097	0,061028	2,338457
72	0,01787	0,02115	0,048645	0,043031	0,068372	0,025572	0,01951	0,046405	2,378481
7	0,024586	0,026993	0,026079	0,079078	0,087306	0,055555	0,02579	0,062004	2,404247
71	0,035981	0,052071	0,108633	0,116377	0,148011	0,071769	0,044026	0,111197	2,525725
49	0,22662	0,194639	0,498048	0,548521	0,67464	0,448597	0,21063	0,542452	2,575382
30	0,219059	0,176526	0,53523	0,508951	0,503828	0,525194	0,197793	0,518301	2,620427
150	0,019019	0,031179	0,068231	0,079137	0,091925	0,02783	0,025099	0,066781	2,660717
140	0,10427	0,048138	0,107126	0,284328	0,216092	0,204903	0,076204	0,203112	2,665375
77	0,328736	0,333931	0,925862	1,05474	0,784744	0,782817	0,331334	0,887041	2,677184
18	0,085325	0,103996	0,237049	0,355337	0,252525	0,283006	0,094661	0,281979	2,978847
47	0,063819		0,223302	0,213186		0,171883	0,063819	0,20279	3,177605
50	0,197968	0,253508	0,721633	0,838063	0,730755	0,638446	0,225738	0,732224	3,243691
43	0,023841	0,021052	0,085608	0,072857	0,079446	0,059009	0,022447	0,07423	3,306955
144	0,07951	0,070183	0,149348	0,384812	0,291711	0,291264	0,074847	0,279284	3,731418
134	0,071176	0,035277	0,224233	0,246062	0,181638	0,149157	0,053227	0,200273	3,762625
25	0,304621	0,367382	1,38381	1,28462	1,15564	1,28847	0,336002	1,278135	3,803956
31	0,085069	0,086046	0,314125	0,370203	0,318765	0,30854	0,085557	0,327908	3,832616
42	0,053075	0,034932	0,183656	0,222322	0,231607	0,098047	0,044004	0,183908	4,179383
115	0,007857	0,024609	0,048916	0,078181	0,096793	0,051796	0,016233	0,068921	4,245823
67	0,096766	0,114558	0,497511	0,56366	0,425266	0,412637	0,105662	0,474769	4,493269
131	0,038628	0,063874	0,307008	0,2757	0,246154	0,186681	0,051251	0,253886	4,953805
1	0,092403	0,167699	1,08239	0,381624	0,59131	0,642842	0,130051	0,674542	5,186748
82	0,0958	0,072323	0,568478	0,467165	0,526774	0,367258	0,084061	0,482419	5,738913
129	0,17463	0,153511	0,974396	0,883362	1,10429	1,35872	0,164071	1,080192	6,583706

