

Cátia Vieira Gil

Licenciada em Biotecnologia



Evolutionary engineering towards the optimization of fruit juice fermentations

Dissertação para obtenção do Grau de Mestre em
Biotecnologia

Orientadora: Professora Doutora Paula Gonçalves, Universidade Nova de Lisboa

Co-orientadora: Professora Doutora Madalena Salema-Oom, Instituto Egas Moniz

Júri:

Presidente: Professor Doutor Carlos Alberto Gomes Salgueiro

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Vogal: Professora Doutora Paula Maria Theriaga Mendes Bernardo Gonçalves



FACULDADE DE
CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Novembro de 2019

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Acknowledgments

This master's thesis had important support and incentives without which this work would not develop into a reality. For this, I will be eternally grateful.

Firstly, I want to thank Professor Paula Gonçalves for “adopting” me as a member of Yeast Genomics Lab, for her support and patience, and for never allowing sadness to take over even when things went wrong. Also, I would like to thank her for the exemplary guidance based on a high and rigorous scientific baggage, which has greatly contributed to the improvement of this work throughout this year.

To my co-advisor Madalena Salema-Oom, I also express my gratitude for the time, suggestions and support given throughout this year.

Furthermore, I would like to thank our lab technician Nicole Soares, for the morning cheerfulness and each Erlenmeyer washed. I could not do anything without her help and dedication. Thank you!

Also, I would like to express my gratitude to PYCC (Portuguese Yeast Culture Collection) for providing the strains for the study and to SUMOL+COMPAL, SA for offering the fruity substrates tested throughout this work

To my FCT colleagues, who were also essential during this year, Beatriz Valpradinhos and Kirill Ispolnov, I would like to thank you for the friendship and unconditional motivation that helped make this challenge a pleasant learning journey.

To all my family, my close friends and “borrowed sister”, with a special affection for my little stars and my “little big” brother, an enormous thank you for the encouragement in every crucial moment of my life.

Of course, I could not fail to thank to my dearest João Nunes for the love, companionship, unconditional support and permanent encouragement, and concern that has always accompanied my work. I have no words to express how grateful I am.

Finally, recognizing that none of this would have been possible on my own, I would like to thank my laboratory colleagues, for being models of knowledge, for their support, friendship, patience and for the endless chocolates. To them I dedicate this modest work.

To all my sincere thanks!

Abstract

The industry has been exploiting the development of new fermented fruit-based products, since they have potential in the increase of nutritional value and health promotion. However, the fermentation of fruity substrates is often disturbed by the presence of aromatic substances, which while giving to the juice a more intense and natural aroma, can be highly inhibitory for microbial growth.

In this context, in the current study, the fermentative performance of the yeasts *Schizosaccharomyces pombe* PYCC 4181 and *Torulaspora delbrueckii* PYCC 4639 was evaluated in orange juices containing different concentrations of orange essential oils. The impact of orange essential oils on yeast growth profiles in various culture media was also estimated. After observing inhibitory effects on the lag phases of yeast growth, different adaptive evolution approaches were adopted for the two yeasts. The purpose of these experiments was to obtain spontaneous mutants through natural selection, which would provide a phenotype of higher resistance to orange essential oils, assuming that a higher tolerance would lead to decreased growth lag times and therefore would accelerate the fermentation process of “fresh” orange juice (not from concentrate-nfc), which has higher concentrations of orange essential oils.

The *T. delbrueckii* cell-based experimental evolution yielded a stable strain (E16900dT), which showed higher tolerance to orange essential oils. This strain fermented orange juice, which had higher concentrations of orange oils, faster than wild-type cells when inoculated on the same substrate. On the other hand, experimental evolutions starting with *Sch. pombe* resulted in strains with unstable phenotypes and, therefore, it was not possible to obtain a stable strain more tolerant to orange essential oils.

Furthermore, in *T. delbrueckii*, differences in gene expression of three genes related to cell wall stability (*PIR3*), occurrence of oxidative stress (*GPX2*), and plasma membrane integrity (*ERG6*) were studied in the presence and absence of orange essential oils in chemically defined culture medium. RT-qPCR results revealed an overexpression of a glutathione enzyme peroxidase (Gpx2p), responsible for the cellular response in the presence of oxidative stress, leading to the conclusion that loss of viability of *T. delbrueckii* in the presence of orange essential oils may be due to oxidative stress.

Keywords: orange essential oils; fermentation; lag phase; adaptive evolution

Resumo

A Indústria tem vindo a explorar o desenvolvimento de novos sumos fermentados à base de fruta, uma vez que estes contribuem para a melhoria do valor nutricional e promoção de saúde. No entanto, o processo fermentativo destes substratos frutados é frequentemente perturbado devido à presença de substâncias aromáticas voláteis de carácter lipofílico, que por um lado conferem ao sumo um aroma mais intenso e natural, mas por outro são substâncias altamente inibitórias para o crescimento microbiano.

Neste contexto, no presente estudo foi avaliado o desempenho fermentativo das leveduras *Schizosaccharomyces pombe* PYCC 4181 e *Torulaspora delbrueckii* PYCC 4639 em sumos de laranja contendo diferentes concentrações de óleos essenciais. Estimou-se também o impacto dos óleos essenciais de laranja nos perfis de crescimento das leveduras em diversos meios de cultura.

Após observação de efeitos inibitórios ao nível da fase de latência de crescimento das leveduras, foram adotadas diferentes abordagens de evolução dirigida, para cada uma das leveduras. Estas experiências tiveram como propósito a obtenção de mutantes espontâneos, através de seleção natural, que conferissem um fenótipo de maior resistência aos óleos essenciais de laranja, assumindo que uma maior tolerância aos óleos levaria a menores tempos de latência no crescimento e, portanto, aceleraria o processo de fermentação do suco de laranja “fresco” (não do concentrado-nfc), que possuía maiores concentrações de óleos essenciais de laranja.

Do ensaio de evolução experimental iniciado com células de *T. delbrueckii* resultou uma estirpe estável (E16900dT), que apresentou uma maior tolerância aos óleos essenciais de laranja. Esta estirpe iniciou a fermentação do sumo de laranja que continha uma maior concentração de óleos essenciais de uma forma mais rápida que as células *wild-type* quando inoculadas no mesmo substrato. Por outro lado, das evoluções experimentais com iniciadas com *Sch. pombe*, resultaram estirpes de fenótipos instáveis e, portanto, não foi possível obter uma estirpe estável mais tolerante aos óleos essenciais de laranja.

Para além disso em *T. delbrueckii* foram estudadas as diferenças de expressão genética de três genes, relacionados com estabilidade da parede celular (*PIR3*), ocorrência de stress oxidativo (*GPX2*) e integridade da membrana plasmática (*ERG6*), na presença/ausência de óleos essenciais de laranja em meio de cultura quimicamente definido. Destes ensaios foi possível concluir que a perda de viabilidade das células de *T. delbrueckii* na presença de óleos essenciais de laranja pode ser devida à ocorrência de stress oxidativo, uma vez que os resultados do RT-qPCR revelaram sobre-expressão da enzima glutathiona peroxidase (*Gpx2p*), que participa na resposta celular na presença de stress oxidativo.

Termos-chave: óleos essenciais de laranja; fermentação; fase de latência; evolução dirigida

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Abbreviations

bp	base pair
cDNA	complementary deoxyribonucleic acid
CFUs	Colony-Forming Units
CO₂	carbon dioxide
CPW	Citrus Processing Wastes
CWI	Cell Wall Integrity
DNA	deoxyribonucleic acid
EO	orange Essential Oils
EO2	EO that were stored used to give continuity to the assays
EO3	deteriorated EO
GMO	Genetically Modified Organisms
mg	milligrams
mL	milliliter
ng	nanograms
ncf	not from concentrate
nm	nanometers
OD	Optical Density
PCR	Polymerase chain reaction
PYCC	Portuguese Yeast Culture Collection Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, Caparica, Portugal.
RNA	ribonucleic acid
rpm	rotations per minute
RT-qPCR	Quantitative Real Time PCR
S.p-A	adaptive evolution carried out by <i>Schizosaccharomyces pombe</i> strain PYCC 4181 in YPD medium
S.p-B	duplicate of adaptive evolution carried out by <i>Schizosaccharomyces pombe</i> strain PYCC 4181 in YPD medium
S.p-C	adaptive evolution carried out by <i>Schizosaccharomyces pombe</i> strain PYCC 41 in YNB medium
T.d-A	adaptive evolution carried out by <i>Torulaspora delbrueckii</i> strain PYCC 4639 in acidified YNB medium
T.d-B	duplicate of adaptive evolution carried out by <i>Torulaspora delbrueckii</i> strain PYCC 4639 in acidified YNB medium
WT	wild type
YNB	Yeast Nitrogen Base
YPD	Yeast Extract Peptone Dextrose
μL	microliters

1. INTRODUCTION

1.1. A general overview of the fermentation process

In the mid-1900s, the term “biotechnology” came into trivial use, especially to support the study of fermentative processes, namely fermenter control and design, and end-product recovery (Crafts-Lighty, 1986). The concept *fermentation* comes from the Latin word “*fermentare*” that means “to boil”. This interesting analogy may be owing to the bubbles of carbon dioxide (CO₂) produced during the alcoholic fermentation, giving an appearance similar to boiling (Kuila and Sharma, 2018). Fermentation as an industrial practice is related to multiple areas of our daily routine, namely food, pharmaceuticals, and beverages on huge scale (Stanley *et al.*, 2010; El-Mansi, 2012; Kuila and Sharma, 2018).

In the industrial setting, the term fermentation may refer to biological processes that do not conform to the biochemical definition of fermentation, although some of the most common are based on alcoholic fermentation. In line with this, the industrial fermentation process is characterized by the biological degradation of the substrate by an appropriate population of microorganisms, under particular conditions (Benedetti and Buratti, 2016; Kuila and Sharma, 2018).

Many important biotechnological processes such as beer and wine production are based on alcoholic fermentation. Alcoholic fermentation yields not only ethanol, CO₂ and NAD⁺, but also important metabolites including organic acids (e.g., acetic, succinic and keto acids) and polyols, namely glycerol and 2,3- butanediol. Other minor metabolites mainly esters, carbonyls, volatile fatty acids, and higher alcohols are also produced throughout the fermentation process with different yeasts (Moreno-Arribas and Polo, 2009).

