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Application of response surface methodology (RSM) to optimize the hybridization efficiency of a PNA probe targeting *Saccharomyces cerevisiae*

Dissertation for Master degree in Bioengineering

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Robert Baden-Powell

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ABSTRACT

The yeast *Saccharomyces cerevisiae* is one of the most important microorganisms in the Biological industry since it is the main responsible for beer, bread and wine production. Currently, quality control of these products has assumed a growing importance with a constant monitoring of the fermentation process. Microbiological monitoring consists both on the control of the necessary organisms to perform the fermentation and on the immediate detection of contaminating and very often spoiling organisms.

Conventional microbial identification such as biochemical tests or differential and selective media cannot be routinely used in industry for rapid detection. As a consequence, the development of rapid detection technologies for food and beverage spoilage yeasts employing molecular biology-based methods has assumed a huge importance in industrial research.

The present work focuses in the development and optimization of a fluorescence *in situ* hybridization method using a PNA probe targeting *S. cerevisiae*. The effects of formamide concentration, time and temperature of hybridization on the FISH signal were optimized by statistical analysis. The response surface methodology (RSM) was used to optimize hybridization efficiency by implementing the Box-Wilson design, also known as central composite design (CCD). According to diagnostic plots, the proposed quadratic model provides an adequate approximation to the real system. Statistical analysis of the results showed that the quadratic terms of these three variables had significant effect. However, no interactions between the three variables were found to contribute to the response at a significant level. The optimal conditions for higher hybridization efficiency were 53.9ºC of temperature, 57.8 min of hybridization time and 43.8% of formamide concentration. Under these conditions, the model predicted a fluorescence intensity of 147 a.u. Verification

of the optimization showed that a fluorescence intensity of 183±13 a.u. was observed under the optimal conditions. Hence, the optimization was successfully achieved and the PNA-FISH method showed to be highly robust.

Subsequently, a simplification of the protocol was carried out. It was shown that the number and concentration of components in the hybridization solution can be reduced to 50 mM Tris-HCl and a hybridization time of 30 min proved to be enough to distinguish a positive sample, presenting a fluorescence intensity of 69±18 a.u. These satisfactory outcomes along with the exclusion of formamide, the simplicity and functionality of the PNA-FISH method may open up new applications at an industrial level.

Key-words: Fluorescence *in situ* hybridization, PNA-FISH, Response Surface Methodology, *Saccharomyces cerevisiae*

RESUMO

A levedura *Saccharomyces cerevisiae* é o microorganismo mais importante na indústria biológica pois é o principal responsável pela produção de cerveja, pão e vinho. Atualmente, o controlo de qualidade destes produtos tem assumido uma importância crescente com a constante monitorização do processo de fermentação. A monitorização microbiológica consiste tanto no controlo dos organismos intervenientes como na deteção imediata de organismos contaminantes e que, muitas vezes, conduzem à deterioração do produto final.

Uma identificação microbiana convencional, como testes bioquímicos ou meios diferenciais e seletivos, não pode ser utilizada diariamente na indústria. Como consequência, o desenvolvimento de tecnologias de deteção rápida com base em métodos de biologia molecular tem sido conduzido tendo como alvo leveduras que deterioram alimentos.

A presente dissertação centra-se na hibridação *in situ* fluorescente com uma sonda de PNA direccionada para *S. cerevisiae*. Os efeitos da concentração de formamida, tempo e temperatura de hibridação no sinal de FISH foram otimizados por análise estatística. A metodologia de superfície de resposta (RSM) foi utilizada para otimizar a eficiência de hibridação através da implementação do desenho experimental *Box-Wilson,* também conhecido como desenho do composto central (DCC). De acordo com gráficos de diagnóstico, o modelo quadrático proposto prevê uma aproximação adequada do sistema real. A análise estatística dos resultados demonstrou que os termos quadráticos das três variáveis apresentam efeito significativo. No entanto, nenhuma interação entre as variáveis contribui para a resposta a nível significativo. As condições ótimas para uma eficiência de hibridação mais elevada são 53.9ºC de temperatura, 57.8 min de tempo de hibridação e uma

concentração de formamida de 43.8%. Sob estas condições, o modelo prevê uma intensidade de fluorescência de 147 u.a. Um ensaio de verificação da otimização demonstrou uma intensidade de fluorescência de 183±13 u.a. sob condições ótimas. A otimização foi alcançada com sucesso e o método de PNA-FISH demonstrou ser altamente robusto.

De seguida, foi realizada uma otimização do protocolo. Demonstrou-se que o número e concentração de componentes na solução de hibridação pode ser reduzido a 50 mM Tris-HCl e um tempo de hibridação de 30 min revelou ser suficiente para distinguir uma amostra positiva, apresentando uma intensidade de fluorescência de 69±18 u.a. Estes resultados, juntamente com a eliminação da formamida do protocolo e a simplicidade e funcionalidade do método de PNA-FISH, podem abrir portas a novas aplicações a nível industrial.

Palavras-chave: Hibridação *in situ* fluorescente, PNA-FISH, Metodologia de superfície de resposta, *Saccharomyces cerevisiae*

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ABBREVIATIONS LIST

2FI	Two-factor interaction
3D	Three dimensional
ANOVA	Analysis of variance
a.u.	Arbitrary units
CCD	Central composite design
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EPS	Extracellular polymeric substance
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
GPI	Glycophosphatidylinositol
GRAS	Generally recognized as safe
ITS	Internal transcribed spacer
LD	Live Dead
OD	Optical density
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PNA	Peptide nucleic acid
QPCR	Quantitative polymerase chain reaction
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RSM	Response surface methodology
YEPD	Yeast extract peptone dextrose

CHAPTER ONE

WORK OUTLINE

Project presentation and motivation

The utilization of microorganism for food production has been part of the cultural evolution of humans for centuries. Although the same organisms are still employed for fermentation processes in a large scale nowadays, the food and beverage production is a highly engineered industrial process (Anon. 2013a). Large fermenters require constant monitoring to assure the quality of the final product and to prevent a break-down directly coupled to loss of money.

Beer, wine and bread production are based upon the fermentation of sugar by the yeast *Saccharomyces cerevisiae*, a commonly used industrial microorganism. Currently, quality control of food products has assumed a growing importance either with the control of the desired organisms as with the immediate detection of contaminating and very often spoiling organisms.

In biological industry an accurate and rapid method for microorganism identification and monitoring is required. Fluorescence *in situ* hybridization (FISH) has been used for the study of the dynamics of yeast populations as it combines the direct and simple visualization of the result with the reliability of molecular methods (Amann and Ludwig 2000; Xufre et al. 2006).

Main Objectives

The present study has as main goal the optimization of a Fluorescence *in situ* hybridization with a Peptide Nucleic Acid (PNA) probe. The targeted microorganism was *S. cerevisiae* mostly as it is without any doubt the most employed microorganism in food and beverage production.

Besides the hybridization optimization with a response surface methodology (RSM) approach, a simplification of the protocol to enable a future adaptation to a microfluidic platform was carried out. In a first stage, a standard RSM experiment with a three-factor layout, the called central composite design (CCD), was performed. Temperature, time and formamide concentration were optimized. Additionally and since the method showed to be relatively robust, several simplification experiments were carried out.

Thesis organization

The present chapter describes the main objectives, context and motivations for the development of this work and serves as a guideline to the overall work presented in the subsequent chapters.

In chapter two, a brief literature review is provided offering thorough information regarding microbial identification in biological industrial and fluorescence *in situ* hybridization technique. The response surface methodology is individually analysed in terms of the standard central composite design and its practical application in Bioengineering.

Chapter three consists in the materials and methods section providing details about the yeast strain and the growth media as well as the hybridization procedures and the probe sequence. It is also described the fluorescence intensity measurement with the ImageJ software.

Chapter four comprises the results and an extensive discussion that encompasses the regression models of response and the model checking adequacy provided by the RSM analysis. This chapter also includes a simplification of the protocol, a discussion about the ImageJ analysis and a *S. cerevisiae* probe specificity testing.

Chapters five and six present concluding remarks and perspectives for further research, respectively. An overview of the developed work and an approach for further research are provided.

CHAPTER TWO

LITERATURE REVIEW

Biological Industry: Bread, beer and wine

Antoine Lavoisier, one of the founders of modern chemistry, was a pioneer in the scientific studies of alcoholic fermentation describing this phenomenon as 'one of the most extraordinary in chemistry' (Barnett 2003). He also defined the chemical reactions that underpin the fermentation of sugars into ethanol and carbon dioxide, estimating the proportions of the elements in sugar, water, and yeast biomass. By the second half of the nineteenth century, Louis Pasteur proved that alcoholic fermentation was a microbial occurrence. Pasteur wrote '…we see that the yeast takes something from the sugar…' and declared indisputably that alcoholic fermentation has a biological basis (Borneman et al. 2013). The yeast *Saccharomyces cerevisiae* was identified as the principal microorganism responsible for the conversion of grape must into wine, the oldest biotechnological endeavour (This et al. 2006).

Furthermore, *S. cerevisiae* is used for bread fermentation throughout the world being very important for bread quality. The fermentative activity of baker's yeast is essential not only for the rising action of the dough by production of CO_2, but also in production of the wide range of aroma compounds identified in bread (Frasse et al. 1992; Birch et al. 2013). The metabolism of yeast originates most of the aroma compounds in the fermented bread such as alcohols, aldehydes and esters. Formation of these aroma compounds in bread is highly influenced by the fermentation temperature, fermentation time and yeast level. Recently, aroma of bread and the choice of yeast strain have attained more focus as a quality criterion for bread (Birch et al. 2013).

Besides the centre stage role of *S. cerevisiae* in wine production and bread baking, it is also the main character in the beer brewing process clarifying its designation as brewer's yeast. On an industrial scale, recent researches are ultimately focused on maintaining the integrity of the final product. The support of the supply of yeast requirements for fermentation has also been investigated (Lodolo et al. 2008).

S. cerevisiae as a model organism

S. cerevisiae has become increasingly important over recent years in biotechnology and is now the most investigated and best characterized eukaryotic microorganism. It has been used as a model eukaryote allowing the understanding of the biology of this cell and hence, ultimately, human biology (Ostergaard et al. 2000).

For several centuries, *S. cerevisiae* has been used in the production of food and alcoholic beverages, and today this organism is also used in a number of different processes within the pharmaceutical industry. *S. cerevisiae* is a very attractive organism to work with since it is non-pathogenic, and due to its extensive application in the production of consumable products, it has been classified as a GRAS organism (generally recognized as safe) (Ostergaard et al. 2000; Murphy and Kavanagh 1999).

Another important feature is the susceptibility of *S. cerevisiae* to genetic modifications by recombinant DNA technology, which has been even further facilitated by the availability of its complete genome sequence, published in 1996 (Goffeau et al. 1996).

Yeast cell wall structure and flocculation

The yeast cell wall is a strong, but elastic, structure that is essential not only for the maintenance of cell shape and integrity, but also for progression through the cell cycle (Levin 2011; Klis et al. 2002). The yeast cell wall has

many functions. First, it provides protection from the exposure to rapid and extreme changes in environment, particularly with respect to osmotic shock (Hohmann 2002; Levin 2011). Additionally, the yeast cell wall is required to establish and maintain cell shape and to protect against mechanical stress. Finally, the cell wall acts as a scaffold for cell-surface proteins (Levin 2011). The polysaccharides that provide the mechanical strength of the cell wall also serve as the attachment matrix for a wide variety of glycoproteins such as sexual agglutination factors and adhesins critical to cell-cell contact during biofilm formation (Douglas et al. 2007; Levin 2011).

S. cerevisiae spends a considerable amount of metabolic energy in cell wall construction which, depending on growth conditions, comprises about 10-25% of the total cell mass (Klis et al. 2006). This structure is composed largely of polysaccharides (~85%) and proteins (~15%) (Lesage and Bussey 2006) consisting specifically of an inner layer of load-bearing, acting as a scaffold for a protective outer layer of mannoproteins that extend into the medium as described in Figure 1 (Klis et al. 2006). The major load-bearing polysaccharide is a moderately branched 1,3-β-glucan. Due to the presence of side-chains, 1,3-β-glucan molecules can only locally associate through hydrogen bonds, resulting in the formation of a highly elastic and continuous three-dimensional network (Klis et al. 2006). At the external face of the 1,3-β-glucan network, highly branched 1,6-β-glucan chains are found, which in turn may be connected to a GPI-modified mannoprotein (Klis et al. 2006; Kollár et al. 1997). Chitin, a polymer of β-1,4-N-actetylglucosamine (GlcNAc), is a minor constituent of the S. cerevisiae cell wall and is concentrated at the bud neck and at the septum (Lesage and Bussey 2006). The lateral walls of the growing bud generally do not contain chitin, demonstrating that chitin is not essential for the mechanical strength of the lateral walls (Klis et al. 2006).