Appendix 1 (cont.) – Intensity relative volumes of spots

Match ID	12h A1	12h A2	48h A1	48h A2	8h+GAPD	+GAPDH+AN	Mean 12h	Mean 48h	48h/12h
37	0,244783	0,238906	1,42222	1,6777	1,74197	1,59438	0,241845	1,609068	6,653314
94	0,054815	0,080703	0,526056	0,458088	0,455041	0,442281	0,067759	0,470367	6,941767
69	0,046319	0,071264	0,544627	0,529732	0,49285	0,311507	0,058792	0,469679	7,988866
152			0,069524	0,095388	0,069386	0,108972		0,085817	
153			0,088179	0,11749	0,128202	0,090339		0,106053	
154			0,099857	0,12157	0,096847	0,131476		0,112437	
155			0,94362	0,846331	0,830818	0,732551		0,83833	
156			0,57497	0,813013		1,13923		0,842404	
157			0,093754	0,310446	0,44067	0,343938		0,297202	
158			0,142338	0,270232	0,248222	0,175526		0,20908	
159			0,10065	0,231019	0,183026	0,15493		0,167406	
160			0,240037	0,450834	0,605986	0,535397		0,458064	
161			0,012834	0,069265	0,034326	0,079004		0,048857	
162			0,031835	0,072914	0,067373	0,028028		0,050037	
163			0,18112	0,14281	0,163869	0,083294		0,142773	
164			0,006441	0,021324	0,022932	0,023097		0,018448	
165			0,098183	0,312894		0,309437		0,240171	
166			0,325354	1,38581	1,12896	0,716414		0,889135	
167			0,441537	0,590004	0,659016	0,941538		0,658024	
168			0,672026	1,16596	0,495679	1,12505		0,864679	
169			0,72561	0,956501	0,615233	1,58032		0,969416	
170			2,0877	1,39892	1,25288	0,532735		1,318059	
171			0,062678	0,021462	0,061073	0,03672		0,045483	
172			0,345952	0,070496	0,448824	0,483767		0,33726	
173			0,012717	0,007725	0,015958	0,005171		0,010393	
174			0,010311	0,115648	0,121451	0,087168		0,083644	
175			1,54228	0,984527	0,696767	0,684165		0,976935	
176			0,191109	0,261299	0,276604	0,475626		0,30116	
177			0,215978	0,989724	1,05572	0,705997		0,741855	
178			0,176357	0,17388	0,014299	0,427579		0,198029	
179			0,019579	0,030109	0,036161	0,014125		0,024994	
180			0,015861	0,029706	0,033714	0,008605		0,021971	
181			0,027415	0,050249	0,05116	0,04893		0,044438	
182			0,004183	0,034385	0,036113	0,030101		0,026195	
183			0,044986	0,055766	0,048553	0,043826		0,048283	
184			0,039137	0,046546	0,031042	0,048607		0,041333	
185			0,034204	0,068534	0,059503	0,056373		0,054654	
186			0,083592		0,122038	0,084601		0,096744	
187			0,100881	0,083088	0,106475	0,083378		0,093455	
188			0,180111	0,187798	0,148288	0,153983		0,167545	
189			0,08599		0,115701	0,090486		0,097392	
190			0,085416	0,047073	0,086967	0,044067		0,065881	
191			0,03601	0,066938	0,036305	0,055622		0,048718	
192			0,282509	0,210338	0,200701	0,267218		0,240192	
193			0,139293	0,088201	0,11455	0,157047		0,124773	
194			0,025916	0,043004	0,056525	0,065835		0,04782	
195			0,083812	0,019531	0,027485	0,025099		0,038982	
196			0,028631	0,027752	0,038287	0,063823		0,039623	
197			0,060461	0,042498	0,043709	0,047		0,048417	
198			0,044544	0,022567	0,020781	0,101841		0,047433	