1.1.1. Yeasts in fermentation

In 1857, Louis Pasteur proved that the alcoholic fermentation process was an outcome of the growth of yeast cells. He achieved the famous conclusion that “*the breakdown of sugar into alcohol and carbonic acid is an action correlating with a vital phenomenon*” (Manchester, 2007; Moreno-Arribas and Polo, 2009).

The fermentation process can be initiated naturally or induced through the addition of yeasts. In the case of spontaneous wine fermentations distinct yeast species such as *Candida*, *Kloeckera*, and *Hanseniaspora*, prevail in the first stages of alcoholic fermentation. Afterward, *Metschnikowia* and *Pichia* dominate the middle stages, and in the last stages of the fermentation process *Saccharomyces* predominates. Besides that, some alternative yeasts, including *Schizosaccharomyces*, *Zygosaccharomyces*, *Torulaspora* and *Kluyveromyces* may also appear during wine fermentation, which can lead to the production of different organoleptic effects (Moreno-Arribas and Polo, 2009).

Moreover, some wineries do not inoculate their fermenters with microorganisms, and the fermentations nevertheless start and are carried out predominantly by *Saccharomyces* (Moreno-Arribas and Polo, 2009). The genus *Saccharomyces* has the ability to efficiently ferment various substrates and are commonly isolated from human (Mackenzie, 1961), fishes (Gatesoupe, 2007), routes of migratory birds (Francesca *et al.*, 2012), wine, bread and beer substrates (Legras *et al.*, 2007; Sampaio and

Gonçalves, 2017), fruits (Abdel-Sater, 2017), trees (Sampaio and Goncalves, 2008), olives (Arroyo-López *et al.*, 2006; Pontes *et al.*, 2019), etc.

Non-Saccharomyces yeasts were deemed as undesirable and problematic yeasts during brewing and winemaking. However, this negative perception has been changing, since several studies have demonstrated their important contribution to the definition of the sensorial quality during spontaneous wine fermentations. As such, in addition to *Saccharomyces*, other yeasts have been exploited for the fermentation of different substrates giving rise to products with different sensory complexities (Tataridis *et al.*, 2013). Two of these *non-Saccharomyces* yeasts are *Schizosaccharomyces pombe* and *Torulaspora delbrueckii*, which were used in this study.

Briefly, the yeast *Torulaspora delbrueckii* is one of the most investigated *non-Saccharomyces* yeasts that aroused the industrial interest by virtue of its high osmotic tolerance (Alves-Araujo *et al.*, 2007). Furthermore, it is characterized by having a great potential in grape fermentation, since it confers favorable characteristics to the sensory character of wine, generating products with distinguished sensory properties and a floral/fruity aroma. Depending on the strain, *Torulaspora delbrueckii* may exhibit a higher demand for nitrogen and oxygen, and is a lower producer of acetoin, acetaldehyde, succinic acid, and polysaccharides, compared to *S. cerevisiae* strains (Tataridis *et al.*, 2013). The other species used in the present work, *Schizosaccharomyces pombe* has several notable metabolic properties, namely urease activity, high production of pyruvic acid, the ability to reduce gluconic acid, and malic dehydrogenase activity, which enables the metabolization of malic acid into ethanol. Since *Schizosaccharomyces pombe* features a notable tolerance for high malic acid concentrations (which can be entirely metabolised without any deleterious effects until concentrations of approximately 20 g/L), these species are used in wineries as a bio-tool for wine deacidification, resulting in the production of high quality wines (Saayman and Viljoen-Bloom, 2006; Saez *et al.*, 2012; Loira *et al.*, 2015, 2018; Benito *et al.*, 2016).

1.1.2. Yeast growth during fermentation

The fermentation of soluble sugars is affected by yeast growth kinetics. Similarly, to other microorganisms, the growth profile of the yeasts has distinct phases, namely a lag phase, a growth acceleration phase, an exponential phase, a stationary phase, and a decline phase. The duration of each phase depends on several factors such as the fermentation temperature, the composition of the fermentation broth, and the amount and quality of inoculum (Moreno-Arribas and Polo, 2009).

In the first phase of the cycle, the yeast population is biochemically active but does not increase. In the course of that period, called the latency phase (lag phase), the cells need to adapt all their enzymatic machinery to the new environmental conditions to develop enzymes that support growth and fermentation (Moreno-Arribas and Polo, 2009). Once the yeasts have adapted, they begin to grow, and the exponential phase begins where yeast population increases exponentially. This stage is strictly conditioned by the concentration of sugars, amino acids, ammonia and other essential nutrients in the broth, temperature, and by the presence of oxygen in the medium (Shuler and Kargi, 2002; Moreno-Arribas and Polo, 2009). After the exponential phase, the rate of cell growth begins to decrease – decelerating phase –. After that, the stationary phase in the fermentation process occurs, followed by

the decline phase. In stationary phase, the number of yeast cells dividing is almost equivalent to the amount of cells that are dying not only because the medium may lack nutrients, but also because the accumulation of high ethanol concentrations and other by-products produced during alcoholic fermentation process are becoming harmful to them (Shuler and Kargi, 2002; Moreno-Arribas and Polo, 2009).

1.2. Fermented products

Fermented foods were present in human nourishment very early on, not because they had planned to create such products, but rather because attempts to preserve raw food often naturally resulted in fermentations (Hutkins, 2006). Nowadays, abundant fermented products are present in the daily life of humankind, namely butter milk, yogurt, wine, beer, bread, among others (Crafts-Lighty, 1986; Kuila and Sharma, 2018). Likewise, diverse products from fermented vegetables and fruits, such as fermented juices, sauerkraut, kimchi and bean curd, are important in meeting the nutritional demands of the world population (Querol and Fleet, 2006; Campos *et al.*, 2016; Kuila and Sharma, 2018).

In general, fermented products have aromas and flavors that are more intense and different than the raw materials from which they were prepared, and have several attributes that determine the integration of these foods in the population's diet, such as long-term food preservation. In addition, the increased nutritional and organoleptic value, as well as the expansion of the spectrum of new products available for consumption, are also features that emerge from the fermentation process (Freitas, 2017).

1.2.1. Fruit-based fermented juices

The vegetables and fruits are natural sources of vitamins, antioxidants, minerals, and fibers, that are essential for the proper functioning of the human body. In line with this, several studies that analyze the nutritional quality and stress the importance of daily consumption of fruits and vegetables have emerged. For that reason, consumers have become more aware of the importance of a balanced diet, resulting in not only in greater concern for what consume, but also a greater demand for quality (Boeing *et al.*, 2012; Di Cagno *et al.*, 2013; Kuila and Sharma, 2018; Rafiq *et al.*, 2018). In this context, in the sight of the need to diversify the offer to the consumer, juice industries have been exploring the development of new beverages that simultaneously present characteristics of quality and that respond to consumer demand. As a result, new fermented beverages based on fruit and vegetables have been emerging. An example of healthy fruit-based fermented beverage that has been widely consumed is fermented beet juice, which may be fermented by probiotic lactic bacteria such as *L. plantarum* C3 or *Lactobacillus acidophilus* LA 39. Apart from being a beverage that preserves nutritional singularities of the raw material, when fermented by these strains, it can also acquire probiotic properties (Kyung, Woodams and Hang, 2005).

Moreover, the use of fruits and vegetables as substrates in the fermentative processes, not only enables the introduction of new and healthy alternative products to the trade, but also allows the recovery of products that are often disposed of as agricultural waste for failing to meet the commercially

required quality standards. An interesting example is the production of a vinegar, endowed with satisfactory nutritional characteristics, from juice of discarded onions (Horiuchi, Kanno and Kobayashi, 1999).

A wide range of substrates may be employed in the fermentation. However, it is necessary to consider if the amount and types of carbohydrates present in the raw material are adequate to support the microbial growth, since each microorganism reacts differently to different substrates (Lea and Piggott, 2003). The raw material used as fermentation substrate in this study was orange juice.

1.2.2. Orange juice

Due to its distinctive, fresh and pleasant flavor, and consumer awareness of the nutritional properties recognized as beneficial for health, orange is a very prestigious fruit with economic and nutritional importance in developing and developed countries (Bermejo and Cano, 2012; Liu, Heying and Tanumihardjo, 2012; John, Muthukumar and Arunagiri, 2017).

Even though oranges are mostly consumed as a fruit, about one third is reserved for the production of orange juices. Orange juice is an acidic drink with a higher antioxidant capacity than other similar juices such as lemon, apple and pineapple juices (Liu, Heying and Tanumihardjo, 2012; Aneja *et al.*, 2014). Regarding the presence of macronutrients, since oranges have a low content of proteins and fats, carbohydrates are the main macronutrient that support the nutritional and caloric value of the orange juice. Glucose, fructose, and sucrose, commonly present in the ratio of 1:1:2, respectively, are the predominant carbohydrates in orange juices. The sweet and delightful taste of orange juice is ensured by the presence of these sugars, trace amounts of others sugars including galactose, mannose, and maltose, and some organic acids, particularly malic, citric and succinic acids (Liu, Heying and Tanumihardjo, 2012; John, Muthukumar and Arunagiri, 2017). The sugar composition of some citrus fruits is listed in Table 1.1.

Various aromatic compounds are additionally synthesized during the orange juice fermentation, as a result of the microbial use of the organic acids present in the fruit. Thereby, a wide range of flavors are produced, giving rise to beverages with different organoleptic properties (Liu, Heying and Tanumihardjo, 2012). The alcoholic fermentation of this orange substrate provides a juice with a profile of flavanones, pigments with provitamin A activity and bioactive compounds enhanced. Thus, this fermented beverage would have an increase in antioxidant content and their biological properties when compared to the original substrate (Escudero-López *et al.*, 2013).