Yeast mannan is an electron-dense and fibrillar outer layer of the wall composed by a varied set of mannoproteins linked to the cell wall polysaccharides (Osumi 1998; Klis et al. 2006).
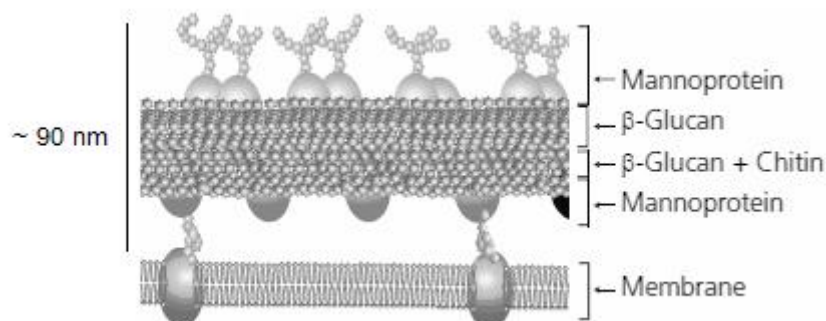
Figure 1 - Structure of the yeast cell wall. The wall is primarily composed of mannoproteins and β-glucan (1→3) and (1→6) (adapted from McClanahan (2009)). Cell-wall thickness has been reported for yeast as approximately 90 nm (Smith et al. 2000).

Yeast flocculation can be defined as an asexual aggregation process of yeast cells into clumps with subsequent fast sedimentation in the medium in which they are suspended; such aggregates are called flocs (Stratford 1992; Soares and Vroman 2003). This process is reversibly dispersed by the action of specific sugars (Masy et al. 1992), salts and EDTA (Soares and Vroman 2003).

The most recognized hypothesis to explain the mechanism of flocculation in *S. cerevisiae* is the lectin-like theory proposed by Miki et al. (1982). According to this model, the flocculation gene FLO1 governs the expression of a lectin present only in flocculent cells. This specific protein, firmly associated with the cell walls of flocculent cells, binds mannose residues present in the cell walls of neighbouring cells. Flocculation was found to be Ca^{2+}-dependent due to the role of calcium ions in the activation of the lectins (Soares and Vroman 2003). Flocculation is a highly complex phenomenon affected by many genetic, physiological and environmental factors that may enhance the survival of yeast cells in starvation conditions (Soares and Vroman 2003).

Requirement for microbial identification at an industrial level

Accurate and rapid microorganism identification is essential in a wide range of applications including microbial forensics, food safety, environmental studies and clinical microbiology. Detection, differentiation and identification of microorganisms can be performed by numerous techniques including those exclusively based on phenotypic, biochemical and immunological features. Nowadays, these assays have been replaced by molecular biology approaches; besides enhancing the sensitivity and specificity of the detection process, they reduce much of the subjectivity inherent to interpreting morphological and biological data (Settanni and Corsetti 2007; Woo et al. 2003).

Focusing in biological industry, serious microbiological problems are caused by yeasts, including *S. cerevisiae*. Particularly in fermented foods and beverages, where the metabolites produced contribute to the flavour, aroma and taste of the final products, it is not easy to define microbial spoilage (Loureiro 2000). In fact, in non-fermented foods, any yeast able to change food sensorial characteristics can be regarded as spoilage yeast. In fermented alcoholic beverages, the spoilage concept is more complex since yeast activity is essential during the fermenting process (Loureiro and Malfeito-Ferreira 2003).

In the wine industry, where alcoholic fermentation occurs in the presence of many yeast species and bacteria (mainly lactic and acetic), the line between beneficial fermenting activity and detrimental spoilage activity is very difficult to draw (Loureiro and Malfeito-Ferreira 2003). Monitoring of spoilage yeasts, such as *S. cerevisiae*, during all phases of winemaking has gained an increasing importance due to the tendency to reduce the use of preservatives, particularly those effective against yeasts such as sulphur dioxide and benzoic acid (Loureiro and Querol 1999; Loureiro and Malfeito-Ferreira 2003).

Currently, yeasts are the most feared cause contaminants leading to wine spoilage. The common spoilage effects are film formation in stored wines, cloudiness or haziness, sediments and gas production in bottled wines, and off-odours and off-tastes at all stages of wine production (Loureiro and Malfeito-Ferreira 2003).

In brewing and winery industries, the discrimination between *S. cerevisiae* fermenting yeasts and ''wild yeasts'' is crucial to assess the microbiological quality of these alcoholic beverages (Loureiro and Malfeito-Ferreira 2003). In this dissertation we will focus in the identification of *S. cerevisiae*, the main microorganism responsible for wine production.

Culture Media and Conventional Phenotypic tests

Conventional laboratory differentiation of yeasts, involving microscopy and biochemical tests, not only requires extensively trained laboratory personnel, but it is also time-consuming and cost-intensive. It depends on several factors difficult to assess, such as the skills of the laboratory staff in examining and identifying the presence and nature of the organisms (Bader et al. 2011; Hay and Jones 2010).

Cultural identification is based on the recognition of specific features, such as macroscopic and microscopic morphology and pigmentation. For some fungi, such as yeast species, rapid biochemical tests or differential and selective media allow a more readily standardized recognition (Hay and Jones 2010). Identification schemes describing the characteristics of a microbial isolate such as colony and cell morphology, nutritional and physical requirements for growth, metabolic characteristics and pathogenicity factors have been developed and improved over many decades (Woo et al. 2003). At this point, even small microbiology laboratories are able to identify isolates to species level using fairly simple traditional test procedures.

Isolation and enumeration media for yeasts are usually complex and nutritionally rich, containing sugar as energy source (e.g., glucose, fructose, sucrose), a digested protein as nitrogen source (e.g., peptone, tryptone, casitone), and a complex supplement (e.g., yeast extract, malt extract) (Loureiro and Malfeito-Ferreira 2003). ()()(30)[57]Several culture media have been developed to allow yeast identification such as Lysine agar and copper sulphate medium. According to van der Aa Kühle and Jespersen (1998), copper sulphate medium was the best medium to discriminate between wild yeasts (including wild *S. cerevisiae*) and fermenting yeasts in lager beers. Heard and Fleet (1986) used Lysine agar to detect non-*Saccharomyces* species, which may be regarded as a hygiene indicator under certain conditions. Thomas and Ackerman (1988) developed a medium with ethanol (11.4% v/v) as a selective agent to detect spoilage yeast in wines verifying the growth of all these yeasts within 72h. Over 77% of the non-spoilers failed to grow in the broth during the same period of time.

Due to the time required and the subjectivity associated, classical identification cannot be routinely used in biological industry. As a consequence, rapid detection and enumeration methods, especially for food and beverage spoilage yeasts, have been developed. Some of the technologies employed include immunological techniques (e.g. ELISA) and molecular biology-based methods.

Nucleic acid-based detection methods

The genetic material of each living system is unique and specific for each species. The design of oligonucleotides that hybridize specifically to target sequences has been the basics to the development of powerful molecular approaches. Techniques like restriction fragment length polymorphism (RFLP) of mitochondrial DNA, restriction enzyme analysis of polymerase chain reaction (PCR)-amplified ribosomal DNA, random amplified polymorphic DNA (RAPD)

assay are now familiar to industry microbiologists (Loureiro and Malfeito-Ferreira 2003).

a. Polymerase Chain Reaction

PCR is a revolutionary method developed by Kary Mullis in the 1980s. This technology is based on using the ability of DNA polymerase to synthesize a new strand of DNA which is complementary to the template strand. The requirement of a primer to initialize DNA polymerase activity makes possible to delineate a specific region of template sequence that the researcher wants to amplify (Anon. 2013b).

The PCR reaction can be divided into three crucial steps. The first one is the denaturation of the double-stranded DNA (dsDNA) molecule at temperatures above 90ºC. Second, oligonucleotide primers bind to the target sequence generally at 50-60ºC - annealing – and, finally, optimal DNA extension occurs at 70-78°C. For conventional PCR methods, the amplification products are analysed by performing a gel electrophoresis followed by an ethidium bromide staining.

PCR methods are particularly promising because of their simplicity, specificity and sensitivity (Martínez et al. 2010). Nevertheless, the choice of target genes and the design of oligonucleotide primers are critical elements in determining the sensitivity of PCR (Yamamoto 2002).

The majority of PCR-based identification methods rely on the amplification of species-specific genes such as: elongation factors, heat-shock proteins, RNA polymerase or ribosomal DNA genes (Johnson et al. 2003; Yamamoto 2002; Johnson 2000). rDNA genes are present in high copy numbers, they contain conserved regions allowing the design of "universal" PCR amplification primers and, at the same time, they exhibit enough genetic differences to allow identification at the species level (Yamamoto 2002; Reyes-

López et al. 2003; Lu et al. 2000). Therefore, rDNA genes are generally considered as ideal targets.

In recent years, a large number of approaches based on PCR techniques have been described as a tool for species identification to assess the quality of products in biological industries. Real-time polymerase chain reaction, also called quantitative polymerase chain reaction (qPCR), is a molecular biology technique based on PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule enabling both detection and quantification (Hierro et al. 2006). qPCR is the abbreviation used for real-time PCR.

Casey and Dobson (2004) developed a qPCR system to differentiate between the common spoilage yeasts, *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Candida krusei*, *Rhodotorula glutinis* and *Saccharomyces cerevisiae*, based on melting peak T_m analysis of the 5.8S rDNA subunit and the adjacent ITS2 (internal transcribed spacer) region of these yeasts.

b. Restriction Fragment Length Polymorphism

Restriction Fragment Length Polymorphism (RFLP) is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases (Anon. 2013c).

A RFLP analysis of ITS region can be applied for rapid identification of spoilage yeasts. Some restriction patterns generated from the region spanning the internal transcribed spacers yielded a unique profile for each species, and could be used as an easy and fast method of routine yeast identification (Caggia et al. 2001).

Isolation of sufficient DNA for RFLP analysis is time-consuming and labour-intensive. However, PCR can be used to amplify small amounts of DNA to the levels required for RFLP analysis (Anon. 2013c). This technique is commonly known as RFLP-PCR.

Ribosomal regions show a low intraspecific polymorphism and a high interspecific variability allowing the classification of *Saccharomyces* species and the identification of several wine yeast species (Esteve-Zarzoso et al. 1999; Loureiro and Malfeito-Ferreira 2003). An RFLP-PCR analysis demonstrated that the complex ITS regions (non-coding and variable) and the 5.8S rRNA gene (coding and conserved) are useful to identify spoilage yeast, since the 5.8S rRNA gene carries great interspecific differences (Esteve-Zarzoso et al. 1999). The 26S rRNA gene has a universally recognized role in yeast taxonomy and recently, it provided the highest correct identification percentage of yeast species associated with honey as described by Carvalho et al. (2010).

Recently, the monitoring of the predominance of the starter yeast strain during industrial wine fermentations has been tested by the analysis of restriction fragment length polymorphism of mitochondrial DNA (mtDNA-RFLP). This method was proposed by Rodríguez et al. (2011) as a response to one of the major challenges for microbiological control in the wine industry allowing a rapid intervention of the wine-producer if the presence of the inoculated yeasts has suffered a sudden decrease in any phase of the fermentation process.

c. Other nucleic acid hybridization methods

The hybridization of complementary DNA oligonucleotides is a basic principle of molecular biology with possible applications in species identification. The method involves essentially the hybridization between the target nucleic acid and a probe (usually labelled with fluorescent or radioactive molecules) and a positive result indicates the presence of the target species (Pereira 2008).

A number of factors are known to limit the widespread application of traditional DNA-DNA hybridization methods: good quality or undegraded DNA are usually required; small changes in experimental conditions may originate different results; non-discrimination between closely related species may occur (due to cross-hybridizations); and it is a time-consuming procedure (Pereira 2008). However, DNA hybridizations methods have the advantage of enabling the simultaneous detection of multiple species in a sample with the use of two or more specific probes either tested in separate reactions or labelled with unique fluorescent dyes.

A widely known nucleic acid hybridization-based approach is the fluorescence *in situ* hybridization (FISH) technique. This technique uses fluorescently labelled probes to detect nucleic acid sequences in whole cells, allowing the direct detection of organisms in complex microbial communities (Pereira 2008; Moter and Göbel 2000). A higher stability and affinity in the hybridization with FISH assays can be achieved by using peptide nucleic acid (PNA) probes. In the next section, the details and advantages of FISH technique as well as of PNA probes will be presented.

Fluorescence *in situ* hybridization

Fluorescence *in situ* hybridization (FISH) is a powerful technique introduced in the late 1980s with numerous applications and it has gained general acceptance as a clinical laboratory tool (Bishop 2010). The FISH method has been used in clinical diagnostic and other fields of microbiology as the characterization of microbial communities and diversity of natural habitats (Levsky and Singer 2003; Amann and Fuchs 2008).

In the last decade, fluorescence *in situ* hybridization became one of the methods of choice for the study of the dynamics of indigenous yeast populations during wine fermentations since it combines the direct visualization

with the reliability of molecular methods (Xufre et al. 2006; Amann and Ludwig 2000; Moter and Göbel 2000).

In wine-related applications, Stender et al. (2001) explored this technique both for the rapid monitoring of lactic acid bacteria and for the detection of the slow growing yeast *Dekkera bruxellensis*, a well-recognized wine spoilage yeast that causes an undesirable flavour.

Recently, Xufre et al. (2006) followed the evolution of the indigenous yeast populations during inoculated wine fermentations of white and red grape musts in a winery at Alentejo, Portugal. In this study, they were developed fluorescent oligonucleotide probes targeted to the D1/D2 region of the 26S rRNA of different yeast species known to be involved in the vinification process.