Appendix 1 (cont.) – Intensity relative volumes of spots

Match ID	12h A1	12h A2	48h A1	48h A2	8h+GAPDH	+GAPDH+AN	Mean 12h	Mean 48h	48h/12h
199			0,110195	0,06765	0,053102	0,067132		0,07452	
200			0,06664	0,036563	0,047085	0,063056		0,053336	
201			0,184487	0,168779	0,180205	0,211263		0,186184	
202			0,023933	0,028063	0,037218	0,026711		0,028981	
203			0,093577	0,063605	0,077511	0,102251		0,084236	
204			0,018224	0,015109	0,015162	0,025044		0,018385	
205			0,085946	0,046395	0,040502	0,123455		0,074075	
206			0,098955	0,159228	0,096579	0,038707		0,098367	
207			0,037899	0,073418	0,095004	0,015705		0,055506	
208			0,13832	0,114739	0,100478	0,131052		0,121147	
209			0,032503	0,030353	0,030268	0,04175		0,033718	
210			0,044611	0,049233	0,116547	0,06839		0,069695	
211			0,274206	0,250753	0,218892	0,262553		0,251601	
212			0,105507	0,07707	0,093254	0,07897		0,0887	
213			0,075343	0,076943	0,055714	0,067167		0,068792	
214			0,11485	0,055435	0,065597	0,053678		0,07239	
215			0,036087	0,010189	0,04924	0,021508		0,029256	
216			0,116431	0,07578	0,083444	0,078013		0,088417	
217			0,048264	0,028124	0,017701	0,02556		0,029912	
218			0,031745	0,018045	0,020099	0,027643		0,024383	
219			0,053067	0,071278	0,054702	0,072498		0,062886	
220			0,073157	0,052159	0,046247	0,03844		0,0525	
221			0,070756	0,050385	0,052497	0,046962		0,05515	
222			0,062432	0,043211	0,02281	0,025006		0,038365	
223			0,093954	0,069404	0,028497	0,060518		0,063093	
224			0,077005	0,047123	0,03455	0,035333		0,048503	
225			0,112194	0,110711	0,091134	0,106176		0,105054	
226			0,068865	0,049256	0,032459	0,051628		0,050552	
227			0,039763	0,031521	0,040306	0,031724		0,035828	
228			0,027943	0,022176	0,026402	0,027264		0,025946	
229			0,109032	0,086339	0,072297	0,066857		0,083631	
230			0,063531	0,059228	0,041578	0,04485		0,052297	
231			0,06156	0,058351	0,038836	0,040149		0,049724	
232			0,024041	0,035914	0,019539	0,021392		0,025221	
233			0,036377	0,038109	0,043959	0,032422		0,037717	
234			0,095855	0,0924	0,074513	0,074925		0,084423	
235			0,067165	0,038455	0,029731	0,061625		0,049244	
236			0,082827	0,047397	0,045199	0,043183		0,054651	
237			1,0594	1,21415	1,15673	1,00302		1,108325	
238			0,220668	0,187626	0,155932	0,154619		0,179711	
239			0,57153	1,18004	1,13114	1,29527		1,044495	
240			0,062797	0,081445	0,165802	0,049707		0,089938	
241			0,110027	0,100173	0,123169	0,089029		0,1056	
242			0,031372	0,025043	0,021716	0,015218		0,023337	
243			0,096803	0,082017	0,069514	0,057618		0,076488	
244			0,133559	0,100292	0,061628	0,076592		0,093018	
245			0,127376	0,210039	0,159308	0,147473		0,161049	
246			0,136998	0,184244	0,146607	0,041273		0,127281	
247			0,108893	0,107474	0,0752	0,046901		0,084617	
248			0,223892	0,166435	0,160507	0,113976		0,166203	

Appendix 1 (cont.) – Intensity relative volumes of spots

Match ID	12h A1	12h A2	48h A1	48h A2	8h+GAPDI	+GAPDH+AM	Mean 12h	Mean 48h	48h/12h
249			0,063323	0,207964	0,08391	0,127255		0,120613	
250			0,680465	0,706116	0,46956	0,69812		0,638565	
251			0,058758	0,127891	0,071794	0,049967		0,077102	
252			0,062959	0,070518	0,07022	0,053297		0,064248	
253			0,366692	0,424111	0,396813	0,369508		0,389281	
254			0,210266	0,175685	0,171661	0,130613		0,172056	
255			0,043598	0,057432	0,059941	0,064599		0,056392	
256			0,131485	0,131044	0,12549	0,091699		0,119929	
257			0,070356	0,08017	0,100888	0,093327		0,086185	
258			0,112772	0,071272	0,090661	0,074322		0,087257	
259			0,120333	0,082231	0,07374	0,054061		0,082591	
260			0,080765	0,103352	0,081785	0,036507		0,075602	
261			0,225023	0,3155	0,226486	0,215842		0,245713	
262			0,078329	0,180191	0,231958	0,147515		0,159498	
263			0,022296	0,172356	0,111013	0,101296		0,10174	
264			0,089451	0,042946	0,070416	0,050925		0,063434	
265			0,035186	0,04793	0,047713	0,054778		0,046401	
266			0,103888	0,092149	0,091862	0,05054		0,08461	
267			0,08182	0,085237	0,057278	0,080327		0,076166	
268			0,049143	0,067656	0,088017	0,064463		0,06732	
269			0,063312	0,03596	0,043933	0,031994		0,0438	
270			0,162706	0,291701	0,253456	0,140405		0,212067	
271			0,081404	0,05395	0,067669	0,045352		0,062094	
272			0,258204	0,261784	0,216633	0,347075		0,270924	
273			0,025594	0,01805		0,015988		0,019878	
274			0,058143	0,062853	0,101003	0,033623		0,063906	
275			0,037801	0,056486	0,051333	0,024558		0,042545	
276			0,02011	0,280572	0,143156	0,249899		0,173434	
277			0,233709	0,27594	0,191134	0,163912		0,216174	
278			0,058904	0,060052	0,025113	0,030057		0,043531	
279			0,051029	0,082958	0,061657	0,04921		0,061213	
280			0,14859	0,125632	0,156523	0,107933		0,13467	
281			0,039806	0,025515	0,039452	0,025203		0,032494	
282			0,080936	0,063182	0,046317	0,071273		0,065427	
283			0,073123	0,112073	0,118147	0,110621		0,103491	
284			0,073531	0,159348	0,123478	0,104261		0,115154	
285			0,145313	0,135195	0,133612	0,092589		0,126677	
286			0,066667	0,102153	0,111732	0,062298		0,085713	
287			0,064385	0,042872	0,059371	0,031466		0,049523	
288			0,190773	0,205945	0,267854	0,146181		0,202688	
289			0,126809	0,352968	0,18922	0,211203		0,22005	
290			0,166282	0,197502	0,122332	0,106504		0,148155	
291			0,375717	0,553378	0,529248	0,755257		0,5534	
292			1,25509	0,984332	0,964018	0,544832		0,937068	
293			0,423328	0,980192	0,42212	0,681847		0,626872	
294			0,431192	0,575933	0,40414	0,369414		0,44517	
295			0,304862	0,968695	0,540502	0,629838		0,610974	
296			0,85034	1,42237	1,0981	1,18353		1,138585	
297			0,375733	0,347992	0,434246	0,26908		0,356763	
298			0,036677	0,04391	0,040741	0,008663		0,032498	