Table 1.1- Sugar composition of citrus juices (Note: The values mentioned are represented by g/100g; Source: (Liu, Heying and Tanumihardjo, 2012))

Fruit	Sugars			Total sugars
	Fructose	Glucose	Sucrose	
Grapefruit	1,75	1,66	2,56	5,97
Tangerine	1,54	1,13	6,53	9,20
Limon	1,35	1,40	0,41	3,16
Orange	2,48	2,03	4,81	9,32

1.3. Examples of fermentation inhibitors

The raw materials used in the alcoholic fermentation, as also the steps preceding the fermentation process, may naturally contain or generate some substances that negatively affect the microorganism's ability to ferment the substrate. These compounds are recognized as "fermentation inhibitors" and depending on their nature can influence cell viability, reduce ethanol yield, and/or hamper/stop the fermentation process (Taherzadeh and Karimi, 2011; Qureshi, Hodge and Vertes, 2014).

Ethanol resulting from alcoholic fermentation is one of the most studied fermentation inhibitors. It is reported that ethanol present in the fermentation medium not only damages plasma membranes, but also diffuses through them, drastically reducing water activity and impairing glucose metabolism (Taherzadeh and Karimi, 2011). Some complex substrates, such as the lignocellulosic biomass, require several pretreatment steps before they are fermented and processed into bioethanol. Depending of the nature of the lignocellulosic structures present in the substrate and the type of pretreatment applied, a variety of inhibitors may be formed, namely furans (including 5-hydroxymethyl-2-furaldehyde and furfural), carboxylic acids (for instance, formic, acetic and levulinic acids), and phenolic compounds (namely, syringaldehyde, vanillin and conferyl aldehyde) (Taherzadeh *et al.*, 2000; Parawira and Tekere, 2011; Taherzadeh and Karimi, 2011; Qureshi, Hodge and Vertes, 2014).

Regarding the presence of fermentation inhibitors in orange juices, the principal limitation in its use as a substrate is the presence of orange essential oils (Mantzouridou, Paraskevopoulou and Lalou, 2015).

1.3.1. Orange essential oils / Limonene

Citrus fruits have a substantial amount of aromatic oils (essential oils), composed by a complex combination of terpenic compounds, namely α -pinene, limonene, linalool, and terpinen-4-ol. The citrus processing wastes (CPW) contains concentrations of citrus oils that average approximately 0,8%, even though the oil concentration can fluctuate between 0,4% and 2%, depending on the citrus variety and the techniques used for oil recovery (Widmer, Zhou and Grohmann, 2010; John, Muthukumar and Arunagiri, 2017). These secondary metabolites are responsible for the peculiar fresh aroma and are commercialized as flavoring agents, pharmaceutical components, and nutritional supplements (John, Muthukumar and Arunagiri, 2017).

Limonene is a cyclic monoterpene, composed by two 5-carbon isoprene units, and is the main component of orange essential oils, where it represents between 70 and 96% of the total. Due to its lipidic nature, limonene is not miscible with water (Menéndez *et al.*, 2002; Brennan *et al.*, 2012; Ciriminna *et al.*, 2014). For the commercial purposes, limonene is often extracted by alkali treatment of citric pulp and peels remaining after orange juice processing, by steam distillation or deterpenation directly from the orange essential oils (John, Muthukumar and Arunagiri, 2017). Its antimicrobial characteristics and pleasant odor make limonene a desirable component for food preservatives, and disinfectants/insecticides (Ciriminna *et al.*, 2014). In addition, its medical action as a dietary anti-cancer tool for mammary carcinogenesis in humans has been reported (Crowell, 1999).

Various studies report that yeast growth inhibition in chemically defined media supplemented with limonene, occurs at very low concentrations, between 0,01-0,20% (v/v) (Mantzouridou, Paraskevopoulou and Lalou, 2015; John, Muthukumar and Arunagiri, 2017). The strong inhibitory effect of orange essential oils (mainly limonene) on yeast growth has been attributed to heterogeneous mechanisms, especially involving changes in fatty acid compositions in the plasma membranes (Sikkema, de Bont and Poolman, 1994; Di Pasqua *et al.*, 2006, 2007; Jidong Liu *et al.*, 2013a), and also at the cell wall level (Brennan, Krömer and Nielsen, 2013). Another study also reported that limonene might cause oxidative stress in *S. cerevisiae* (Jidong Liu *et al.*, 2013a). Although all these cellular disorders have been observed after limonene exposure, the mechanism of inhibition by orange essential oils remains ambiguous (Chubukov *et al.*, 2015).

1.4. Strategies to overcome fermentation inhibitors

Several strategies are implemented with the purpose of abolishing the toxic effects caused by different fermentation inhibitors. Regarding the choice of substrate, it must contain a composition suitable for yeast growth, and several fundamental parameters should be monitored during fermentation, in particular the concentrations of ethanol, sugars and salts, so that they remain below the tolerance limit of the microorganism. In pretreatment or hydrolysis of substrates, in particular in lignocellulosic substrates, a crucial point, as far as possible, is to prevent the formation of inhibitors. (Taherzadeh and Karimi, 2011).

1.4.1. Fermentation Strategies

Modeling the mode of operation of the fermentative process may help in the reduction of the harmful effects of fermentation inhibitors. The fermentative process can be performed in three modes, namely continuous, fed-batch, and batch. The batch process is characterized by the fact that the materials are fed to the fermentation system once. On the other hand, in the continuous and fed-batch processes, a defined volume of medium is continually added to the fermentation vessel. With this in mind, in batch mode, at the beginning of the fermentation process, the sugars and the inhibitors are in high concentrations, while in the fed-batch and continuous modes their concentrations may be kept low. The combination of a suitable operating mode, as well the *in situ* detoxification by microorganisms that have the capacity to transform some inhibitory compounds at low concentrations, increases the productivity of the fed-batch and continuous fermentations (Lidén, Niklasson and Taherzadeh, 1999; Taherzadeh *et al.*, 1999; Taherzadeh and Karimi, 2011).

Fermentation operations at high cell density also allow a better efficiency of inhibitor conversion, thereby reducing the toxicity of the medium (Taherzadeh *et al.*, 1999; Taherzadeh and Karimi, 2011).

Furthermore, another strategy used to remedy the inhibition effects of the substrates is the use of encapsulated/immobilized cells during the fermentative process. In this case, the cells are imprisoned in a semipermeable matrix, which creates a suitable microenvironment allowing the cells to tolerate media with toxic compounds. A successfully practical example of this technique was the use of

encapsulated *S. cerevisiae* for ethanol production from dilute-acid hydrolysates (Talebnia, Niklasson and Taherzadeh, 2005).

1.4.2. Detoxification of the Substrate

Detoxification processes usually increase waste production, require specific equipment, and may reduce fermentable sugar content. Consequently, this approach must be considered only if the microorganism's fermentation ability is compromised and cannot proceed without it (Taherzadeh and Karimi, 2011). Several detoxification processes have been studied using lignocellulosic hydrolysates for bioethanol and chemical production (Paes and Almeida, 2014).

1.4.2.1. Physical Detoxification

When using physical methods, the toxic compounds present in the substrate are removed without addition of enzymes or chemical compounds. In this category fall methods such as vacuum evaporation and membrane separations. Vacuum evaporation is the simplest procedure for reducing the content of volatile compounds. This method has some advantages, namely the efficient reduction of the content of volatile substances, and the increase of sugar concentration in the media, which may help yield a higher concentration of ethanol (Mussatto and Roberto, 2004; Taherzadeh and Karimi, 2011). Improved fermentability after vacuum evaporation treatment was observed in lignocellulose hydrolysates of spruce, where a reduction of 90% of the initial content of furfural was achieved (Larsson *et al.*, 1999).

1.4.2.2. Chemical detoxification

Chemical detoxification consists in the elimination of toxic substances by the addition of chemical compounds. This strategy includes techniques such as adsorption on activated charcoal (Converti, Perego and Domínguez, 1999), ion exchange resins (Larsson *et al.*, 1999), neutralization (Chandel *et al.*, 2007), calcium hydroxide overliming (Millati, Niklasson and Taherzadeh, 2002) and solvent extraction, namely extraction with ethyl acetate (Wilson, Deschatelets and Nishikawa, 1989).

Among these strategies, adsorption on activated charcoal and calcium hydroxide overliming are the most commonly used techniques to detoxify complex substrates, in particular lignocellulosic hydrolysates, with satisfactory results (Taherzadeh and Karimi, 2011). One of the disadvantages reported with respect to the chemical detoxification of lignocellulosic hydrolysates is the loss of part of the sugars, which may lead to low ethanol yields (Taherzadeh and Karimi, 2011).

1.4.2.3. Biological detoxification

As an alternative to chemical and physical detoxification described above, arises the use of microorganisms and/or enzymes for detoxification of the substrates. Biological methods of detoxification require microbial cells or enzymes to interact with the inhibitory compounds existing in the media, and

change their molecular structure consequently forming less toxic compounds (Mussatto and Roberto, 2004; Parawira and Tekere, 2011).

Some specific enzymes, such as peroxidase and laccase enzymes from white-rot fungus *Trametes versicolor*, enable the detoxification of wood hydrolysates by oxidative polymerization of phenolic compounds (Jönsson *et al.*, 1998). One of the advantages of using enzymes in substrate detoxification is the high catalytic efficiency of pure enzymes. Thereby, the detoxification process may develop more rapidly than with microorganisms. Conversely, the use of enzymes entails some drawbacks, namely the prolonged incubation demanded for detoxification, as well the high monetary cost of enzyme production (Parawira and Tekere, 2011).

Furthermore, the use of microorganisms also had good results in the selective removal of inhibitors from lignocellulose biomass, before fermentation with *S. cerevisiae* for ethanol production. (Okuda *et al.*, 2008) tested the bacteria *Ureibacillus thermosphaericus*, and the chromatographic analysis revealed that the thermophilic bacteria degraded the furfural and the phenolic compounds present in the waste house wood hydrolysates. Besides, the bacteria grow speedily and use less than 5% of the total sugars present in the biomass (Okuda *et al.*, 2008; Parawira and Tekere, 2011). The major advantages of this method include mild reaction conditions, once the use of toxic chemicals is avoided, being environmentally friendly with a lower energy demand, less generated waste, an efficient method to reduce fermentation lag times associated to toxicity of the inhibitory compounds, and the possibility to implement on the same vessel and simultaneously with fermentations. On the other hand, disadvantages include long incubation periods with the detoxifying microorganisms, which may result in the consumption of the fermentable sugars that could be used for the production of ethanol (Parawira and Tekere, 2011; Taherzadeh and Karimi, 2011).