Andorra et al. (2011) analysed *Saccharomyces cerevisiae* and *Hanseniaspora guilliermondii* populations during alcoholic fermentations by plating and culture-independent methods, such as fluorescence *in situ* hybridization (FISH) and quantitative PCR (qPCR). Species-specific FISH probes labeled with fluorescein (FITC) were used to directly hybridize *S. cerevisiae* and *H. guilliermondii* cells from single and mixed cultures that were enumerated by epifluorescence microscopy and flow cytometry. FISH and qPCR revealed the presence of high populations (10^7-10^8 cells/ml) throughout fermentations. Flow-FISH uses flow cytometry to perform FISH automatically using per-cell fluorescence measurements and allowing a high resolution and highly automated analysis of mixed microbial populations (Amann et al. 1990). The main advantage of this technique is its sensitivity since it can detect one cell in a million.

FISH method - How does it work?

Fluorescence *in situ* hybridization detects nucleic acid sequences by a fluorescently labelled probe that hybridizes specifically to its complementary

target sequence of sample ribosomal RNA (rRNA) within the intact cell (Amann and Fuchs 2008; Moter and Göbel 2000). Figure 2 demonstrates the basic steps of FISH method. The oligonucleotide probes are covalently linked at the 5′-end to a single fluorescent dye molecule (Amann and Ludwig 2000).
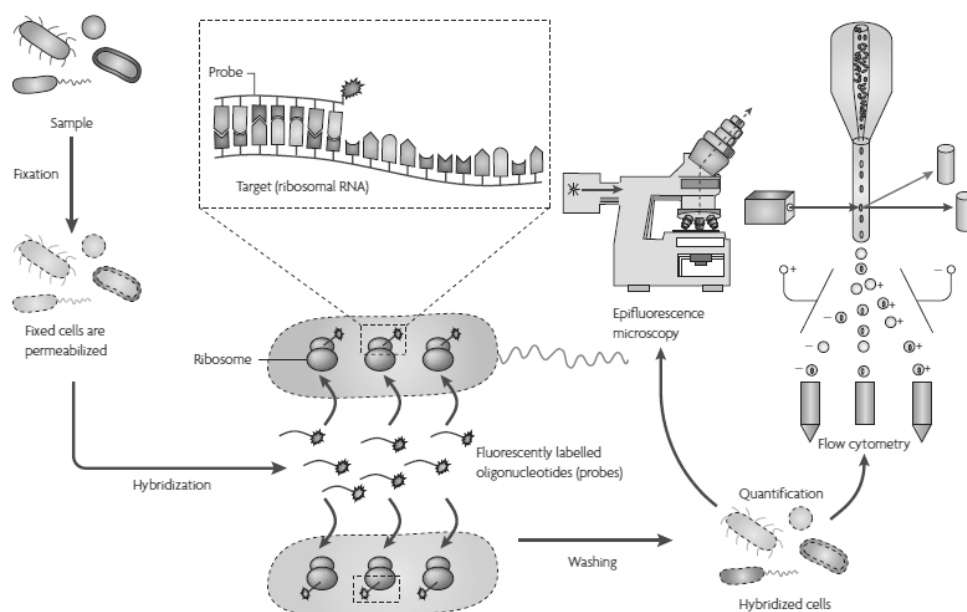
Figure 2 - Basic steps of fluorescence *in situ* hybridization: fixation, hybridization, washing and quantification by either epifluorescence microscopy or flow cytometry (Amann and Fuchs 2008).

rRNAs are the main target molecules for FISH for several reasons: they can be found in all living organisms, they are relatively stable, they include both variable and highly conserved sequence domains and they occur in high copy numbers that can range from a few hundred to 100,000 per cell (Stender et al. 2002; Amann and Ludwig 2000; Amann and Fuchs 2008).

The first step of FISH using rRNA-targeted oligonucleotide probes is the fixation and permeabilization of the sample. This treatment is crucial for a quantitative FISH assay not only stabilizing cell morphology, but also permeabilizing as many cells as possible to allow the labelled oligonucleotides to diffuse to their intracellular rRNA target molecules (Amann and Fuchs 2008). Formaldehyde and ethanol continue to be the main fixatives used, but there is still no standard permeabilization protocol for all microbial cells. The specific

composition of the microbial cell wall is considered in the FISH optimization protocol introducing modifications such as enzymatic digestion of thick peptidoglycan layers by lysozyme, digestion of proteinaceous cell walls by proteases, the use of detergents and even short-term incubations in hydrochloric acid (Amann and Fuchs 2008; Roller et al. 1994). Membrane integrity is intrinsically linked to cell viability and, consequently, fixed FISH-stained cells are no longer viable.

The following step is hybridization, which consists on the incubation with a probe during which the labelled oligonucleotide diffuses to its intracellular targets and forms specific hybrids. During hybridization, temperature, pH, ionic strength and formamide concentration should be correctly defined and optimized to guarantee that the probe accesses and hybridizes with the target sequence (Cerqueira et al. 2008). The unbound probe is then washed away. Hence only specifically targeted cells retain the probes under the appropriate stringency conditions in the hybridization and washing steps. At this point, the sample is ready for single-cell identification and quantification by either epifluorescence microscopy or flow cytometry, which also allows for fluorescence-activated cell sorting (Sekar et al. 2004; Amann and Fuchs 2008).

If an oligonucleotide probe is properly designed, failure to detect target cells by FISH is most often caused by lack of cell permeabilization, low cellular ribosome content or inaccessibility of the probe binding site based on the higher-order structure of the ribosome (Amann and Fuchs 2008).

Peptide Nucleic Acid FISH

Peptide nucleic acid (PNA) probes are synthetic DNA mimics developed in the early 1990s (Nielsen et al. 1994), where the negatively charged sugar-phosphate backbone of DNA is replaced by an achiral, neutral polyamide backbone formed by repetitive units of N-(2-aminoethyl) glycine (Figure 3)

(Stender et al. 2002). They can hybridize to complementary nucleic acid targets obeying the Watson–Crick base pairing rules (Perry-O'Keefe et al. 2001). The lack of electrostatic repulsion, due to the uncharged nature of the PNA backbone is perhaps the main reason responsible for its properties, such as the higher specificity and more rapid hybridization kinetics compared to traditional DNA probes (Cerqueira et al. 2008).
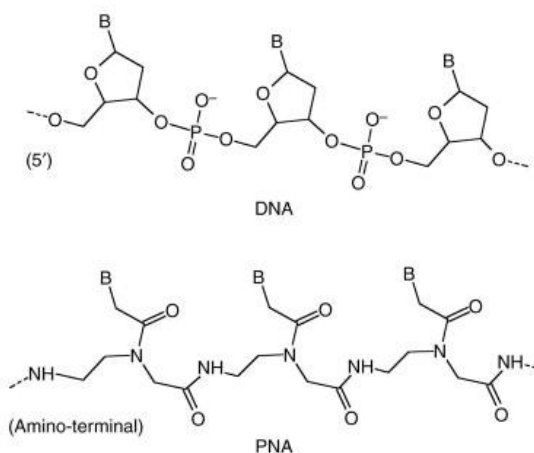
Figure 3 - Chemical structures of DNA and PNA. In PNA, the sugar phosphate backbone of DNA is replaced by a polyamide backbone, keeping the space between the nucleotide bases the same (Nielsen 2001).

The improved thermal stability compared with DNA/DNA duplexes implies that the melting temperature (Tm) for PNA/DNA duplexes is higher than for DNA/DNA (Perry-O'Keefe et al. 2001; Nielsen 2001). This increased Tm enables the synthesis of PNA probes shorter than most DNA probes. In fact, sequences of approximately 15 bp have been found to be optimal for PNA probes which contrast with probes of 20-24 bp for DNA. The higher specificity of PNA may be explained by the effect on the Tm of a single-base mismatch that will have much more impact in PNA/DNA hybridization than in DNA/DNA hybridization (Cerqueira et al. 2008).

In addition, PNA probes hybridize efficiently under low salt concentrations, a condition that promotes the target of nucleic acids with a high degree of secondary structure such as rRNA (Perry-O'Keefe et al. 2001; Cerqueira et al. 2008). The unnatural PNA backbone also means that PNA is

not degraded by ubiquitous enzymes, such as nucleases and proteases (Demidov et al. 1994; Stender et al. 2002). Finally, diffusion through the cell membrane and naturally occurring microstructures such as the EPS biofilm matrix might be easier, even in Gram positive bacteria, due to the hydrophobic character of PNA as compared to DNA (Drobniewski et al. 2000).

The utilization of PNA probes appears to have a particularly promising future in the rapid identification of yeast species.

Response Surface Methodology

To improve the performance of the systems and to increase the yield of the processes without increasing the cost, the called optimization, has assumed an emergent importance in industrial research and development (Baş and Boyacı 2007). The one-variable-at-a-time method consists of a parameter change to define the optimal operating conditions while keeping the others at a constant level. This method is time-consuming and it does not include interactive effects among the variables. Eventually, it does not depict the complete effects of the parameters on the process. In order to overcome this problem, optimization studies can be carried out using response surface design software (Baş and Boyacı 2007).

Response surface methodology (RSM) is a collection of mathematical and statistical techniques useful for developing, improving, and optimizing processes. The objective is to optimize a response of interest which is influenced by several independent variables (Anderson-Cook et al. 2009; Baş and Boyacı 2007). An experiment is a sequence of designed tests, called *runs*, in which changes are made in the input variables in order to identify the reasons for changes in the output response.

A standard RSM design

RSM can be used to define the relationships between the response and the independent variables offering a large amount of information from a small number of experiments. In RSM it is possible to observe the interaction effect of the parameters on the response. In addition to analysing the effect of the independent variables, this experimental methodology also generates a mathematical model. The graphical perspective of the mathematical model has led to the term *Response Surface Methodology* (Baş and Boyacı 2007).

The relationship between the response and the input is given in Eq. (1):

$$\eta = f(x_1, x_2, \ldots, x_n) + \varepsilon \tag{1}$$

where η is the response, f is the unknown function of response, $x_1, x_2, \ldots, x_n$ denote the independent variables, also called natural variables and n is the number of the independent variables. The measurement error is an example of other source of variability represented by the statistical error ε. It is generally assumed that ε has a normal distribution (Baş and Boyacı 2007).

Response surface methodology allows the approximation of a complex unknown function with a low-order polynomial, usually either a first-order model (linear equation) or a second-order model (quadratic equation) (Anderson-Cook et al. 2009). To fit the data to a second order polynomial is the major drawback of RSM as not all the systems containing curvature are well accommodated by this function. For example, simple enzyme kinetics is defined by the Michaelis–Menten equation which defines a rectangular hyperbola through the origin (Baş and Boyacı 2007).

A standard RSM design consists of a central composite design (CCD) that fits a quadratic surface, which usually works well for process optimization. The three-factor layout for this CCD is represented in Figure 4. It is composed of a star design with axial points at $\pm\alpha$ (stars), a 2^k factorial design points at ± 1

(vertices) and a common centre point of the two designs (Stat-Ease 2010). CCD is also known as Box-Wilson design.
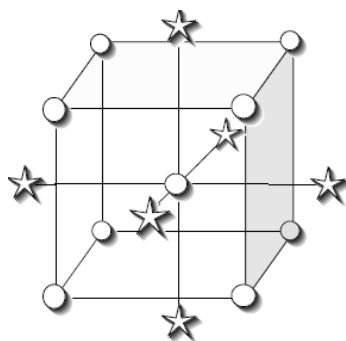
Figure 4 - Central Composite Design (CCD) for three factors (Stat-Ease 2010).

Practical application in Bioengineering

In recent years, RSM has been very popular for optimization studies in a wide range of research areas. Some examples of the RSM applications performed for optimization of biochemical process are pectin hydrolysis using pectolytic enzymes (Rodríguez-Nogales et al. 2007), coagulation–flocculation process for a paper-recycling wastewater treatment (Wang et al. 2007), aerobic biodegradation of dichloromethane (Wu et al. 2009) and determination of reaction parameters for damaged starch assay (Boyacı et al. 2004).

Beg et al. (2003) optimized the alkaline protease production from *Bacillus mojavensis* in a bioreactor. The effects of casamino acids concentration, glucose concentration, inoculum age, incubation time, and agitation rate on response were investigated. Firstly, the effect of the independent parameters on protease production in shake flask cultures was determined using RSM and then optimum parameters were used in a bioreactor. Alkaline protease production in *B. mojavensis* was improved up to 4.2-fold in a 14L bioreactor during validation of a predicted statistical model. The final enzyme yield in the bioreactor was 2389 Uml^{-1} obtained within 10-12 h compared to 558 Uml^{-1} after 24 h in shake flask cultures.

Gonçalves et al. (2012) enhanced the production of pectinases by recombinant *Penicillium griseoroseum* T20 using Response Surface Methodology. The independent variables studied were the concentration of the carbon source sucrose and the cultivation time. The *P. griseoroseum* T20 strain presented an increase in pectin lyase (PL) production of more than 400 fold compared to the wild type when cultivated in commercial sucrose and yeast extract. This optimization confirmed the large potential of the industrial application of response surface designs. The optimum conditions were reached in a less laborious and less costly manner, detecting, further, the interaction among several factors.

Yeasts play a prominent role in wine fermentations. Different factors can affect this process directly influencing the growth rate of the microorganisms and the final composition, quality and flavour of wine (Torija et al. 2003). Arroyo-López et al. (2009) studied the effect of temperature, pH and sugar concentration on the growth parameters of *S. cerevisiae* T73 by means of response surface methodology based in a central composite design. In the case of the maximum specific growth rate (μ_{max}), the temperature was the most important variable, although the effect of sugar concentration was also significant ($p < 0.05$).