Appendix 1 (cont.) – Intensity relative volumes of spots

Match ID	12h A1	12h A2	48h A1	48h A2	8h+GAPD	+GAPDH+AN	Mean 12h	Mean 48h	48h/12h
299			0,223164	0,524898	0,387679	0,530453		0,416549	
300			0,124784	0,187097	0,174305	0,09492		0,145276	
301			0,03687	0,081801	0,080608	0,086618		0,071474	
302			0,117288	0,11004	0,08812	0,07692		0,098092	
303			0,017314	0,022781	0,018059	0,015852		0,018502	
304			0,290844	0,298472	0,022676	0,652039		0,316008	
305			0,0194	0,019492	0,02149	0,014723		0,018776	
306			0,132329	0,172041	0,183518	0,121033		0,15223	
307			0,225894	0,23835	0,297331	0,185541		0,236779	
308			0,095759	0,101211	0,12276	0,105237		0,106242	
309			0,148706	0,206658	0,238154	0,209309		0,200707	
310			0,124465	0,184086	0,173249	0,095866		0,144417	
311			0,018192	0,013917	0,01428	0,005576		0,012991	
312			0,103786	0,134475	0,127847	0,076833		0,110735	
313			0,200956	0,582102	0,359789	0,22773		0,342644	
314			0,401227	0,197032	0,065088	0,12622		0,197392	
315			0,020321	0,025867	0,032246	0,028084		0,02663	
316			0,048017	0,092365	0,062503	0,044156		0,06176	
317			0,025304	0,040409	0,031149	0,014677		0,027885	
318			0,063488	0,030426	0,03375	0,017418		0,036271	
319			0,002984	0,003401	0,006109	0,001806		0,003575	
320			0,018328	0,026659	0,026527	0,015577		0,021773	
321			0,025098	0,037681	0,05259	0,021064		0,034108	
322			0,042073	0,064659	0,064751	0,041625		0,053277	
334			0,007916	0,049585	0,03112	0,020274		0,027224	
335			0,002221	0,023146	0,028972	0,003417		0,014439	
336					7,21786	6,14883		6,683345	
323			2,00302	2,26228	4,24408			2,83646	
324			0,472964	1,09795	0,563787			0,711567	
325			0,01459	0,237758	0,088456			0,113601	
326			0,258003	0,072682	0,182973			0,171219	
327			0,076586	0,055886	0,059615			0,064029	
328			0,013763		0,038009			0,025886	
329			0,088198		0,385951			0,237074	
330			0,031882	0,194525				0,113204	
331			0,186571	0,321562				0,254067	
332			0,063699	0,039063				0,051381	
333			0,064968	0,039541				0,052254	
337	0,100601	0,102262					0,101432		
338	0,133498	0,11805					0,125774		
339	0,055862	0,057593					0,056728		
340	0,048109	0,048981					0,048545		
341	0,138711	0,184334					0,161523		
342	0,046406	0,027219					0,036813		
343	0,036614	0,027141					0,031877		
344	0,045727	0,060175					0,052951		