1.4.2.4. Combined Detoxification methods

A single detoxification process might not be sufficient to make a given recalcitrant substrate fermentable for microorganisms. As an example, to detoxify dilute-acid hydrolysate of hazelnut shell, the most effective method was reported to be the combination of 3% of sodium hydroxide for delignification, and subsequent detoxification by overliming with calcium oxide followed by two treatments with charcoal. That procedure resulted in increased cellulosic ethanol production from hazelnut shells (Arslan and Eken-Saraçoğlu, 2010). Notwithstanding, the use of two processes increases the costs of detoxification, and may result in significant loss of sugars (Taherzadeh and Karimi, 2011).

1.4.3. Microorganisms Modification

1.4.3.1. Genetic / Metabolic Engineering

The success of an industrial fermentation process using substrates with undesired compounds depends on the microorganism tolerance and its ability to metabolize those compounds. When such strains are not available, genetic manipulation strategies can be implemented to improve the yeast response in the presence of such substances (Paes and Almeida, 2014). Therefore, the basis of this approach is to utilize biotechnological techniques, such as genetic engineering (also called recombinant

DNA technology) to operate a targeted manipulation of the microorganism's genome in order to create a genetically adjusted microorganism with an increased resistance to the inhibitors (Paes and Almeida, 2014).

A study conducted by (Alriksson, Horváth and Jönsson, 2010) investigated the possibility to manipulate *S. cerevisiae*, in an effort to increase its resistance to several inhibitory compounds by overexpressing genes encoding for multidrug-resistance (MDR). The authors achieved promising results concerning an improvement in resistance to inhibitory compounds in lignocellulosic hydrolyzates. Another example is the microbial engineering to improve biofuel tolerance using efflux pump operons cloned into plasmids. The authors efficiently identified pumps that improved the survival of microorganisms in the presence of toxic substrate (Dunlop *et al.*, 2014).

Hence, the identification of enzymes responsible for the transformation of inhibitory compounds, with subsequent genetic engineering of the microorganisms, seems to be a very auspicious method to get efficient strains with increased tolerance to the inhibitory substrates (Almeida *et al.*, 2007). Although the identification of possible genes or enzymes requires some time, this strategy is advantageous, since the added or improved features may be replicable for generations (Paes and Almeida, 2014).

1.4.3.2. Evolutionary engineering

Adaptive evolution has become an extensively exploited vehicle to generate new biological functions and attain strain improvement in several industrial areas, namely for several industrial stress conditions (Çakar *et al.*, 2012; Paes and Almeida, 2014; Wang *et al.*, 2014). Although genetic engineering has had good results in improving yeast performance, an obstacle that arises is the poor consumer acceptance of genetically modified organisms (GMO). Therewith, adaptive evolution is becoming an emerging method for the development of novel yeast strains. Among the various areas, the main focus of adaptive evolution research has been to improve the efficiency of bioethanol production based on lignocellulosic substrates (Çakar *et al.*, 2012).

Laboratory adaptive evolution is a method, which consists of long-term cultivation of cells under defined selective pressure. During adaptive evolution, various genetic alterations occur through the several successive transfers to new medium with selective pressure, and populations with improved fitness become larger (Nevoigt, 2008; Parawira and Tekere, 2011).

Several studies have emerged in which performance enhancement was attained by evolutionary engineering assays. For example, successful adaptation studies have been performed to create strains with improved tolerance for furfural present in lignocellulosic hydrolysates, by selecting strains which can remain viable and therefore grow in an inhibitory environment (Heer and Sauer, 2008). Others authors have used this technique not only to improve the performance of *S. cerevisiae* in media containing limonene, but also to investigate the mechanisms that trigger this toxicity and to point out genetic targets for posterior tolerance engineering (Brennan *et al.*, 2015).

The advantage of using this approach is that a detailed insight into the true mechanism of action of inhibitors and their interactions with the several biochemical networks is not necessary. On the other hand, a well-designed selection procedure is required and crucial (Almeida *et al.*, 2007; Koppram, Albers and Olsson, 2012).

1.5. Main objectives of this work

Food industry has been exploring the creation of new fruit juice-based products, in order to respond to the progressive consumer demand for products that promote their well-being and health. However, sometimes in these substrates there are compounds that prevent fermentation, which is the case of the presence of orange essential oils in natural orange juice. Thus, in view of the possible expansion of fruit substrates and yeasts employed in the development of fermented beverages, the specific objectives of the present study were:

- Evaluation of the fermentative performance of wild type *Torulaspora delbrueckii* strain PYCC 4639 and *Schizosaccharomyces pombe* strain PYCC 4181 yeasts in two orange juices substrates: “fresh” orange juice and juice prepared from concentrate (“concentrate” orange juice).
- Confirmation the likely identity of the inhibitory component present in “fresh” orange juice whose presence delays the fermentation and influence growth parameters.
- To understand how yeast cells respond in the presence of the inhibitory compound to get insight into the mechanism involved in the cell response of this stress: cell viability and quantitative differential expression of *GPX2*, *PIR3* and *ERG6* genes.
- To obtain, by adaptive evolution, yeast strains with better performance regarding the fermentation of “fresh” orange juice substrates, and subsequent physiological characterization of evolved strains compared to the original strains.

2. MATERIALS AND METHODS

2.1. Evaluation of the fermentative performance of wild-type *T. delbrueckii* and *S. pombe* on fruit substrates

2.1.1. Yeast strains and preservation

The yeasts used in this study were *Torulaspora delbrueckii* strain PYCC 4639 (Figure 2.1-(A)) and *Schizosaccharomyces pombe* strain PYCC 4181 (Figure 2.1-(B)) provided by the Portuguese Yeast Culture Collection (PYCC). For long-term storage, the yeasts were maintained in 2 mL cryotube vials with 40%(v/v) glycerol at -80°C, and for short-term storage in YPD solid medium (2% peptone (Bacto™); 2% glucose (Kirsch Pharma, COD. 10888, NO.1052); 1% yeast extract (Bacto™)) and 2% of agar (LABCHEM)) at 25°C.

The orange and apple juices, and the orange essential oils (EO) used in the assays were kindly provided by SUMOL+COMPAL, SA.

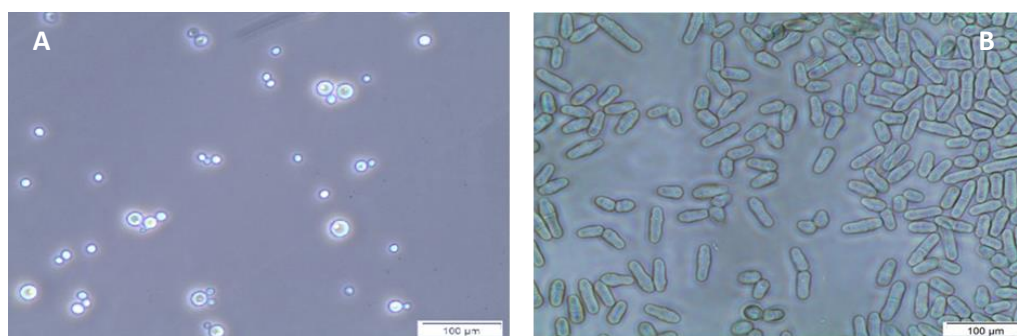


Figure 2.1- Yeasts used in the current study. (A): *Torulaspora delbrueckii* PYCC 4639 viewed under the microscope (OLYMPUS BK50; Objective: 40 X; Phase 2). **(B):** *Schizosaccharomyces pombe* PYCC 4181 viewed under the microscope (OLYMPUS BK50; Objective: 40 X; Phase 1).

2.1.2. Microbial culturing and fermentation procedure in orange juice

Fermentation assays were performed using two different types of orange juice, a “fresh” orange juice that naturally contains a high concentration of orange EO, and a “concentrate” orange juice, which unlike the previous one, due to industrial evaporation inherent to juice concentration, has a lower content of orange EO. The “concentrate” orange juice was diluted with sterile distilled water to obtain 13°Brix.

To prepare the inoculum, each yeast was inoculated in 200 mL of YPD liquid medium (2% peptone (Bacto™); 2% glucose (Kirsch Pharma, COD.10888, NO.1052) and 1% yeast extract (Bacto™)) and incubated overnight at 25°C with orbital shaking at 150 rpm (Sartorius CERTOMAT® U). The cells were subsequently washed twice with sterile distilled water, centrifuged for 5 minutes (3000 rpm, SIGMA) and suspended to a final cell density of 5×10^6 cell/mL in 100 mL of orange juice. The fermentation assays were carried out at 25°C with orbital shaking at 150 rpm, in 500 mL Erlenmeyer flasks capped with cotton. Initial and final cells were microscopically counted using a hemocytometer. The fermentative performance was monitored using a refractometer (ATAGO, PAL-1), as shown in Figure 2.2, where the Brix scale provides a means to monitor the concentration of all soluble solids in the sample. All orange juice fermentations carried out by these yeast cells started at $\approx 13^\circ$ Brix, and

when the degree stabilized at 5°Brix, the fermentations were taken as finished. The onset of the juice fermentation was assumed when a decrease of $\approx 0,5^\circ$ Brix was observed. The fermentation rates were calculated by determining the slopes of the produced fermentation curves.

In addition, the pH of “fresh” orange juice was also measured in the beginning and end of fermentation, using a pH electrode (PHM82 STANDARD).

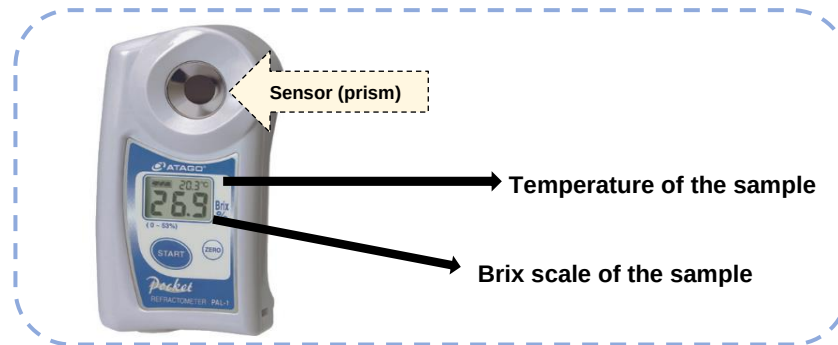


Figure 2.2- Refractometer used to monitor fermentation assays. The refractometer is an optical instrument of high precision, portable and of simple handling. It is based on the refraction of light when passing through a prism and thus allows the determination of the concentration of sugar in the most varied substances.