Based on these examples, we can affirm that RSM is a useful tool for the optimization of chemical and biochemical process with a huge impact on an industrial scale.

CHAPTER THREE

MATERIALS AND METHODS

Yeast strain, inoculum cultures and growth media

Saccharomyces cerevisiae PYCC3507 was kindly provided by Drª Manuela Rodrigues of the Department of Biology of the University of Minho. Yeasts were maintained on YEPD medium (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose) at 30ºC for at least 24h. For YEPD-agar medium, 2% (w/v) agar was added to the previous formulation. Yeast extract, dextrose and agar are from Merck, Darmstadt, Germany and peptone is from Liofilchem, Teramo, Italy.

The *S. cerevisiae* preculture was prepared by transferring biomass of one YEPD-agar plate into 50 ml of YEPD medium in 100 ml Erlenmeyer flasks which were incubated for 16 h (Andorra et al. 2011) at 30ºC (VELP Scientifica Incubator, FOC 225E model, Usmate, Italy) and 160 rpm (IKA KS 130 basic shaker, Vidrolab, Portugal), under aerobic conditions. Then, 100 ml of YEPD medium in 250 ml Erlenmeyer flasks were inoculated with 2 ml of inoculum, as described by Sekavov et al. (2005), allowing yeast proliferation until the exponential growth phase. The cultivations were carried out at 30°C, 160 rpm and under aerobic conditions.

S. cerevisiae growth phase

According to Hoshino et al. (2008) the intensity of probe-conferred fluorescence in *E. coli* cells presents lower intensities in the stationary phase that might be explained by reduced permeability of the cells due to structural changes in the cell wall. To assure the growth phase of *S. cerevisiae*, a growth

curve was constructed following the optical density at 600 nm, using a spectrophotometer (VWR V-1200). This yeast grows with a specific growth rate of 0.40 h^{-1} corresponding to a doubling time of 1.87 h on YEPD medium. To the FISH experiments, *S. cerevisiae* culture was grown until the mid-log phase with an OD$_{600nm}$ of approximately 0.80 what, according to Bergman (2001) corresponds to a cell density of 2.4x10^7 cells/ml. The *S. cerevisiae* growth curve may be found in Appendix I.

Experimental design and statistical analysis

The central composite design (CCD), which is the standard RSM, was selected for the optimization of the hybridization parameters. Temperature (x_1), time (x_2) and formamide concentration (x_3) were selected as three independent variables on the hybridization efficiency of a PNA probe targeting *S. cerevisiae*. Table 1 shows the maximum and minimum levels of variables defined for trials in the central composite design.

Table 1- Experimental levels of variables tested for fluorescence intensity.

Independent variables		Range and level				
		-α	-1	0	+1	+α
x_1	Temperature (ºC)	29.77	40.00	55.00	70.00	80.23
x_2	Time (min)	9.55	30.00	60.00	90.00	110.45
x_3	[Formamide] (%v/v)	0.00*	19.00	47.00	75.00	94.09

* The value defined by the software was -0.09.

Fluorescence intensity was selected as the dependent variable. The response variable was fitted by a second-order model in the form of quadratic polynomial equation as described in equation 2 (Wang et al. 2007):

$$Y_m = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i}^{i<j} \sum_j b_{ij} X_i X_j \tag{2}$$

where Y_m is the response variable to be modelled; X_i and X_j the independent variables which influence Y_m; b_0, b_i, b_{ii} and b_{ij} are the offset terms, the *i*th linear

coefficient, the quadratic coefficient and the *ij*th interaction coefficient, respectively.

The fitted polynomial equation was expressed as surface and contour plots in order to visualize the relationship between the response and experimental levels of each factor and to obtain the optimum conditions (Lu et al. 2008). The optimization study was performed with Design-Expert 8 software developed by Stat-Ease, Inc. (Minneapolis).

Probe sequence

A probe targeting *S. cerevisiae* had already been designed by Meireles (2012) with Primrose Software (v2.17 - 2012) developed by Dr. K.E. Ashelford, Cardiff University, UK and ordered to Panagene Inc. The probe targets *S. cerevisiae* 26S rRNA (Appendix II) and it has the following characteristics: Alexa 594-OO-AGGCTATAATACTTACC (sequence 5' to 3'), being HPLC purified > 90%. The theoretical specificity is 91.15% calculated as the number of *S. cerevisiae* hits / total hits in all detected microorganisms. This probe presents a sensitivity of 89.47% calculated as the number of *S. cerevisiae* hits / total *S. cerevisiae* sequences in database.

Table 2 - Probe thermodynamic parameters calculated by Meireles (2012).

Probe	ΔH (Kcal/mol)	ΔS (Kcal/K)	ΔG (Kcal/mol)	Tm (ºC)	Tm PNA (ºC)
AGGCTATAATACTTACC	-115	-321.7	-15.26	62.4	66.2

Hybridization in suspension

The hybridization method was based on the procedure of Guimarães et al. (2007) with slight modifications. First, 1ml of the *S. cerevisiae* culture with an OD_{600nm} of approximately 0.8 was pelleted by centrifugation at 10,000 g for 5 minutes (Centrifuge 5418, eppendorf, USA), resuspended in 400 µl of 4%

(wt/vol) paraformaldehyde (Acros Organics, UK) and fixed for 1 h. Then, 500 µl of 50% (vol/vol) ethanol was added to the fixed cells incubating for at least 30 min at -20ºC. Subsequently, 200 µl of the fixed cells aliquot was pelleted by centrifugation and resuspended in 100 µl of hybridization solution with 200 nM of PNA probe and incubated at 53ºC for 60 min in an oven (FD 23, Binder, Germany). After hybridization, cells were centrifuged at 10,000 g for 5 min, and 500 µl of wash solution was added (incubation at 53ºC for 30 min). Washed suspension was pelleted by centrifugation and resuspended in 500 µl of sterile water. Finally, 20 µl of the cell suspension were spread on a microscope slide. Samples were allowed to air dry. The sample is then ready to be observed in a fluorescent microscope. The composition of the hybridization and washing solutions may be found in Appendix III.

To overcome the high degree of flocculation of *S. cerevisiae*, some modifications to the standard protocol were performed. The modifications consisted of a pre-fixation washing with 500 µl of physiologic serum (0.9% NaCl) + 0.05% (vol/vol) Tween 80 (Liofilchem, Teramo, Italy) and a final resuspension in 30 mM EDTA (Panreac Quimica, Spain) instead of water.

For every experiment, a negative control was performed simultaneously, where all the steps described above were carried out, but where no probe was added during the hybridization procedure.

Hybridization procedure on slides

The standard PNA-FISH protocol was performed as referred by Guimarães et al. (2007) with some adjustments. An inoculum of *S. cerevisiae* was prepared by standard procedures and immersed in 4% (wt/vol) paraformaldehyde followed by 50% (vol/vol) ethanol for 15 minutes each and allowed to air dry. Samples were then covered with 20 µl of hybridization solution containing 200 nM PNA probe. A simplified hybridization solution was

also tested. The detailed composition of these solutions may be found in Appendix III. Samples were covered with coverslips, placed in moist chambers and incubated for 60 minutes at 55ºC in an oven (FD 23, Binder, Germany). Subsequently, coverslips were removed and the slides were submerged in a pre-warmed washing solution. Washing was performed at 55ºC for 30 minutes and the slides allowed to air dry. The sample is then ready to be observed in a fluorescent microscope.

Microscopic visualization

The fluorescence signal was acquired using a Leica DM LB2 (Leica Microsystems, Germany) epifluorescence microscope equipped with a Live/Dead Filter sensitive to the Alexa Fluor 594 molecule attached to the PNA probe (Excitation 530 to 550 nm; Barrier 570 nm; Emission LP 591 nm). A N2.1 Filter (Excitation 515 to 560 nm; Barrier 580 nm; Emission LP 590 nm) was used to confirm the absence of autofluorescence of *S. cerevisiae* cells in the red emission range.

Fluorescence intensity measurement

The technique of analysis was based on free available software, the ImageJ NIH, which is widely used for confocal microscope fluorescent image analysis (Rasband 2006; Casanova-Molla et al. 2011). The main steps are schematized in Figure 5. The RGB image was separated into three 8–bit grayscale images containing the red, green and blue components of the original (option: Split Channels). Due to the red fluorescent probe that target S. cerevisiae, the red channel was duplicated. The next step was the definition of a specific threshold, selecting B&W and Dark Background and adjusting manually the value. In order to minimize the variations, it was necessary to use a method that reduces the difference for each measurement. The method consists in defining the threshold smaller as the disappearance of the background, and no

disappearance of cell fragments. When the intensity values are very high, the default threshold was accepted. This binary image was processed assuming black cells on a white background.
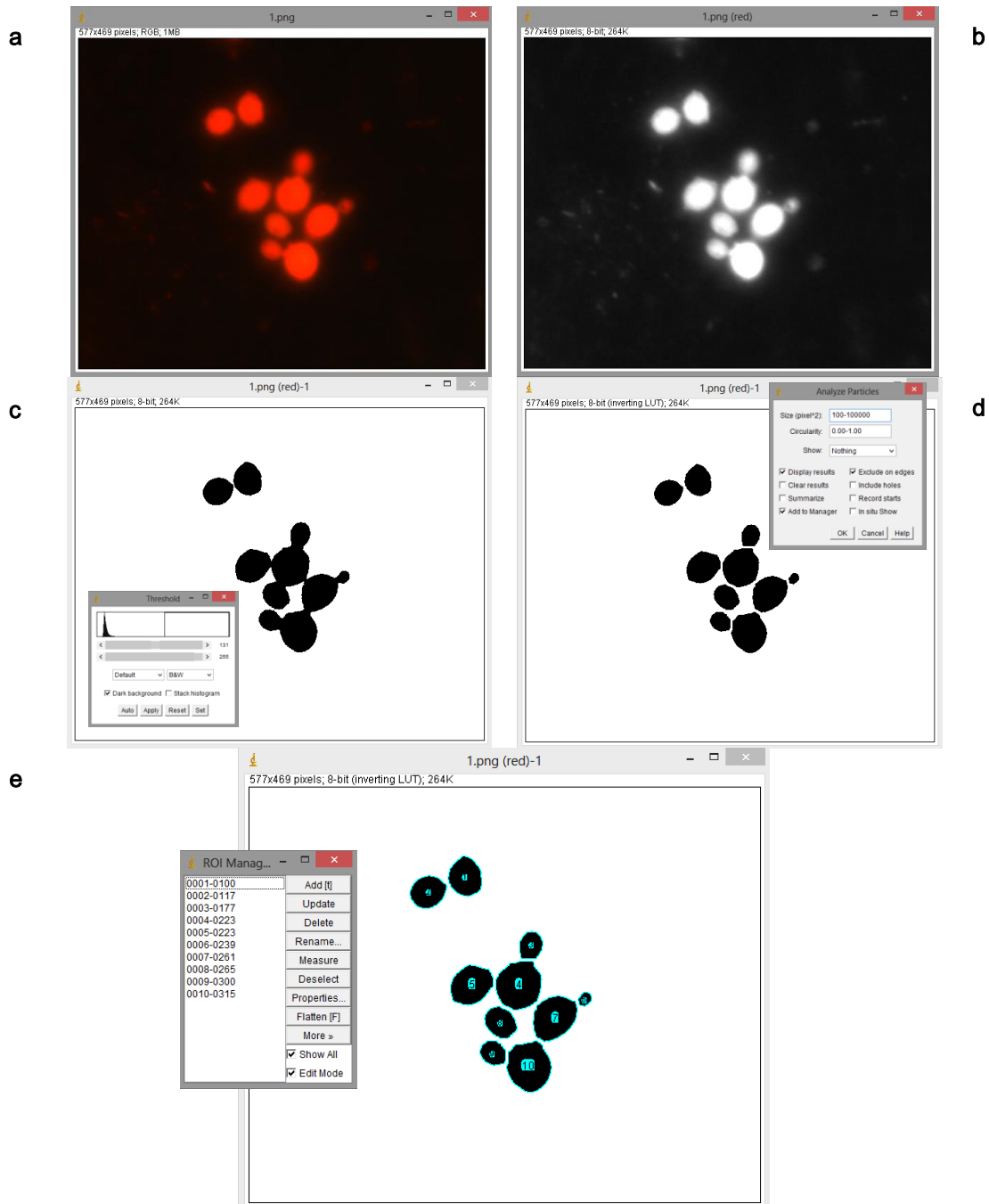
Figure 5 – Fluorescence intensity measurement with ImageJ software. a) Open image; b) Split channels; c) Adjust threshold; d) Watershed, Erode and Analyse Particles; e) Save data from ROI manager.

Watershed segmentation was selected to separate nearby cells allowing an automatically separation or cutting apart cells that touch. Then, the option Erode removes pixels from the edges of cells in the binary image. Redirecting to

the duplicated red channel image, the option Analyse particles was selected. The following parameters were defined after the analysis of several images. Size: 100-100000; Circularity: 0.00-1.00

The results are shown in a new window, ROI manager, and then saved as .txt file. It was also developed an automatic analysis of the results using MATLAB that makes the average, standard deviation and count the number of cells in each image and saves an excel document with these parameters of all the images placed in the folder.