Appendix 2 – Statistical analysis

Table A.2 Fold and p-value

Fold >/= 5
p value </= 0,05

Table A.3 ANOVA values and spots overexpressed

Match ID	Max	Match Count	12hours	48hours	Anova	Wilcoxon	olmogoro	</=0,2	>/=5	validated spots
33	0,674541	2	0,130051	0,674541	0,070264	0	1	0	1	0
37	1,60907	2	0,241844	1,60907	1,92E-04	0	1	0	1	1
69	0,469679	2	0,058792	0,469679	0,007152	0	1	0	1	1
82	0,482419	2	0,084061	0,482419	0,003773	0	1	0	1	1
94	0,470367	2	0,067759	0,470367	1,65E-04	0	1	0	1	1
129	1,08019	2	0,16407	1,08019	0,004113	0	1	0	1	1

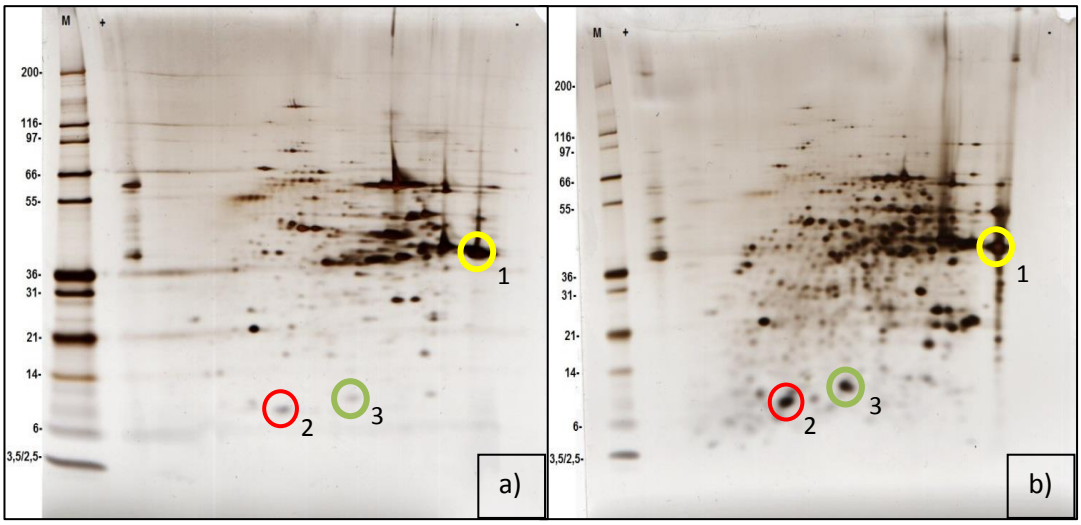
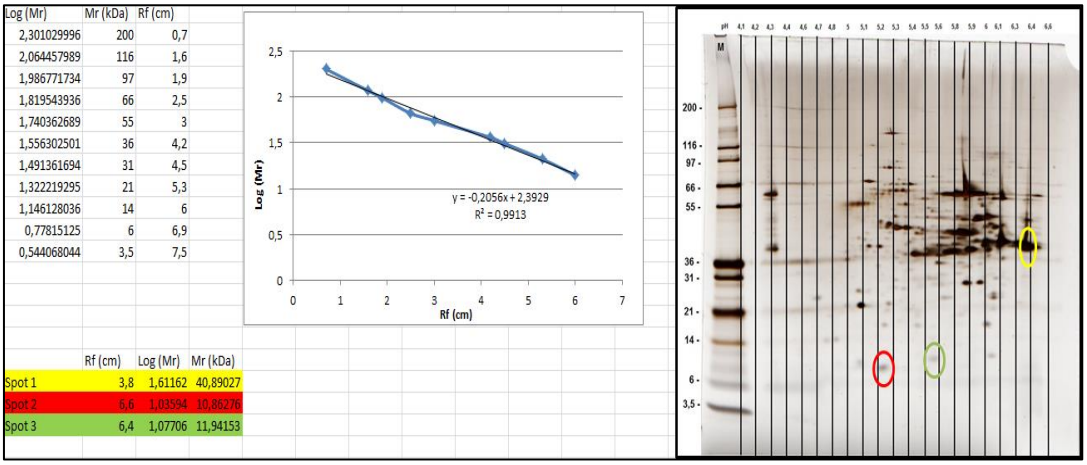