2.1.3. Microbial culturing and fermentation procedure in apple juice

Fermentation of apple juice was carried out in a similar way as the fermentation assay with orange juice (described in the previous section), with few modifications. Briefly, the concentrate apple juice was diluted in sterile water so that the °Brix was approximately equal to 13 and thus similar to the concentration of sugars present in “fresh” orange juice.

In 500 mL Erlenmeyer, 200 mL of YPD liquid medium were inoculated with *Sch. pombe* and incubated overnight at 25°C with orbital shaking at 150 rpm. The cells were subsequently washed twice with sterile distilled water, centrifuged for 5 minutes (3000 rpm, SIGMA) and suspended to the final concentration of 1×10^6 cell/mL in 100 mL of clarified diluted apple juice. The fermentation assays were carried out at 25°C with orbital shaking at 150 rpm, in 500 mL Erlenmeyer flasks capped with cotton. Microbial growth was monitored by measuring the optical density (OD_{640nm}) using a spectrophotometer (Ultrospec 3100 pro) and the fermentative performance was monitored using a refractometer (ATAGO, PAL-1). When the degree stabilized at 5°Brix, the fermentations were taken as finished. The onset of the juice fermentation was assumed when a decrease of $\approx 0,5^\circ$ Brix was observed. Fermentation and growth rates were calculated by determining the slopes of the produced fermentation/growth curves.

In addition, the pH of apple juice was also measured in the beginning and end of fermentation, using a pH electrode (PHM82 STANDARD).

2.2. Effects of orange essential oils on growth parameters

Growth assays in YPD and YNB liquid medium and solid medium were performed in order to clarify how yeast growth is impacted by the presence of orange EO at various concentrations.

2.2.1. Growth assays in liquid medium with orange essential oils

T. delbrueckii and *Sch. pombe* were pre-grown overnight in 200 mL of YNB liquid medium (0,4xYNB (Difco)) supplemented with 1,5% (w/v) glucose (Kirsch Pharma, COD. 10888, NO.1052) and YPD liquid medium at 25°C with orbital shaking at 150 rpm. The cells were subsequently washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and resuspended in sterile water. Appropriate volumes of orange EO ($\approx$ 6; 7; 23; 34; 102 μ L) were directly and aseptically added to 100 mL of YNB and YPD liquid medium, resulting at a concentration range from 50 to 900 mg/L. The broths containing orange EO were immediately inoculated with approximately 5×10^6 cells/mL and incubated at 25°C with orbital shaking at 150 rpm, in 500 mL Erlenmeyer flasks capped with cotton. Samples were taken during growth at 1-2h intervals and OD_{640nm} of each culture was measured using a spectrophotometer (Ultrospec 3100 pro). The specific growth rates were calculated by determining the slopes ($\Delta \ln OD_{640nm} / \Delta \text{time}$) in the exponential growth phase. The length of the lag phase was considered to be the time that the culture remained at the initial absorbance.

An additional *T. delbrueckii* growth assay was also performed. *T. delbrueckii* was pre-grown overnight in 200 mL of YNB liquid medium at 25°C with orbital shaking at 150 rpm. The cells were subsequently washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and resuspended in sterile water. Then, approximately 5×10^6 cells/mL were inoculated in six 500 mL Erlenmeyer flasks, containing 100 mL of YNB liquid medium without orange EO, and incubated at 25°C with orbital shaking at 150 rpm. After reaching the mid-exponential phase, appropriate volumes of orange EO ($\approx$ 6; 7; 23; 34; 102 μ L) were aseptically added to the broths. Microbial growth was monitored by measuring the OD_{640nm} for 10 hours.

2.2.2. Growth assays in solid medium with orange essential oils

Additionally, YNB and YPD agar plates were prepared with the same range of orange EO concentrations used in the growth assays in liquid medium. Spotting tests were performed to evaluate the ability of cells to grow on agar plates with orange EO.

In 100 mL Erlenmeyer, 20 mL of YPD liquid medium were inoculated with *T. delbrueckii* and *Sch. Pombe* (one strain in each 100 mL Erlenmeyer) and incubated at 25°C with orbital shaking at 150 rpm, until reach an OD_{640nm} $\approx$ 0,3. Then, the cells were washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and resuspended in sterile water to prepare serial dilutions (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4}). 5 μ L of each dilution was inoculated as shown in Figure 2.3, in YPD solid medium with orange EO (50, 60, 200, 300, 900 mg/L), and incubated at 25°C. Colony growth was visually monitored after 24 and 48 hours.

The same method was implemented in YNB solid medium with orange EO. However, in this medium, colony growth was visually monitored after 24, 48, 60, 108, 132 and 228 hours.

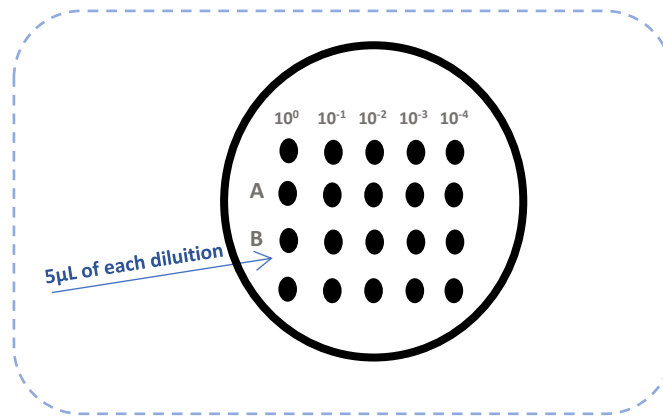


Figure 2.3-Arrangement of inoculated drops in spotting test. A and B represent different strains.

2.3. Cell viability

2.3.1. Growth assays for cell viability assessment

Cell viability was estimated during *T. delbrueckii* growth assays in YNB liquid media, supplemented with various orange EO concentrations, using staining with methylene blue, spreading in YPD plates (colony-forming units) and spotting test. The growth assays performed for viability assessment are described in the previous section “2.2.1. Growth assays in liquid medium with orange essential oils”.

2.3.2. Methylene Blue

For the staining with methylene blue, aliquots were taken during the growth of the cultures, and a drop of the cell suspension was mixed with a drop of 0,1% methylene blue (1 mg of methylene blue powder (Difco™) dissolved in 10 mL of sterile water). The solution was incubated for 5 minutes at room temperature in the hemocytometer. Cell viability was examined under the microscope (OLYMPUS BK50T) assuming that the cells that presented a blue color were those that failed to reduce the dye, and therefore were not viable. On the other hand, viable cells were colorless.

The total cells were counted using the hemocytometer and the formula $\frac{\text{total cells}(t=x) - \text{blue cells}(t=x)}{\text{total cells}(t=x)} \times 100$ was applied to determine the percentage of viable cells. Adapted from (Kwolek-Mirek and Zadrag-tecza, 2014).

2.3.3. Colony-Forming Units (CFUs)

Colony Forming Units (CFUs) is a microbiological term used to denote the number of viable cells that exist in a certain cell suspension, inferred from the number of colonies forming upon inoculation of a defined volume of a cell suspension.

During growth of the cultures, aliquots were taken to perform appropriate serial dilutions. 100 μL samples of the appropriate dilution were inoculated by spreading with a glass spreader in YPD solid

medium and the plates incubated at 25°C. CFUs were counted after 2 days. Adapted from (Kwolek-Mirek and Zadrag-tecza, 2014).

2.3.4. Spotting test

This assay was performed to obtain a growth pattern of cultures supplemented with various concentrations of orange EO. Thus, the serial dilutions carried out for the CFUs counting assay were re-used, and 5 µL of each dilution were inoculated in YPD plates and incubated at 25°C. The YPD plates were visually inspected 48 h after inoculation. Adapted from (Kwolek-Mirek and Zadrag-tecza, 2014).

2.4. Analysis of quantitative differential expression of *GPX2*, *PIR3* and *ERG6* ortholog genes in *T. delbrueckii*

2.4.1. Search of *ACT1*, *GPX2*, *PIR3* and *ERG6* ortholog genes in *T. delbrueckii*

The search for *GPX2*, *PIR3* and *ERG6* orthologous genes and the internal control (*ACT1*) was conducted using the Yeast Gene Order Browser (YGOB, v.7 accessed on March 20, 2019 in the website: <http://ygob.ucd.ie/>), that was designed to facilitate the comparative genomic visualization. Moreover, it features a "visual browser" that generates an image of the syntenic context surrounding a particular gene. The support of the YGOB software is based on its curated database of homology assignments among the various deposited genomes (Byrne and Wolfe, 2005).

2.4.2. Extraction and purification of the genomic DNA of *T. delbrueckii*

The genomic DNA of *T. delbrueckii* was extracted by the Phenol-Chloroform method. First, 4 loopfuls of *T. delbrueckii* grown in YPD solid medium were used to prepare a cell suspension in tubes containing $\approx$ 200 µL glass beads and 1,8 mL of sterile water. The tubes were then centrifuged using a microcentrifuge (Heraeus™Fresco™17) for 3 minutes at 13000 rpm to pellet de cells. The generated pellet was stored at -20° C for 1,5 hours. Genomic DNA from *T. delbrueckii* was extracted using the TE-Phenol + Chloroform (1:1), with a phenol solution (Sigma-Aldrich®) equilibrated with a layer of TE buffer (10mM of Tris-HCl +1mM of EDTA pH=8). In short, 250 µL of TE-Phenol and 250 µL of chloroform were added to the pellet. Thereafter, 500 µL of lysis buffer solution composed by 100mM NaCl, 10mM Tris-HCl (pH=8,0), 1mM EDTA, 2% Triton X-100 and 1% of sodium dodecyl sulphate (SDS) was then added to the mix. Cell lysis was performed by mechanical lysis with glass beads at 1000 rpm (IKA®, Vibrax VXR basic), for 20 minutes. The genomic DNA was precipitated at - 20°C with ethanol (100%), washed with ethanol (70%) and dissolved in 50 µL of a TE+ RNase solution (1X TE + 50 µg / mL RNase). Lastly, the DNA was incubated at 55°C for 15 minutes. The extracted genomic DNA was quantified by NanoDrop (ND-1000 Spectrophotometer).