Statistics

Data were calculated with the mean and standard deviation. Student's two-sided t-tests were performed to compare data sets using p-values <0.05 to determine statistically significance (McDonald 2009).

CHAPTER FOUR

RESULTS AND DISCUSSION

Before the determination of the optimal conditions to the fluorescence *in situ* hybridization by response surface methodology, several additional experiments were performed in order to define the best assay conditions since the composition of the hybridization solution and the optical density that corresponds to *Saccharomyces cerevisiae* exponential phase.

Simplification of the hybridization solution - I

The optimization of the FISH protocol started with the comparison of simplified and complex hybridization solutions. The difference between them is the presence of NaCl, sodium pyrophosphate, polyvinylpyrrolidone, Ficol and disodium EDTA in the complex one. Theoretically, the high salt concentration, or ionic strength, stabilizes secondary structures of rRNA and increases the reaction rate (Azevedo 2005). Sodium pyrophosphate, polyvinylpyrrolidone and Ficol are high-molecular weight polymers that compose the Denhardt's solution. This mixture works as a blocking reagent for preventing the unspecific binding of nucleic acids in the hybridization step. EDTA is a chelating agent removing free divalent cations that strongly stabilize PNA-RNA duplexes (Azevedo 2005). According to Meireles (2012), 53ºC is the optimal temperature for hybridization in suspension. As such, this temperature was selected as the start point. The simplified hybridization solution shows greater fluorescence intensity contrarily to the expected, suggesting that in these conditions the complex solution can be substituted for this one with an improvement in the final signal.
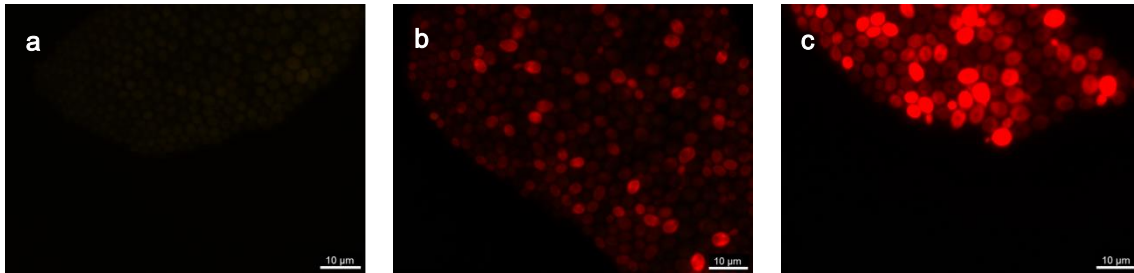
Figure 6 - Fluorescence Microscope results for in suspension PNA-FISH (30% formamide) at 53ºC for 1h - Hybridization solution optimization. a) Negative control; b) Standard hybridization solution; c) Simplified hybridization solution.

Deflocculating assay

An important observation is the tendency of *S. cerevisiae* to form aggregates what will difficult its analysis by the ImageJ software. Yeast flocculation is enhanced in the presence of starvation conditions and, in this particular case, may be due to the sequential centrifugations of the in suspension FISH protocol. Consequently, the yeast flocculation was the first hurdle to overcome. Several experiments were tested since sonication and pre-fixation washings until the hybridization method on slide. The main goal was to decrease the degree of flocculation.

Sonication assays between 1 and 45 minutes were performed with and without an additional step of filtration (10 μm). Yeast cells were placed in an ultrasound water bath in order to disrupt possible clumps and to obtain a single-cell suspension to be used in optimal conditions during FISH experiments. Increasing the sonication time, smaller circular clumps were observed but the dispersion was not enough to perform an adequate image analysis. Interestingly, after 45 minutes of sonication, yeast cells maintain their shape relatively defined, probably as a result of organism cell walls which are highly resistant. It is possible that sonication physically weakens the yeast cell wall; in the absence of chemical cell wall stressors, the sonication treatment had negligible effect on viability (Islahudin et al. 2013). No structural differences and advantages were observed by adding filtration.

On slide PNA-FISH was also tested because it is clearly well documented and widely implemented comparing to the suspension procedure. Nevertheless, the suspension procedure is preferable as it allows an anticipated fixation followed by the storage of fixated *S. cerevisiae* at -20ºC until the experiment. Considering the Response Surface Methodology assay, it comprises three blocks (two of them with 6 assays and the other one with 8) and the storage of the fixated microorganism will facilitate the experiment. According to Meireles (2012), 55ºC is the optimal temperature for PNA-FISH on slide. The FISH protocol on slide showed relatively greater fluorescence intensity (Figure 7) perhaps due to the fact that the suspension procedure demands more manipulation due to the numerous centrifugation and resuspension steps, situation that may overexpose the sample to the light, resulting in loss of signal.
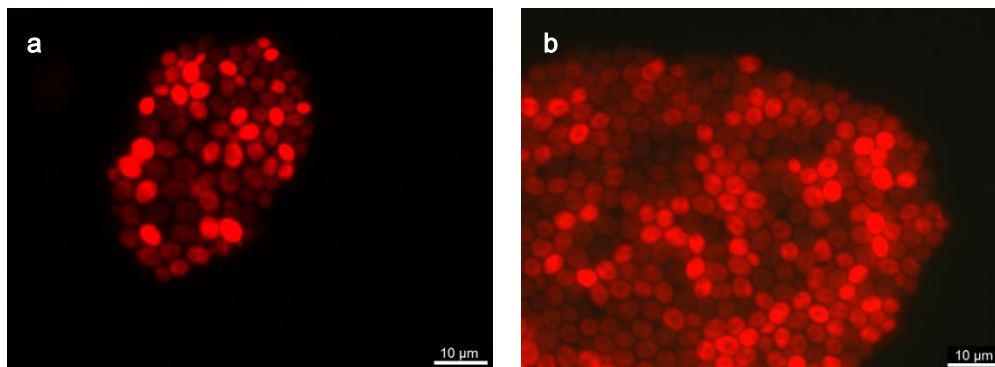
Figure 7- Fluorescence Microscope results for PNA-FISH for 1h (Simplified hybridization solution; 30% formamide). a) In suspension at 53ºC; b) On slide at 55ºC.

Flocculation of yeast cells has been shown to be dependent on flocculins, lectin-like proteins in the cell wall of the yeast cells encoded by genes in the FLO-gene family, which bind to carbohydrates present in the cell wall of neighboring yeast cells (Johan et al. 2012). Several combinations between pre-fixation washings and final resuspensions of 1x PBS, physiologic serum + 0.05% (vol/vol) Tween 80 and 30 mM EDTA were tested to reverse this process. Tween 80, also known as polysorbate 80, is a non-ionic surfactant that is used as an emulsifier and dispersing agent (Kopec et al. 2008). It was tested

if the depletion of *S. cerevisiae* flocculation could be induced by the presence of 0.05% Tween 80. In Johan et al. (2012) work, *Saccharomyces cerevisiae* cells were washed with 30 mM EDTA to ensure complete floc dispersion. This option was also experimented.

The best deflocculation result was obtained conjugating a pre-fixation washing with 0.05% (vol/vol) Tween 80 and a final resuspension in 30 mM EDTA. Within these conditions, *S. cerevisiae* cells were significantly dispersed allowing the fluorescence image analysis (Figure 8).
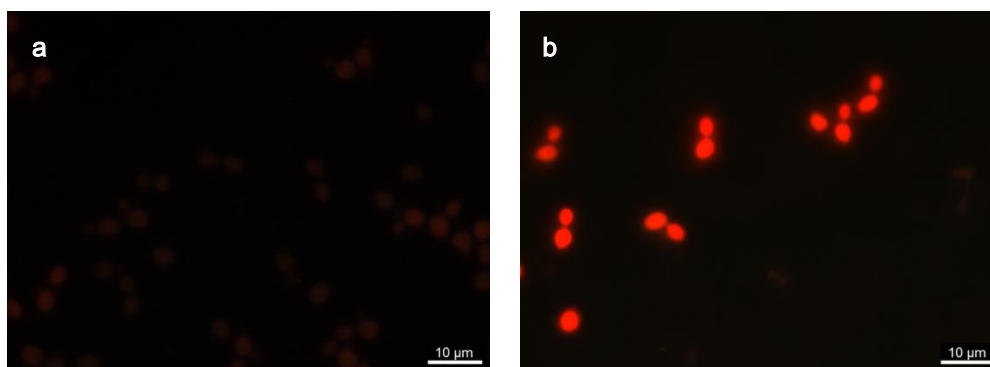
Figure 8 - Fluorescence Microscope results for in suspension PNA-FISH (Simplified hybridization solution with 30% formamide) at 53ºC for 1h - Defflocculation optimization: pre-fixation washing with 0.05% Tween 80 (in physiologic serum) and final resuspension in 30 mM EDTA. a) Negative control; b) Positive.

In order to allow an accurate ImageJ analysis of the RSM assay, a definition of the appropriate filter and microscope parameters was performed. N2.1 is a red filter that confirms the validity of this technique since the negative control is completely black. In fact, it is important to assure that negative control has cells. So, it was selected the Live/Dead Filter to proceed. However, in each experiment it was always made a confirmation with N2.1 Filter. For the LD filter, the microscope parameters chosen to the RSM assay was: exposure 3.0 s; gain 1.0x; saturation 1.50 and gamma 1.00.

Response Surface Methodology assay

Regression models of response

For RSM based on the Box-Wilson design, used for the optimization of fluorescence *in situ* hybridization targeting *S. cerevisiae*, 20 experimental runs with different combinations of three factors were carried out. The variables used for the factorial analysis were temperature (x_1), time (x_2) and formamide concentration (x_3).

Table 3 - Box-Wilson experiments design matrix with experimental values of hybridization efficiency

Run	Variables			Response
	x_1 Temperature (ºC)	x_2 Time (min)	x_3 [Formamide] (%v/v)	y Fluorescence intensity (a.u.)
1	40	30	9	118
2	55	60	47	136
3	40	90	75	88
4	55	60	47	143
5	70	90	19	59
6	70	30	75	42
7	70	30	19	55
8	40	90	19	66
9	55	60	47	166
10	70	90	75	59
11	40	30	75	54
12	55	60	47	173
13	29.8	60	47	52
14	55	60	0	85
15	55	110.5	47	81
16	80.2	60	47	91
17	55	60	47	150
18	55	60	94.1	84
19	55	60	47	105
20	55	9.5	47	95

The experimental responses for the 20 runs are presented in Table 3, which shows considerable variation in the hybridization efficiency depending on the three independent variables. The maximum fluorescence intensity (173 a.u.) was achieved in run number 12, while the minimum fluorescence intensity (42 a.u.) was observed in run number 6. The center point of the design was repeated six times for estimation of error resulting in a fluorescence intensity of 145±24 a.u. The standard deviation reflects the non-homogeneity of the hybridization process since the distribution of the signal is not uniform among the same sample in spite of the same growth phase of *S. cerevisiae* cells. Additionally, the user-dependent threshold definition may partly explain the standard deviation value.

Table 4 - Summary of ANOVA parameters

Source	Sequential p-value	Lack of Fit p-value	R-squared	
Linear	0.9765	0.0609	0.0142	
2FI[a]	0.8974	0.0429	0.0640	
Quadratic	**0.0053**	**0.1960**	**0.7935**	Suggested
Cubic	0.3139	0.1480	0.9229	Aliased

a - Two-factor interaction

The summary of data analysis for the response surface model may be consulted in Table 4. The suggested model is the quadratic one due to the significant sequential p-value (<0.05) and the relatively high R-squared (~0.8). In this case, the linear and the 2FI (two-factor interaction) models definitely can be ruled out, because its Lack of Fit p-value falls below 0.05. The quadratic model, identified earlier as the likely model, does not show significant lack of fit. The cubic model is aliased, so it should not be chosen, despite the highest R-squared.

The "Lack of Fit F-value" of 3.02 implies the Lack of Fit is not significant relative to the pure error (Table 5). There is a 19.60% chance that a "Lack of Fit

F-value" this large could occur due to noise. As we want the model to fit, a non-significant lack of fit is intended.

Table 5 - Lack of Fit tests

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F	
Linear	28954.93	11	2632.27	7.58	0.0609	
2FI	27438.10	8	3429.76	9.88	0.0429	
Quadratic	**5241.63**	**5**	**1048.33**	**3.02**	**0.1960**	Suggested
Cubic	1303.41	1	1303.41	3.75	0.1480	Aliased
Pure Error	1041.46	3	347.15			

The analysis of variance (ANOVA) for the quadratic model is shown in Table 6. The Model F-value of 3.42 implies the model is significant. There is only a 4.89% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.05 indicate model terms are significant. In this case, x_1^2, x_2^2 and x_3^2 are significant model terms. Values greater than 0.10 indicate the model terms are not significant.