Figure A.1 Spot 1 - GAPDH is not in the statistical analysis; spot 2 is not overexpressed; spot 3 is overexpressed

Table A.4 ANOVA values and spots underexpressed

Match ID	Max	Match Count	12hours	48hours	Anova	Wilcoxon	olmogoro	</=0,2	>/=5	validated spots
4	5,70645	2	5,70645	1,1222	5,95E-04	0	1	1	0	1
8	0,195315	2	0,195315	0,01828	0,027416	0	1	1	0	1
9	0,349832	2	0,349832	0,036489	0,005975	0	1	1	0	1
10	0,494632	2	0,494632	0,044806	0,004016	0	1	1	0	1
11	0,409556	2	0,409556	0,05076	0,073236	0	1	1	0	0
40	0,880209	2	0,880209	0,122324	1,09E-04	0	1	1	0	1
59	5,08397	2	5,08397	0,332929	5,47E-06	0	1	1	0	1
84	0,75779	2	0,75779	0,099189	1,25E-05	0	1	1	0	1
85	0,201579	2	0,201579	0,023344	1,55E-04	0	1	1	0	1
105	1,09346	2	1,09346	0,069103	0,111967	0	1	1	0	0
107	0,438145	2	0,438145	0,014784	2,35E-04	0	1	1	0	1
113	0,030407	2	0,030407	0,005227	0,002954	0	1	1	0	1
118	0,63479	2	0,63479	0,089562	1,95E-05	0	1	1	0	1
119	0,177879	2	0,177879	0,026434	1,42E-04	0	1	1	0	1
124	0,331859	2	0,331859	0,065788	5,37E-04	0	1	1	0	1
125	0,378832	2	0,378832	0,063463	0,002609	0	1	1	0	1
126	0,039334	2	0,039334	0,005994	5,52E-05	0	1	1	0	1
139	0,063547	2	0,063547	0,007878	0,003662	0	1	1	0	1

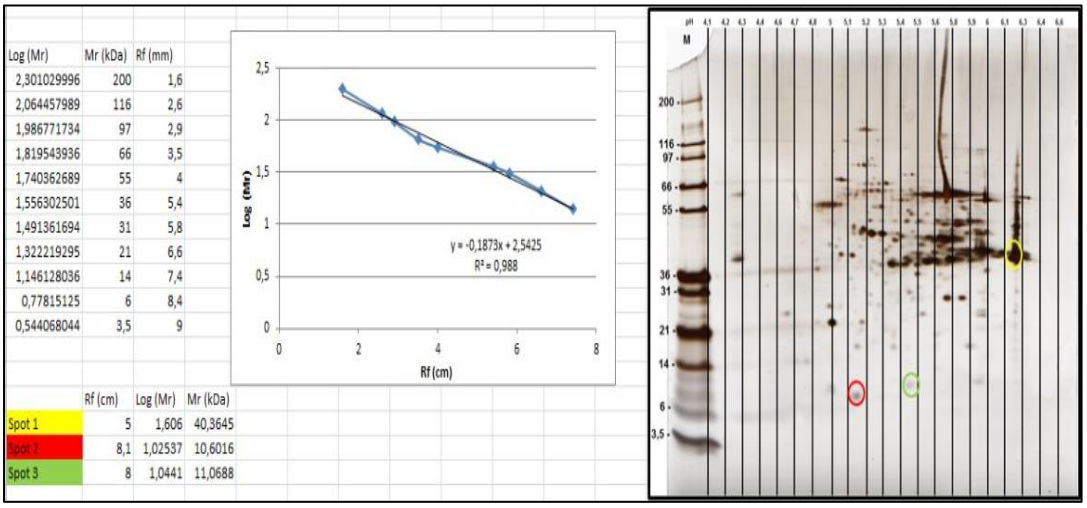
Appendix 3 – Relative molecular masses of the spots