2.4.3. Amplification of orthologous genes *ACT1*, *GPX2*, *PIR3* and *ERG6*

PCR reactions were performed to amplify fragments of the *ACT1*, *GPX2*, *PIR3* and *ERG6* orthologous genes. The primers used were designed based on the orthologous gene sequences which were retrieved from the YGOB. The complete list of primers used in this study can be found in Table 6.1, in “Appendix”. All PCR reactions were performed using DreamTaq thermosensitive DNA Polymerase (Thermo Scientific). The PCR products were analyzed by electrophoresis in 1% agarose gel using an ultra-sensitive fluorescent nucleic acid dye GelRed (Biotium), and GeneRuler (Thermo Scientific) as a molecular weight marker.

2.4.4. Extraction and purification of total RNA of *T. delbrueckii*

For analysis of differential expression of the *GPX2*, *PIR3* and *ERG6* genes, *T. delbrueckii* was exposed for two hours to orange EO prior to RNA extraction. To that end, *T. delbrueckii* was pre-grown overnight in 200 mL of YPD liquid medium at 25°C. Then, approximately 5 x10⁶ cells/mL were re-inoculated in two 500 mL Erlenmeyer flasks containing 100 mL of the same medium, at 25°C with orbital shaking at 150 rpm. After reaching the mid-exponential growth, in one 500 mL Erlenmeyer flask, 23 µL of orange EO were aseptically added, resulting in a concentration of 200 mg/L. After two hours of exposure the cells were collected in 50 mL falcon tubes, washed once in ice-cold water and kept at -80°C for RNA extraction. Total RNA was obtained using a TRIzol/chloroform method. Cells were transferred to 2 mL eppendorf with glass beads, a microcentrifugation was performed using a microcentrifuge (Heraeus™Fresco™ 17) during 10 minutes at 8000 rpm at 4°C and then the supernatant was discarded with the micropipette. Immediately after, 1 mL of TRIzol reagent (Ambion) was added to each eppendorf and cell lysis was started, with 6 cycles of 30s vortex (Labnet International) and 30 seconds on ice. The homogenates were incubated for 5 minutes at 30°C to allow for complete dissociation of nucleoprotein complexes. Then, 0,2 mL of chloroform were added (per 1 ml of TRI Reagent used) and the samples were vigorously agitated for 15 seconds and incubated at 30°C for 3 minutes. The samples were then centrifuged at 12,000 x g for 15 minutes at 4°C. After this centrifugation, the samples presented two phases: a lower phase containing the TRIzol reagent and chloroform, an interface with proteins and cellular debris, and a colorless upper aqueous phase containing the RNA. The aqueous phase was transferred to a sterile and cold 1,5 mL eppendorf and one volume of isopropanol was added for precipitation of the RNA. Samples were then incubated at 30 °C for 10 minutes and centrifuged for 15 minutes at 12000 x g at 4°C. The supernatant was discarded with the micropipette and the RNA pellet was washed with 75% ethanol. The pellets were centrifuged for 5 minutes at 7500 x g at 4°C, and the ethanol removed by inversion. The RNA pellet was air-dried and dissolved in 40 µL of Water Nuclease Free (Sigma-Aldrich®). Adapted from (Rio *et al.*, 2010). Total RNA was subsequently purified using the RNA Clean & Concentrator-5 kit (Zymo Research) following the manufacture’s guidelines. To check if the RNA was clean of DNA and ready for cDNA synthesis, PCR reactions were performed with total RNA.

2.4.5. cDNA Synthesis

To obtain total cDNA from *T. delbrueckii*, total RNA was used for reverse transcription with 100U of Maxima H Minus Reverse Transcriptase (Thermo Fisher Scientific), 0,5 mM of dNTPs mix, an Oligo(dT)₂₀ as primer, 4 µL of 5X RT Buffer (Thermo Fisher Scientific) and 20U of RiboLock RNase Inhibitor (Thermo Fisher Scientific) following the manufacturer's guidelines.

2.4.6. Quantitative Real Time PCR (RT-qPCR)

The Two-step RT-qPCR method uncouples cDNA synthesis and subsequent qPCR. Thus, cDNA is first synthesized and used as the template for the qPCR reaction. The PCR reactions with specific primers (Table 6.1, in "Appendix") were performed using Rotor-GeneTM6000 (Corbett Research), with 50 ng of each cDNA synthesized and 10 µL of the 2X SensiFAST SYBR[®] No-ROX Mix from Bioline. The program run in the Rotor- GeneTM 6000, is described in Table 6.2, in "Appendix". Both genomic DNA (50 ng) and total RNA (50 ng) samples were used as positive and negative controls, respectively. A denaturation curve analysis was included at the end of the program to confirm the specificity of the reactions and check for possible DNA contaminations.

Data produced after RT-qPCR was analyzed using (Rotor-Gene 6000 Series Software 1.7) software. The expression fold change was calculated using $(1 + E)^{-\Delta\Delta CT}$ method, where ΔCT is the difference between the CT obtained for a particular gene and the housekeeping gene (*ACT1*). The double ΔCT ($\Delta\Delta CT$) is the difference between the ΔCT of the control assay and ΔCT obtained for the situation in which the cells were exposed to the orange EO. E is the efficiency of target amplification. Therefore, considering that the primer efficiency is roughly 1, the formula becomes $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001).

2.5. Experimental evolutions

The ratio of OD_{640nm} at 10 and 0 hours (relative fitness) was used as a metric to elucidate the progression of the experimental evolution assays.

2.5.1. Experimental evolution of *T. delbrueckii* in culture medium

T. delbrueckii was transferred from glycerol stocks stored at -80°C to YPD solid medium and incubated at 25°C. Two loopfulls of cells were inoculated in 100 mL of YNB liquid medium and the culture was left to grow overnight. Then, cells were washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and resuspended in sterile water. The appropriate volume of inoculum was used to inoculate 100 mL of acidified YNB liquid medium (pH≈3,5-3,6) to target OD_{640nm} ≈ 0,1. After optimizing the procedure, 100 mg/L of orange EO was added aseptically to the broth culture, immediately after inoculation. The orange EO-challenged cultures were incubated at 25°C with orbital shaking at 150 rpm, in 500 mL Erlenmeyer flasks capped with cotton. After about 1,5 generations (OD_{640nm} ≈ 0,3), a new transfer was performed to new acidified YNB liquid medium with orange EO (100 mg/L). For this, the appropriate volume of the previous culture was used to inoculate 100 mL of acidified YNB liquid medium

to a target $OD_{640nm} \approx 0,1$. This procedure was successively replicated, and the working volume was maintained constant during the sequential transfers. The evolutionary assays were conducted in duplicate (*T.d-A* culture and *T.d-B* culture).

Contaminations were verified daily by microscopic observation and at 2-week intervals by plating the cultures on YPD plates. To preserve the putative evolved yeasts strains developed during the course of evolution, at the end of each week, 1 mL aliquots of the populations were stored at -80°C in 40 % (v/v) of glycerol solution.

2.5.2. Experimental evolution of *Sch. pombe* in culture medium

The method applied to conduct the experimental evolution assays of *Sch. pombe* was similar to that used in the evolutionary assays of *T. delbrueckii*, with a few adjustments. As such, *Sch. pombe* was similarly transferred from glycerol stocks stored at -80°C to YPD plates and incubated at 25°C . Two loopfulls of cells were inoculated in 100 mL of YPD liquid medium and the culture was left to grow overnight. Then, cells were washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and resuspended in sterile water. The appropriate volume of inoculum was used to inoculate the evolution experiments to an initial $OD_{640nm} \approx 0,4$ in two different media, in 100 mL of YNB liquid medium (without adjustment of the original pH of the medium), and in YPD liquid medium supplemented with chloramphenicol. After optimizing the procedure, 35 mg/L ($\approx 4\mu\text{L}$) and 60 mg/L of orange EO ($\approx 7\mu\text{L}$) were added aseptically and respectively to each broth culture, immediately after inoculation. The orange EO-challenged cultures were incubated at 25°C with orbital shaking at 150 rpm, in 500 mL Erlenmeyer flasks capped with cotton. Likewise, after the cell population multiplied about 1,5 generations ($OD_{640nm} \approx 1,2$) in both evolutionary media, a transfer to fresh medium with orange EO was performed and cells would resume growth. For this, the appropriate volume of previous cultures was used to inoculate 100 mL of fresh medium to a target $OD_{640nm} \approx 0,4$. This procedure was successively replicated, and the working volume was maintained constant during the sequential transfers. After 94 passages, the orange EO load in YNB liquid medium increased to $\approx 6\mu\text{L}$ (50 mg/L) remaining in this concentration until the end of the evolutionary assay. The evolutionary assays in YPD liquid medium supplemented with chloramphenicol were conducted in duplicate (*S.p-A* culture and *S.p-B* culture). For evolution conducted in YNB liquid media, only one evolution experiment was performed (*S.p-C* culture).

Contaminations were verified daily by microscopic observation and at 2-week intervals by plating the cultures on YPD plates. To preserve the putative evolved yeasts strains developed during the course of evolution, at the end of each week, 1 mL aliquots of the populations were stored at -80°C in 40 % (v/v) of glycerol solution.