Table 6 - ANOVA results of the quadratic model

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F	
Model	24144.99	9	2682.78	3.42	0.0489	Significant [a]
x_1 – Temperature	161.14	1	161.14	0.21	0.6626	
x_2 – Time	31.31	1	31.31	0.040	0.8467	
x_3 – [Formamide]	239.24	1	239.24	0.30	0.5961	
$x_1 x_2$	188.08	1	188.08	0.24	0.6377	
$x_1 x_3$	101.89	1	101.89	0.13	0.7280	
$x_2 x_3$	1226.86	1	1226.86	1.56	0.2467	
x_1^2	11340.27	1	11340.27	14.44	0.0052	Significant [a]
x_2^2	7224.60	1	7224.60	9.20	0.0162	Significant [a]
x_3^2	7984.85	1	7984.85	10.17	0.0128	Significant [a]
Residual	6283.08	8	785.39			
Lack of Fit	5241.63	5	1048.33	3.02	0.1960	Not significant [a]
Pure Error	1041.46	3	347.15			

a - at 5% level (P < 0.05)

The p-values are used as a tool to check the significance of each of the coefficients which, in turn, are necessary to understand the pattern of the mutual interactions between the best variables (Li et al. 2007). The smaller the p-value, the greater the significance of the corresponding coefficient. P-values suggest that the independent variables x_1, x_2, x_3 have not a significant effect on the hybridization efficiency. No interactions between the variables were found to contribute to the response at a significant level. However, the quadratic term of these three variables had a significant effect.

The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared. Usually, the higher the value of CV, the lower the consistency of experiment is. Here, a CV of 29.31 indicated a reasonable precision and reliability of the experiments. The precision of a model can be checked by the determination coefficient (R^2) and correlation coefficient (R). The determination coefficient implies that the sample variation of 79.35% for hybridization efficiency was attributed to the independent variables, and only about 20.65% of the total variation cannot be explained by the model.

A regression model having an R^2 value higher than 0.9 is considered to have a very high correlation (Li et al. 2007). In this experiment, a high determination coefficient of approximately 0.8 shows a good adjustment of the model to the experimental data.

The closer the value of R to 1, the better the correlation between the experimental and predicted values. Here, the value of R (0.8908) indicates a close agreement between the experimental results and the theoretical values predicted by the following model equation.

Another ANOVA parameter is the adequate precision that represents a measure of the range in predicted response relative to its associated error, in other words a signal to noise ratio. A ratio greater than 4 is desirable. This

model presents a ratio of 4.320 indicating an adequate signal. This model can be used to navigate the design space. Therefore, the quadratic model was selected in this optimization study.

By applying multiple regression analysis on the experimental data, the following second order polynomial equation was found to describe the hybridization efficiency of the *S. cerevisiae* PNA probe:

$$y = 146.20 - 3.43x_1 - 1.51x_2 - 4.19x_3 + 4.85x_1x_2 + 3.57x_1x_3 + 12.38x_2x_3$$

$$-28.06x_1^2 - 22.40x_2^2 - 23.55x_3^2 \tag{3}$$

where y is the predicted response (fluorescence intensity); x_1, x_2, x_3 are coded values of temperature, time and formamide concentration, respectively.

Comparison of observed and predicted hybridization efficiency

A regression model can be used to predict future observations on the response y (hybridization efficiency) corresponding to particular values of the independent variables.
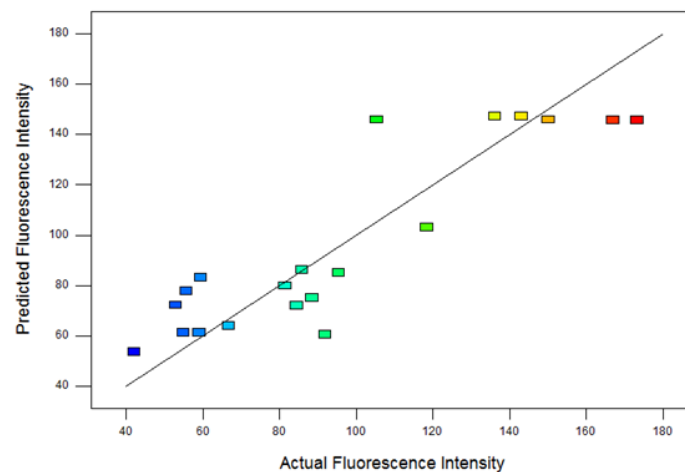

Figure 9- Observed fluorescence intensity vs the predicted fluorescence intensity - Diagnostic plot.

Figure 9 shows observed fluorescence intensity versus those from the empirical model demonstrating graphically the determination coefficient of 79.35% for the hybridization efficiency. Actual values are the measured

response data for a particular run, and the predicted values were evaluated from the model and generated by using the approximating functions. This graph indicates an adequate agreement between real data and the one obtained from the model.

Determination of optimum conditions

The 3D response surface plots described by the regression model illustrate the effects of the independent variables and the interactive effects of each independent variable on the response (Li et al. 2007). The optimal values of the independent variables could be easily understood from the 3D response surface plots and the corresponding contour plot.

Regarding fluorescence *in situ* hybridization, it is important to introduce the concept of stringency as the extent to which hybridization can occur between nucleic acids with mismatched sequences. At high stringency, duplexes form only between strands with perfect complementarity while lower stringency allows annealing between strands with some degree of mismatch between bases. In order to maximize the confidence and specificity of these assays, high stringency hybridization conditions were investigated. Typically, they can be achieved by reducing NaCl concentration or increasing temperature. Formamide is a denaturant routinely used in hybridization techniques because it enables hybrids to be formed at lower temperatures adjusting stringency conditions (Yilmaz et al. 2012). A probe specificity testing with strains with only one mismatch should be done to assess stringency.

According to Figure 10, this study suggests that at a standard hybridization time of 60 minutes, the hybridization efficiency increased gradually as the formamide concentration increased. However, increasing this concentration beyond approximately 45% decreased the hybridization efficiency. Formamide interferes with the binding kinetics of the probe enhancing the hybridization until a determined threshold. Extremely high

formamide concentrations decreased substantially the hybridization efficiency. This could be explained by the increased viscosity that results in reduced hybridization sensitivity. Everything indicates that formamide acts at the level of the cell envelope and the cell wall thickness can determine the optimal formamide concentration in hybridization experiments (Santos R. S., Guimarães N., Madureira P., Azevedo N., personal communication, June 2013). Previous researches point in this direction since the greater the thickness of bacteria cell wall, the higher the optimal formamide concentration. The cell wall of *S. cerevisiae* has about 90 nm of thickness (Smith et al. 2000) and the optimal formamide concentration according to the RSM analysis is 43.8% (vol/vol), supporting the previous hypothesis.
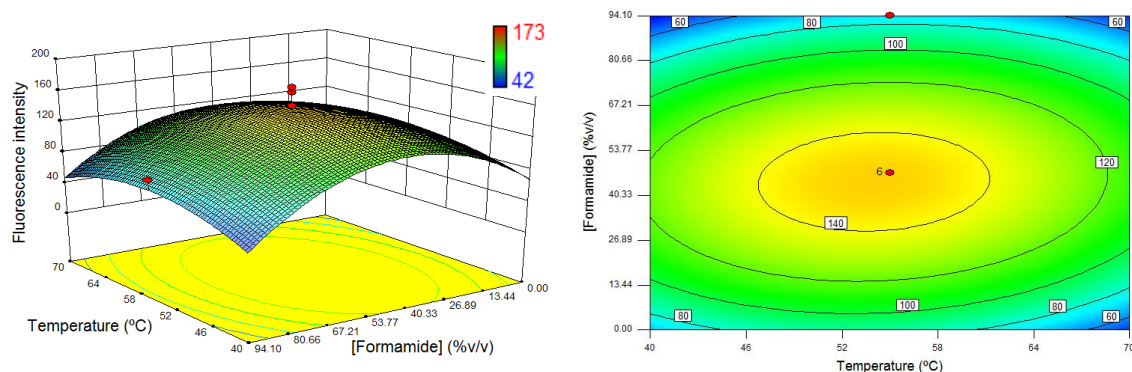
Figure 10 - Response surface plot and corresponding contour plot of the combined effects of formamide concentration and temperature on the hybridization efficiency of a *S. cerevisiae* PNA probe with a constant hybridization time of 60 min. Red points - Design points above predicted value.

According to the quadratic model, the temperature of hybridization is not significant since setting the time of hybridization and formamide concentration at 60 min and 47%, respectively, the fluorescent intensity is greater than 120 in a range of temperatures comprised between 45 and 65ºC. The PNA-FISH method targeting *S. cerevisiae* is robust as it allows the identification of this yeast in a wide range of hybridization conditions. A probe specificity testing should be done to assess the real robustness of the PNA-FISH method.

Figure 11 depicts the effects of temperature and time of hybridization on the fluorescence intensity (the measurement of hybridization efficiency), while formamide concentration was fixed at 0 (-α), 47 and 94.1% (+α).

0% Formamide

47% Formamide
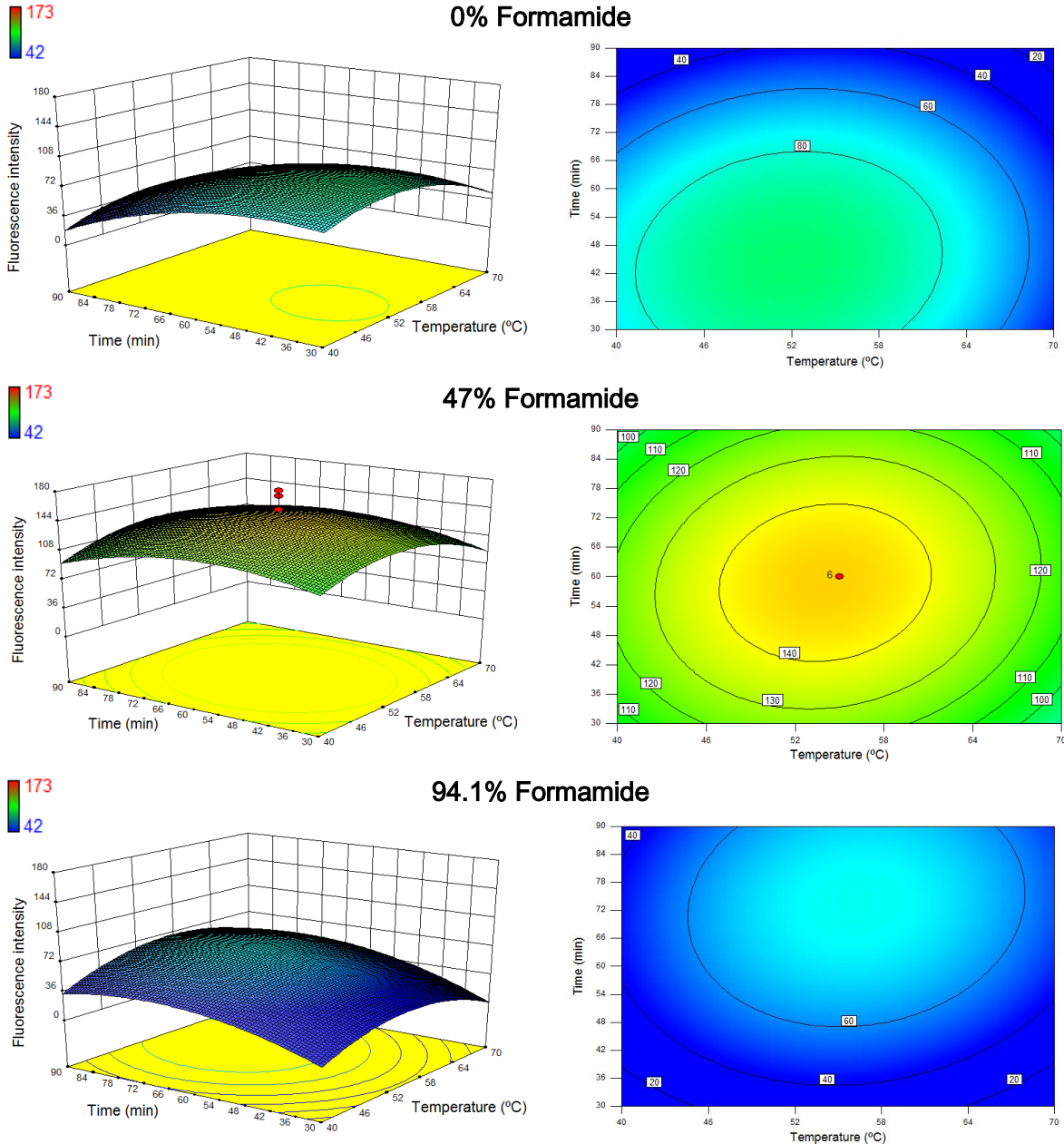
94.1% Formamide

Figure 11 - Response surface plot and corresponding contour plot of the combined effects of time and temperature on the hybridization efficiency of a *S. cerevisiae* PNA probe with constant Formamide concentration. Red points - Design points above predicted value.

Analysing the 3D response surface plot of 0% formamide, the initial idea is that the hybridization efficiency is very weak compared to the middle point of 47% formamide. Nevertheless, a closer look at the counter plot indicates that

even without a denaturing agent the hybridization is possible and clearly distinguished compared with a negative control (fluorescence intensity = 30±3 a.u.). Without a denaturing agent, the optimal ranges of temperature and time of hybridization were respectively 45-55ºC and 30-60 min, presenting a fluorescence intensity higher than 80. The optimal formamide concentration should be around the middle point of 47% since the hybridization efficiency is higher, presenting some design points above the predicted value. At 94.1% formamide, the hybridization efficiency substantially decreased being almost null at a temperature below 46ºC and during less than 48 min.