Log (Mr)	Mr (kDa)	Rf (cm)
2,301029996	200	0,7
2,064457989	116	1,6
1,986771734	97	1,9
1,819543936	66	2,5
1,740362689	55	3
1,556302501	36	4,2
1,491361694	31	4,5
1,322219295	21	5,3
1,146128036	14	6
0,77815125	6	6,9
0,544068044	3,5	7,5

	Rf (cm)	Log (Mr)	Mr (kDa)
Spot 1	3,8	1,61162	40,89027
Spot 2	6,6	1,03594	10,86276
Spot 3	6,4	1,07706	11,94153

Figure A2 Relative molecular masses of spots 1, 2, 3 in 2DE gel of membrane proteins extracted from cells at 12 h of growth. Replicate a).

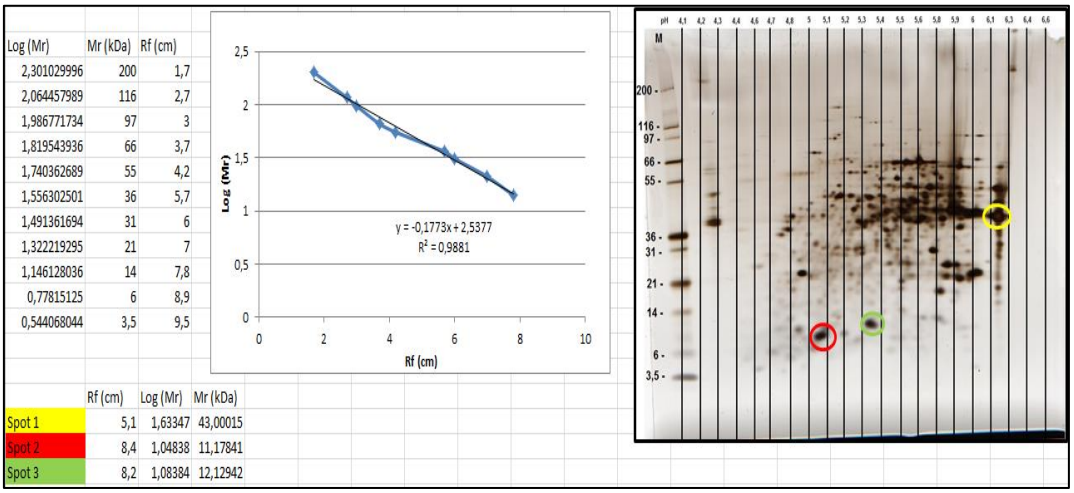


Log (Mr)	Mr (kDa)	Rf (mm)
2,301029996	200	1,6
2,064457989	116	2,6
1,986771734	97	2,9
1,819543936	66	3,5
1,740362689	55	4
1,556302501	36	5,4
1,491361694	31	5,8
1,322219295	21	6,6
1,146128036	14	7,4
0,77815125	6	8,4
0,544068044	3,5	9

	Rf (cm)	Log (Mr)	Mr (kDa)
Spot 1	5	1,606	40,3645
Spot 2	8,1	1,02537	10,6016
Spot 3	8	1,0441	11,0688

Figure A3 Relative molecular masses of spots 1, 2, 3 in 2DE gel of membrane proteins extracted from cells at 12 h of growth. Replicate b).

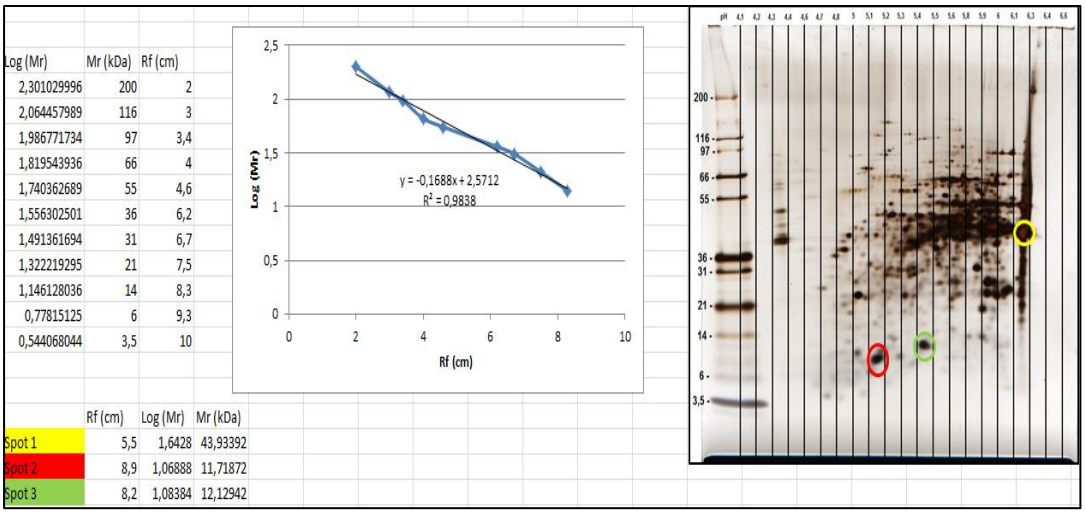
Appendix 3 – Relative molecular masses of the spots (cont.)