2.5.3. Experimental evolution in orange juice substrate

For *Sch. pombe*, a different experimental evolution approach was conducted using orange juice, naturally containing orange EO ("*fresh*" orange juice), as depicted in Figure 2.4. The *Sch. pombe* cells were pre-grown overnight in 100 mL of YPD liquid medium at 25°C with orbital shaking at 150 rpm. Then, cells were washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and

resuspended in sterile water. The appropriate volume of inoculum was used to inoculate 100 mL of “fresh” orange juice, so that the initial concentration of cells in the juice was 5×10^6 cells/mL ($OD_{640nm} \approx 0,45-0,5$). Under these conditions, fermentation of the substrate was initiated and monitored using the refractometer (ATAGO, PAL-1). The experimental evolution assays in juice were incubated at 25°C with orbital shaking at 150 rpm, in 500 mL Erlenmeyer flasks capped with cotton. After fermentation began (decrease of approximately 0,5°Brix), 100 μ L sample of the culture broth was immediately plated on YPD plates. After incubation of the YPD plates at 25°C for 72h, a further fermentation in orange substrate was initiated. For that, a scraping of the cells grown on the YPD plate was performed, and the cells were resuspended in sterile water. Approximately 5×10^6 cells/mL were re-inoculated in “fresh” orange juice and this method successively replicated (Figure 2.4). 2 weekly sequential transfers were performed during approximately 4 months.

Absence of contaminants was verified by observing inoculated YPD plates at the beginning of the fermentation and after the transfers.

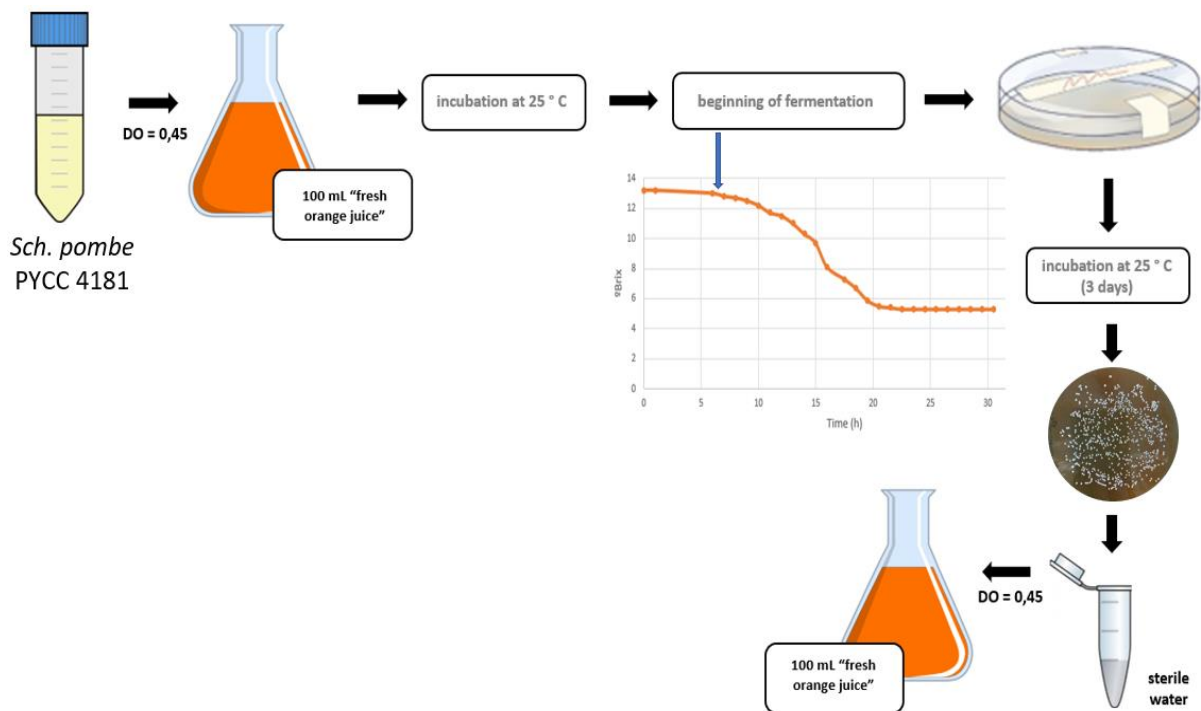


Figure 2.4-Flowchart relative to the adaptive evolution assay in orange juice substrate conducted by *Sch. Pombe*.

2.6. Screening of evolved mutants

For strain screening, a sample of the *T.d-B* and *S.p-B* cultures was appropriately diluted and spread in YNB solid medium supplemented with 300 mg/L of orange EO (with pH set to $\approx 3,5$ for *T.d-B* culture), and the plates were incubated at 25°C until colonies appeared. To elucidate the phenotypic differences between the single-colony isolates, the largest colonies (19-20) and 3 of the smallest colonies were tested for their ability to grow in the experimental evolution media. Each colony was

restreaked 4 times in medium without selective pressure to eliminate possible unstable point mutations that may have arisen during the evolution.

Pre-cultures of each colony isolated from *T.d-B* culture were grown overnight in 10 mL of acidified YNB liquid medium at 25°C with orbital shaking at 150 rpm. Then, appropriate volume of inoculum was re-inoculated in 10 mL of acidified YNB liquid medium supplemented with 100 mg/L of orange EO, to an initial $OD_{640nm} \approx 0,1$. Likewise pre-cultures of each colony isolated from *S.p-B* culture were grown overnight in 10 mL of YPD liquid medium, and the appropriate volume of inoculum was re-inoculated into 10 mL of YPD liquid medium supplemented with 60 mg/L of orange EO, to an initial $OD_{640nm} \approx 0,4$. The cultures were incubated at 25°C with orbital shaking at 150 rpm, in 50 mL Erlenmeyer flasks capped with cotton. Growth was monitored for 10 hours using a spectrophotometer at 640nm. Mutants with stable improved relative fitness compared to the WT parent cells were stored at -80°C in 40 % (v/v) glycerol.

Regarding the screening of *Sch. pombe* mutants resulting from adaptive evolution in orange substrate, plates with “fresh” orange juice with 2% of agar were prepared. Similarly, to assess the presence of possible mutants with improved tolerance to the substrate, 8 large and 1 small colony were selected. Each colony was grown overnight in 10 mL of YPD liquid medium at 25°C with orbital shaking at 150 rpm. Then, cells were washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and resuspended in sterile water. 5×10^6 cells/mL were inoculated in 10 mL of “fresh” orange juice and incubated at 25°C with orbital shaking at 150 rpm, in 50 mL Erlenmeyer flasks capped with cotton. The fermentations were monitored for 10 hours using the refractometer (ATAGO, PAL-1). The mutants with improved fermentation lag time were stored at -80°C in 40 % (v/v) glycerol.

2.7. Fermentative performance of the evolved mutants

After selecting the strains that had a stable phenotype, their fermentative performance was evaluated in “fresh” orange juice. For that, each selected clone was grown overnight in 10 mL of YPD liquid medium at 25°C with orbital shaking at 150 rpm. The cells were subsequently washed twice with sterile water, centrifuged for 5 minutes (3000 rpm, SIGMA) and resuspended in sterile water. Then, 5×10^6 cells/mL were inoculated in 10 mL of “fresh” orange juice (50 mL Erlenmeyer flasks capped with cotton) and incubated at 25°C with orbital shaking at 150 rpm. The time required for the fermentation onset was determined by monitoring each fermentation for 10 hours using the refractometer (ATAGO, PAL-1). The onset of the juice fermentation was assumed when a decrease of $\approx 0,5^\circ$ Brix was observed.

3. RESULTS AND DISCUSSION

3.1. Evaluation of the fermentative performance of wild-type *T. delbrueckii* and *S. pombe*

The fermentative performance of *Torulaspora delbrueckii* PYCC 4639 (*T. delbrueckii*) and *Schizosaccharomyces pombe* PYCC 4181 (*Sch. pombe*) was evaluated in orange juices. Both yeasts are crabtree positive i.e., they ferment the sugars present in the substrates to ethanol, with the corresponding release of CO₂, in the presence of O₂ (Rodicio and Heinisch, 2009).

Preliminary fermentations with both species were carried out in two different unpasteurized orange juices under aerobic conditions (Erlenmeyer flasks stoppered with cotton). “Fresh” orange juice was one of the two substrates used in this study and is characterized by naturally containing a high concentration of orange essential oils (EO), known to have inhibitory effects on microbial growth (Tserennadmid *et al.*, 2011). The other orange substrate used in the present work was a “concentrate” orange juice, which unlike the previous one, has a low concentration of orange EO. The fermentation processes were monitored using a refractometer, which according to the guidelines that accompanied the instrument, is used to measure the refractive index of a solution, enabling the estimation the total sugar concentration of the samples. Since fermentation is accompanied by sugar consumption, it was possible to monitor fermentations using this device.

The fermentation profiles obtained after each orange juice fermentation conducted by *T. delbrueckii* and *Sch. pombe* are depicted respectively, in Figure 3.1 and Figure 3.2. For both yeasts, a lengthened delay was observed in the onset fermentation in fermentations of “fresh” orange juice, when compared with fermentations conducted with juice prepared from concentrate (Figure 3.1 and Figure 3.2). In order to gather support to the hypothesis that the delay in the onset of the fermentation of “fresh” orange juice was due to a difference in orange EO content between the two types of juices, another fermentation was performed for both yeasts. For this additional fermentation, approximately 250mg/L of orange EO was added to the “concentrate” juice, in order to simulate the orange EO concentration of the “fresh” orange juice.

Regarding the fermentations conducted by *T. delbrueckii* (Figure 3.1), it was observed that in “concentrate” orange juice, “fresh” orange juice and “concentrate” orange juice supplemented with orange EO, fermentations started approximately at 7,5h; 12,5h and 16,5h after inoculation, respectively. On the other hand, in the orange juice fermentations carried out by *Sch. pombe*, it was ascertained that in “concentrate” orange juice, “fresh” orange juice and “concentrate” orange juice supplemented with orange EO, the fermentation processes initiated approximately at 6h; 9,5h and 16,5h after inoculation, respectively (Figure 3.2). In light of these results, it was observed that *Sch. pombe* was able to start the fermentation process in “fresh” orange juice faster than *T. delbrueckii*. Furthermore, it was possible to confirm that the orange EO caused a delay in the onset of fermentation like that observed for “fresh” orange juice albeit more pronounced, for both species. Since the amount of orange EO added to the “concentrate” orange juice was intended to compensate the difference in orange EO content between the two types of orange juices, the lengthier delay was unexpected (Figure 3.1 and Figure 3.2).