Furthermore, response surface methodology (RSM) was employed to determinate the optimal hybridization conditions which would present the highest estimated fluorescence intensity. Simultaneous optimization of all parameters is possible by combining them into a single objective function, the desirability function, which basically represents the relationship of all parameters that are to be optimized (Aksezer 2008). Desirabilities range from zero to one for any given response. A value of one represents the ideal case. A zero indicates that one or more responses fall outside desirable limits. The optimal parameters are described in the Table 7 presenting an acceptable desirability of approximately 0.8.

Table 7- Optimal hybridization parameters.

Optimal conditions	Temperature (ºC)	Time (min)	[Formamide] (%v/v)	Fluorescence intensity (a.u.)	Desirability
	53.9	57.8	43.8	147	0.797

Model adequacy checking

Usually, it is essential to check the fitted model to ensure that it provides an adequate approximation to the real system. The residuals from the least squares fit show how well the model satisfies the assumptions of the analysis of variance playing an important role in judging model adequacy (Li et al. 2007).

Residuals are the difference between actual and predicted values for each point. Studentized residuals are the residuals divided by the estimated standard deviation of that residual. It measures the number of standard deviations separating the actual and predicted values.

The normal probability plot indicates whether the residuals follow a normal distribution, in which case the points will follow a straight line (Korbahti and Rauf 2008). The normality assumption was satisfied as the residual plot approximated along the straight line indicating that no response transformation was needed (Figure 12a).
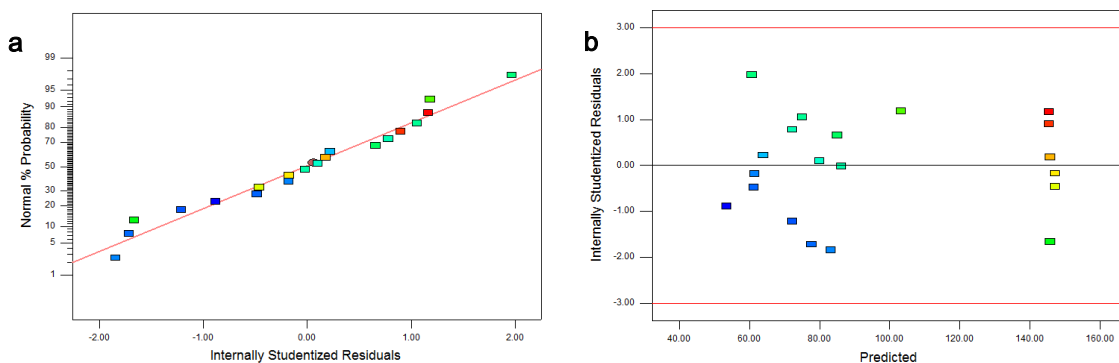
Figure 12 - Diagnostic plots. a) Normal probability of internally studentized residuals; b) Plot of internally studentized residuals vs predicted response.

Figure 12b presents a plot of residuals versus the predicted response. The residuals distribute somewhat randomly on the display, suggesting that the variance of the original observation is constant for all values of the response.

The outlier t is a measure of how many standard deviations the actual value deviates from the predicted value. Most of the standard residuals should lie in the interval of ±3.50 and any observation with a standardized residual outside of this interval is potentially unusual with respect to its observed response (Korbahti and Rauf 2008). According to Figure 13, the approximation of the fitted model to the response surface was fairly good with no data recording error since all the outlier t values are located below the interval of

±3.50. Both of the diagnostic plots are satisfactory, so the empirical model is adequate to describe the hybridization efficiency by response surface.
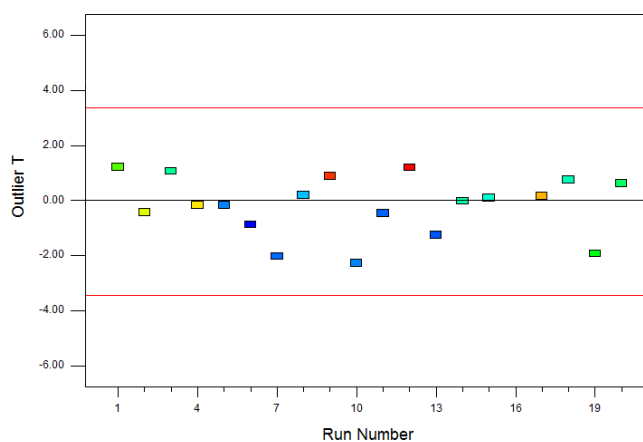
Figure 13 - The outlier t test plot of *Saccharomyces cerevisiae* hybridization.

Validation of optimized parameters

In order to verify the optimization results, an experiment was performed under the predicted optimal conditions. Design-expert software predicted a fluorescence intensity of 147 a.u. at a hybridization temperature of 53.9ºC for 57.8 min in a 43.8% formamide solution. The experimental values presented a fluorescence intensity of 184±14 a.u. which is consistent with the results obtained from RSM. The design points above the predicted value can somewhat support this high fluorescence.
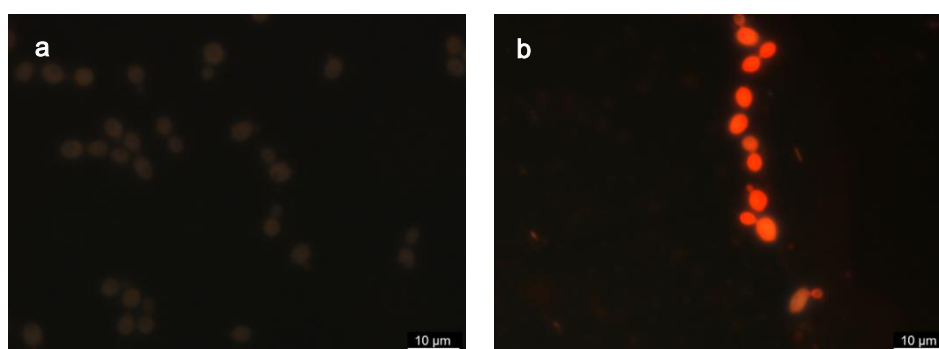
Figure 14 - Fluorescence Microscope results for in suspension PNA-FISH (Simplified hybridization solution with 43.8% formamide) at 53.9ºC for 57.8 min - Optimization Result. a) Negative control; c) Positive.

Denaturing agent – Is it required?

Although the well-known toxicity of formamide, it remains the preferred solvent to lower the melting point and annealing temperature of nucleic acid strands in *in situ* hybridization (Matthiesen and Hansen 2012). Unlike formamide, urea is non-toxic and has been indicated as an additional permeabilizer that could increase the FISH signal (Lawson et al. 2012). The exclusion of formamide may open up new applications, such as simplified FISH analysis in a microfluidic platform.

Urea was tested as a substitute for formamide in the hybridization buffer at 0.5, 2 and 4 M. Higher urea concentrations were not tested since, according to Simard et al. (2001), result in reduced hybridization sensitivity, possibly due to the solution's viscosity that increases as a function of urea concentration.
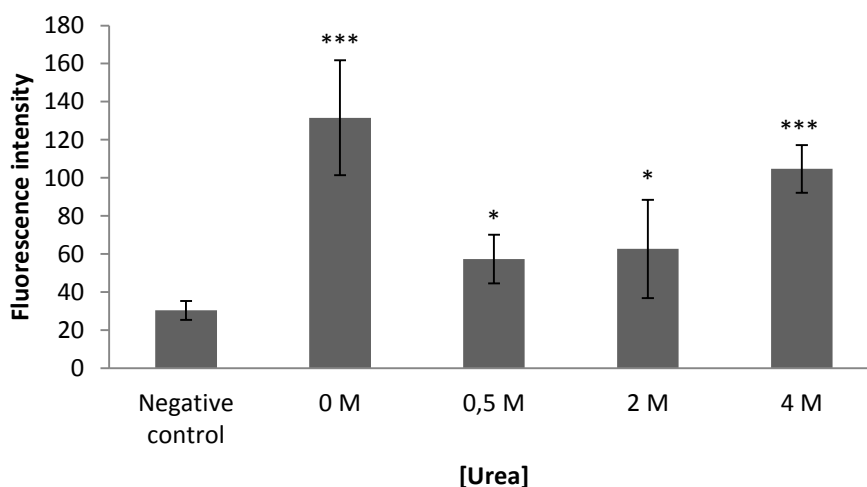
Figure 15 - Influence of different concentrations of urea in FISH signal. *: $p < 0.05$ and ***: $p < 0.005$ relative to the negative control. All experiments were performed in triplicate.

Interestingly, the signal was significantly higher ($p < 0.005$) with 0 M urea suggesting that the denaturing agent is not essential for the hybridization to occur. The fluorescence intensity increased from 0.5 M to 4 M urea, but always below the signal without denaturing agent as presented in Figure 15. The signal

was highest at 0 and 4 M urea, so these concentrations were chosen for further testing. Triplicate samples were always performed.

Fixation as a crucial step

In order to reduce the total time needed to perform the suspension procedure, a simplification of the fixation step was also tested. Fixation is one of the most crucial steps to assure an accurate and successful FISH result (Amann and Fuchs 2008) and can be based on either dehydration by alcohols (methanol or ethanol) or cross-linking by paraformaldehyde (Stadler et al. 2010). Additionally to the standard procedure of 4% (wt/vol) paraformaldehyde during 1 h followed by 30 min of membrane permeabilization in 50% (vol/vol) ethanol at -20ºC, it was performed a reduced fixation step only with ethanol. All experiments were performed in triplicate.
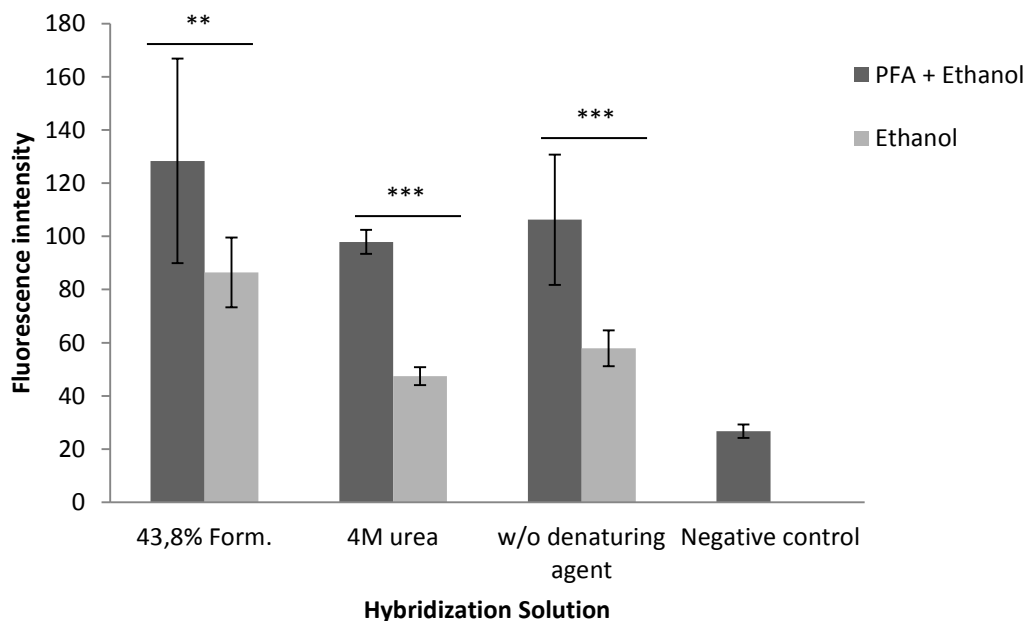
Figure 16 - Effect of the fixation procedure and the presence of a denaturing agent in the hybridization efficiency. . **: $p < 0.01$ and ***: $p < 0.005$. All experiments were performed in triplicate.

The hybridization occurred even with the simplified fixation; however the incubation with paraformaldehyde showed to be important for an efficient hybridization with a substantial increase in the FISH signal. In the assay with 0

and 4 M urea it was verified an increase of signal of 45.52% and 51.56%, respectively, with the standard fixation procedure (Figure 16). In accordance with these data, the standard fixation protocol was maintained. Regarding the composition of the hybridization buffer, the supposed additional permeabilizer effect of urea described by Lawson et al. (2012) was not confirmed again.

The idea was to compare the influence of optimal formamide and urea concentration with a hybridization solution without any denaturing agent, since the objective was to simplify the method. At this point, the decision was to proceed without any denaturing agent because it was verified a significant difference ($p < 0.005$) of FISH signal of 74.80% between this sample and the negative control. The sample subjected to a hybridization solution without any denaturing agent presented a fluorescence intensity of 106±25 a.u.

Simplification of the hybridization solution - II

The following approach was to further reduce the components of the hybridization solution removing dextran sulphate and Triton X-100. According to Azevedo (2005), dextran sulphate accelerates the rate of nucleic acid hybridization by decreasing the volume of solvent available to the probe and Triton X-100, as a detergent, affects membrane permeabilization.
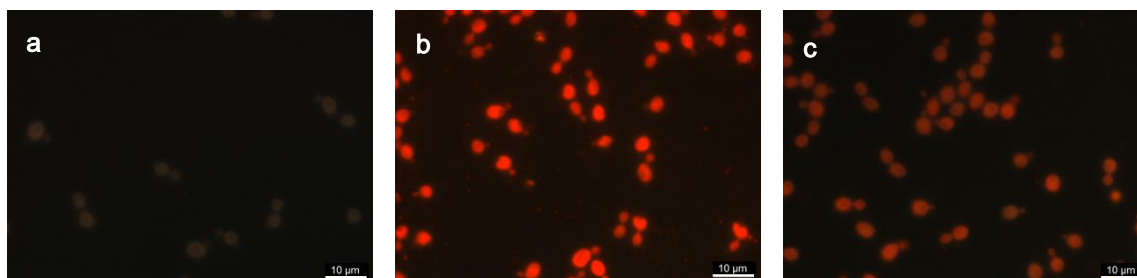
Figure 17 - Fluorescence Microscope results for PNA-FISH at 53.9ºC for 58 min - Simplification of the protocol. a) Negative control; b) Simplified hybridization solution 0M urea; c) Simplified hybridization solution 0M urea without dextran and Triton X-100.