Log (Mr)	Mr (kDa)	Rf (cm)
2,301029996	200	1,7
2,064457989	116	2,7
1,986771734	97	3
1,819543936	66	3,7
1,740362689	55	4,2
1,556302501	36	5,7
1,491361694	31	6
1,322219295	21	7
1,146128036	14	7,8
0,77815125	6	8,9
0,544068044	3,5	9,5

	Rf (cm)	Log (Mr)	Mr (kDa)
Spot 1	5,1	1,63347	43,00015
Spot 2	8,4	1,04838	11,17841
Spot 3	8,2	1,08384	12,12942

Figure A.4 Relative molecular masses of spots 1, 2, 3 in 2DE gel of membrane proteins extracted from cells at 48h of growth. Replicate a).



Log (Mr)	Mr (kDa)	Rf (cm)
2,301029996	200	2
2,064457989	116	3
1,986771734	97	3,4
1,819543936	66	4
1,740362689	55	4,6
1,556302501	36	6,2
1,491361694	31	6,7
1,322219295	21	7,5
1,146128036	14	8,3
0,77815125	6	9,3
0,544068044	3,5	10

	Rf (cm)	Log (Mr)	Mr (kDa)
Spot 1	5,5	1,6428	43,93392
Spot 2	8,9	1,06888	11,71872
Spot 3	8,2	1,08384	12,12942

Figure A.5 Relative molecular masses of spots 1, 2, 3 in 2DE gel of membrane proteins extracted from cells at 48h of growth. Replicate b).

Appendix 4 – Protocol optimization

Prior to results obtained in proteomic analysis, several experiments were performed in an attempt to separate and detect GAPDH proteins and its derivative small molecular weight peptides (data not shown). Considering the three isoforms of GAPDH and their p*I*'s (from the SWISS-2DPAGE database - http://world-2dpage.expasy.org/swiss-2dpage) between 6.59-6.98, we selected the IPG gel strip 4-7 pH linear gradient (7 cm long) in order to resolve the GAPDH proteins in their respective p*I*'s. Although it was possible to detect the entire protein, we were not able to separate the GAPDH isoforms according to their p*I*'s since the gel strip ends at pH 6.55. For this reason, we tried IPG gel strips with non-linear 3-10 pH gradient (7 and 13 cm long) but with no success. We decided to continue the work using IPG gel strip 4-7 pH gradient, 7 cm long.

Work previously done by the research group found that the most suitable polyacrilamide gels for the second dimension to detect the AMPs with low MW are the precast 4-12% Bis-Tris gels (NuPAGE® Novex® Bis-Tris ZOOM® protein gels). In the beginning of the work these precast 4-12% Bis-Tris gels were not available in the laboratory, therefore we initially used 4-12% Bis-Tris gels with Tricine SDS running buffer, which is supposed to be suitable for separation of small peptides. However, this combination of Bis-Tris gels and Tricine SDS running buffer was not compatible. We then tried several other protocols based on different running buffers such as Tricine-SDS-PAGE by Schägger (2006) and protocols with higher concentration of bis-acrylamide gels, in order to resolve the AMPs with MW. Though, none of the protocols worked out (data not shown). After several attempts, we found that the most suitable running buffer for electrophoresic separation of small peptides was the MES running buffer with the precast 4-12% Bis-Tris gels.