The new solution presented only 50 mM Tris-HCl (pH 7.5) acting as a buffer to control the pH of the hybridization, as variations in pH significantly

affect hybridization temperature (Azevedo 2005). All experiments were performed in triplicate.

Despite the marked decrease in FISH signal, the hybridization occurred even with a probe in a greatly simplified hybridization buffer of Tris-HCl (Figure 17) suggesting that this simplification is valid. A hybridization step in these conditions for only 30 min was also tested demonstrating a fluorescence intensity of 69±18 a.u. compared to 86±8 a.u. for 60 min hybridization (Figure 18).
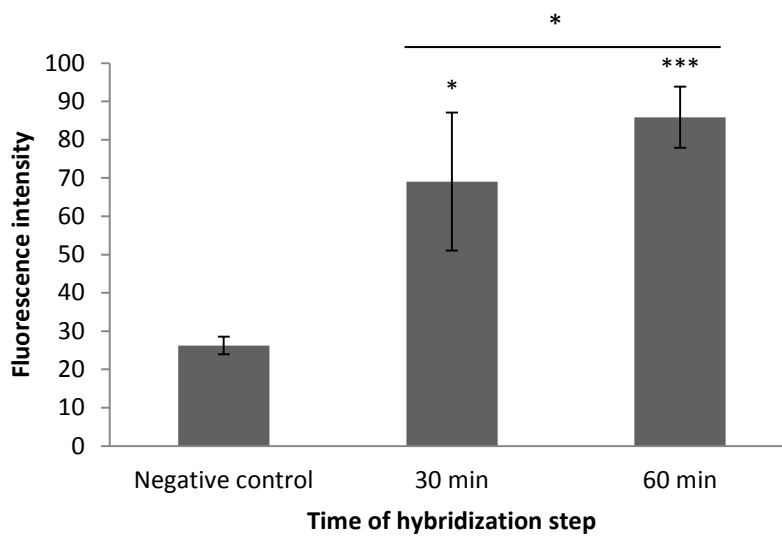
Figure 18 - Effect of the time of hybridization in the FISH signal. Simplified hybridization buffer only with 50 mM Tris-HCl. *: p < 0.05 and ***: p < 0.005 relative to the negative control. All experiments were performed in triplicate.

ImageJ analysis

The developed ImageJ procedure to analyse fluorescence microscopy images demonstrated to be relatively simple and accurate. However, it is not completely automated since the threshold of each image has to be processed individually. For images with very distinguishable intensity values, the results appear to be consistent. The failure of this analysis seems to be images with

very close intensity values in which this method may not be precise enough to obtain differences.

In the present study, the ImageJ analysis showed differences in the fluorescence intensity according to several hybridization conditions. To complete this work and to assess the real validity of image analysis, flow cytometry can be used to confirm the obtained data.

S. cerevisiae probe specificity – preliminary testing

Following optimization of the hybridization conditions, the specificity of the PNA probe was tested in the optimal conditions found by RSM using 3 *S. cerevisiae* strains (IGC 2608[T], IGC 3507 and PYCC 4072) and 1 *S. bayanus* IGC 4568[T]. Apart from the *S. bayanus* that was detected with lower fluorescence intensity, the remaining *S. cerevisiae* strains were detected with the expected fluorescence intensity levels (data not shown). It is possible that a small cross-reaction may have occurred in the *S. bayanus* hybridization experiment. Additional assays are needed to assess the real specificity of the probe including yeast species typically presents in wine and other *S. cerevisiae* strains. Negative controls were also always performed for this experiment.

Chapter Five

Concluding Remarks

The present area of research has assumed an increasing importance in modern biological industries with the increase of quality control requirements and the continuous focus on final product excellence. Beer and wine spoilage organisms include several so-called wild yeasts, of which *Saccharomyces* species are generally considered the most important. For this reason, the monitoring of wild yeast contamination is crucial in the production process of food and beverage industries.

The scope of this dissertation consisted in the optimization of *Saccharomyces cerevisiae* detection by fluorescence *in situ* hybridization. With a response surface methodology approach, the maximum hybridization efficiency of 147 a.u. was predicted to occur at a hybridization temperature of 53.9ºC, for 57.8 min in a 43.8% formamide solution. The proposed quadratic model was subjected to a model adequacy checking. The diagnostic plots are satisfactory, so the empirical model is adequate to describe the hybridization efficiency by response surface since it provides an adequate approximation to the real system. Statistical analysis of the results showed that the quadratic terms of these three variables had a significant effect. However, no interactions between the three variables were found to contribute to the response at a significant level.

The PNA-FISH method targeting *S. cerevisiae* proved to be highly robust, and as such a simplification of the protocol was carried out. The removal of the paraformaldehyde incubation during the fixation step proved to decrease substantially the hybridization efficiency. Additionally, we demonstrated that a

simplification of the hybridization solution is possible removing both Triton X-100 and dextran sulphate. The obtained FISH signal for a hybridization of 30 min had a fluorescence intensity of 69±18 a.u.

To prove the robustness of our method, a preliminary probe specificity assay was performed. The probe bound to all *S. cerevisiae* strains; nevertheless it bound to the *S. bayanus* strain presenting relatively lower FISH signal. In the future research, an extent specificity assay should be performed.

CHAPTER SIX

PERSPECTIVES FOR FURTHER RESEARCH

Throughout this work, it was noticed that additional experiments would be valuable to complement the topic addressed in this dissertation. However, due to time constraints and material resources some tests were not possible to perform. As an example, a flow cytometry assay of the optimal hybridization conditions should be performed in order to validate the quadratic proposed model. It would be also interesting to assess the Flow-FISH signal of the most simplified hybridization solution comparing to the negative control. In order to further reduce the total time needed to perform the suspension procedure, several tests should be performed to assess if the necessary time to each step could be condensed maintaining the final result clearly distinguishable from a negative control. Taken into account the adaptation of this concept to equipment for biological industry, the analysis time should be maintained as short as possible.

In terms of simplicity and functionality, the development of a miniaturized platform integrating microfluidic PNA-FISH for cell detection emerged as a possible ultimate goal of the present dissertation. Recently, FISH-based microfluidic technique has been introduced due to the associated reduced cost, improved performance, automation and high speed. It also offers a number of advantages such as lower amounts of sample and reagents required disposability, compact size and computerization (Liu et al. 2011; Devadhasan et al. 2011). Combining multiple operations onto a single device is an attractive approach for automating FISH analysis on an industrial scale, ensuring the monitoring of yeast species. As an example, in wine production, a microfluidic

device (µFISH) will enable the early knowledge of the microbiological conditions and the application of corrective measures before spoiling becomes irreparable (Bottari et al. 2006). A better monitoring of the fermentation process will prevent the risk of alteration, leading therefore to a better quality of the final product.

The continuation of this work depends on the design of adequate microfluidic geometry, enhancing as much as possible the detectability of the sample while trying to maintain the analysis time as short as possible.

Despite the successful optimization approach, studies at industrial level are needed to define the real efficacy of FISH method and to determine the specific threshold that leads to the spoilage of the final product.

Regarding the robustness of the PNA-FISH method, an extent probe specificity assay should be performed and a new target should be tested: 5.8S rRNA gene, according to Esteve-Zarzoso et al. (1999) useful to identify spoilage yeast, since it carries greater interspecific differences than the 18S and 26S rRNA genes.

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APPENDIX

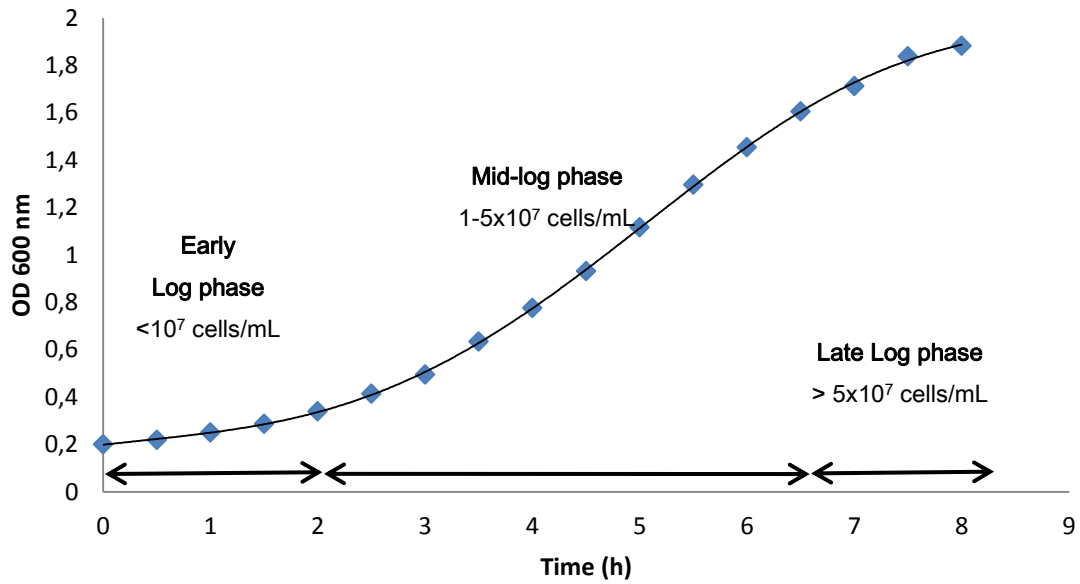## I – *Saccharomyces cerevisiae* Growth Curve

Figure 19 - *Saccharomyces cerevisiae* growth curve and corresponding cell density, according to (Bergman 2001).
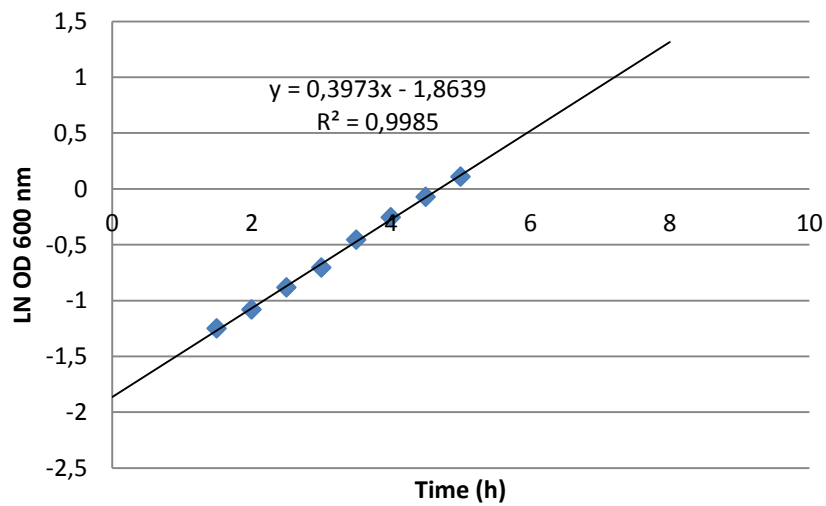

Figure 20- Determination of growth rate and doubling time of *S. cerevisiae*.

II – *S. cerevisiae* genome BLAST

Query sequence: AGGCTATAATACTTACC (probe)

Description: 26S ribosomal RNA gene

Table 8 - BLAST significant alignment.

Score	Expect	Identities	Gaps	Strand
34.2 bits (17)	0.010	17/17 (100%)	0/17 (0%)	Plus/Minus

III – Composition of FISH Solutions

Table 9 - Standard hybridization solution (pH 7.5)

	Manufacturer
10 mM Sodium Chloride	Sigma-Aldrich, USA
10% (wt/vol) Dextran Sulfate	Fisher Scientific, UK
30%(vol/vol) Formamide	Acros Organics, UK
0.1% (wt/vol) sodium pyrophosphate	Acros Organics, UK
0.2% (wt/vol) polyvinylpyrrolidone	Sigma-Aldrich, USA
0.2% (wt/vol) Ficol	Fisher Bioreagents, UK
5 mM Disodium EDTA	Panreac Quimica, Spain
0.1% (vol/vol) Triton X-100	Panreac Quimica, Spain
50 mM Tris-HCl	Fisher Scientific, UK

Table 10 - Simplified hybridization solution (pH 7.5)

	Manufacturer
10% (wt/vol) Dextran Sulfate	Fisher Scientific, UK
30%(vol/vol) Formamide	Acros Organics, UK
0.1% (vol/vol) Triton X-100	Panreac Quimica, Spain
50 mM Tris-HCl	Fisher Scientific, UK

Table 11 - Washing solution (pH 10)

	Manufacturer
5 mM Tris Base	Fisher Scientific, UK
15 mM Sodium Chloride	Sigma-Aldrich, USA
1% (vol/vol) Triton X-100	Panreac Quimica, Spain