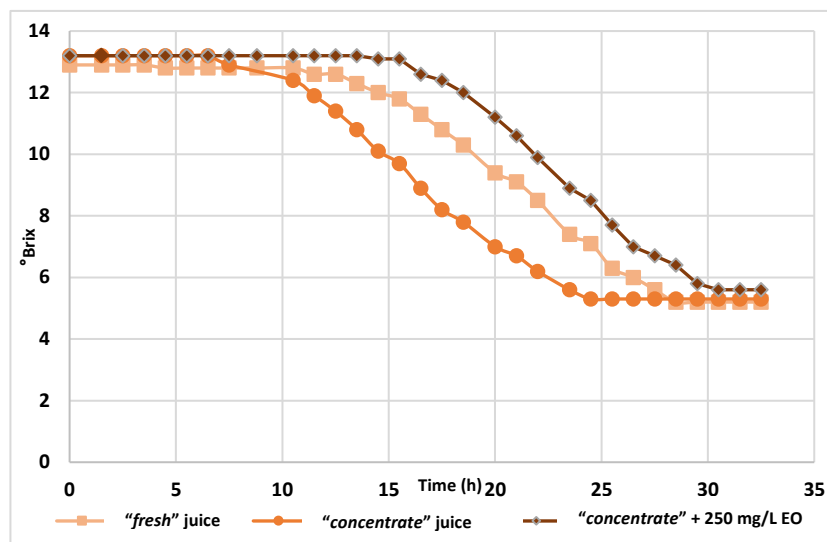


Figure 3.1- Fermentation profile of *T. delbrueckii* represented by the Brix variation as a function of time, in the different types of orange juice. Light orange line corresponds to the “fresh” orange juice fermentation, the orange line to the fermentation of the “concentrate” orange juice and the brown line concerns to the fermentation of the “concentrate” orange juice to which was added about 250 mg/L of orange EO.

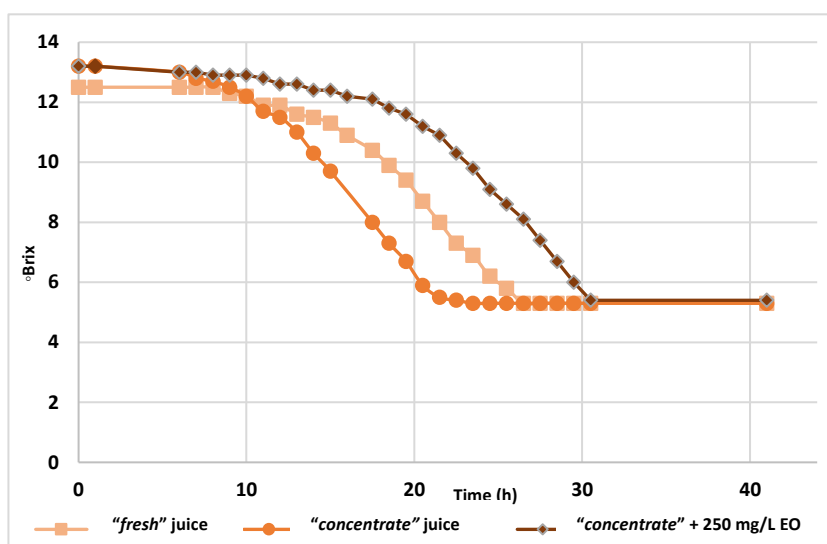


Figure 3.2- Fermentation profile of *Sch. pombe* represented by the Brix variation as a function of time, in the different types of orange juice. Light orange line corresponds to the “fresh” orange juice fermentation, the orange line to the fermentation of the “concentrate” orange juice and the brown line concerns to the fermentation of the “concentrate” orange juice to which was added about 250 mg/L of orange EO.

At the moment of inoculation and the end of the fermentation processes, the cells were counted, and the fermentation rates were calculated. The results of cell count and calculated fermentation rates for each orange juice are shown in Table 3.1 and Table 3.3. As shown in Table 3.1, at the end of each orange juice fermentation, *T. delbrueckii* grew roughly two orders of magnitude, relative to the initial inoculum. On the other hand, the fermentations conducted by *Sch. pombe* generated a lower number of cells (approximately 4 times less) compared to the fermentations carried out by *T. delbrueckii* (Table 3.3).

3. RESULTS AND DISCUSSION

Table 3.1- Initial and final cell concentrations and calculated fermentation rates for the orange juice fermentations carried out by *T. delbrueckii*.

<i>T. delbrueckii</i> Fermentations	Initial cell concentration (cell/mL)	Final cell concentration (cell/mL)	Fermentation rate (°Brix/h)
“fresh” orange juice	1,1x10 ⁶	5,4x10 ⁸	-0,5
“concentrate” orange juice	3,8x10 ⁶	4,8x10 ⁸	-0,5
“concentrate” orange juice + 250 mg/L orange EO	5,5x10 ⁶	7,7x10 ⁸	-0,5

Table 3.2- pH values of **“fresh”** orange juice before and after fermentation by *T. delbrueckii*.

<i>T. delbrueckii</i> Fermentations	pH value
fermentation beginning	3,43
fermentation end	3,39

Table 3.3- Initial and final cell concentrations and calculated fermentation rates for the orange juice fermentations carried out *Sch. pombe*.

<i>Sch. pombe</i> Fermentations	Initial cell concentration (cell/mL)	Final cell concentration (cell/mL)	Fermentation rate (°Brix/h)
“fresh” orange juice	3,6x10 ⁶	9,2x10 ⁷	-0,6
“concentrate” orange juice	5,9x10 ⁶	1,6x10 ⁸	-0,6
“concentrate” orange juice + 250 mg/L orange EO	4,2x10 ⁶	1,4x10 ⁸	-0,6

Table 3.4- pH values of **“fresh”** orange juice before and after fermentation by *Sch. pombe*.

<i>Sch. pombe</i> Fermentations	pH value
fermentation beginning	3,60
fermentation end	3,19

Another fact portrayed in these fermentative assays was that fermentation rates did not appear to be affected by the presence of large amounts of orange EO (Table 3.1 and Table 3.3). As shown in Table 3.1, all *T. delbrueckii* fermentation rates were identical to each other. Similarly, all *Sch. pombe* fermentation rates were identical, and slightly higher than those displayed in *T. delbrueckii* juice fermentations (Table 3.3).

Taking all these observations into account, the results suggest that the orange EO affected yeast growth during orange juice fermentations, leading to a delay in the onset of the fermentations.

The fermentation of orange substrates has been subject of study with other yeasts. One of these studies used *S. cerevisiae*, isolated from sugar cane in Brazil, to produce an orange distilled beverage from a 11°Brix autoclaved orange must. This fermentative process ended 24 hours after inoculation, similarly to our results concerning the fermentation of the **“fresh”** orange juice performed by both strains (Santos *et al.*, 2013). In another report, a fermentation of a commercial pasteurized juice was conducted by *Pichia kluyveri*, which lasted for 9-11 days (Escudero-López *et al.*, 2013). It is important to note that in the latter case 5 liters of juice were fermented in PVC tanks, and this larger fermentation volume, may have lengthened the fermentation.

At the beginning and end of the “fresh” orange juice fermentations, the pH was measured to assess whether the fermentation process altered this quality parameter. The measured pH values are shown in Table 3.2 and Table 3.4, respectively for *T. delbrueckii* and *Sch. pombe* fermentations. The initial pH values measured varied between 3,43-3,64 respectively, and are identical with those reported in the literature regarding orange substrate before fermentation (Corazza, Rodrigues and Nozaki, 2001; Escudero-López *et al.*, 2013). As observed in Tables 3.2 and 3.4, and also noted by other authors (Okunowo, Okotore and Osuntoki, 2005; Escudero-López *et al.*, 2013), the pH of orange juice tended to decrease after fermentation, possibly due to the production/transformation of organic acids, which can reshape the sensory properties of the juices. Regarding the fermentation by *T. delbrueckii*, (Table 3.2) an insignificant decrease of the initial pH was observed (only 0,04 decrease). *Torulaspora delbrueckii* is reported to have an influence on wine quality performing fermentations with positive effects in aroma and taste. Also, this *non-Saccharomyces* yeast is described for its lower production of acetaldehyde, ethyl acetate, glycerol, acetoin, and acetic acid, and when used in mixed or sequential fermentations with *S. cerevisiae*, allows to model volatile acidity (Ciani and Picciotti, 1995; Bely *et al.*, 2008; Loira *et al.*, 2015). On the other hand, “fresh” orange juice after fermentation by *Sch. pombe* exhibited a reduction of 0,42 pH units, reaching a much lower pH value than that achieved after fermentation by *T. delbrueckii* (Table 3.4 and Table 3.2). Species that constitute the genus *Schizosaccharomyces* are limited for winemaking use, since some strains produce unsatisfactory sensory deviations, namely high production of acetic acid (a strain-dependent characteristic), which is responsible for increasing volatile acidity (Loira *et al.*, 2015; Mylona *et al.*, 2016). Beyond this, *Schizosaccharomyces pombe* has the ability to transform the malic acid into ethanol due to their particular metabolism of maloalcoholic fermentation, which also impacts acidity during fermentation and is important for winemaking (Minnaar *et al.*, 2017). With these facts in mind, it was possible to hypothesize (although no measurements have been made) that the pH of the juice fermented by *Sch. pombe* has decreased due to the production of acetic acid, and, on the other hand, since *Torulaspora delbrueckii* is noted for its lower production of acids, the pH of the juice remained for that reason practically unchanged after fermentation.

For curiosity and since the substrate was available in the laboratory for alternative studies, another fermentation, this time using clarified apple juice, was performed by *Sch. pombe*. The fermentation and growth profiles in the clarified apple juice are depicted in Figure 3.3. In Figure 3.3-(B) is observed that, similarly to the fermentation performed by *Sch. pombe* in “fresh” orange juice, apple juice was fermented with a delay at the fermentation onset of approximately 14 hours. However, during this fermentation delay, cell growth did not appear to be harmed, since the cells entered into exponential phase immediately after inoculation into apple juice. Also, in Figure 3.3-(A) is depicted that the fermentation process occurred when cell growth was in exponential and stationary phase. This result was similar to that reported for apple juice fermentations conducted with commercial *S. cerevisiae* (Herrero *et al.*, 1999), and for the fermentation of natural cashew apple juice with *Acinetobacter calcoaceticus* (Rocha *et al.*, 2006).

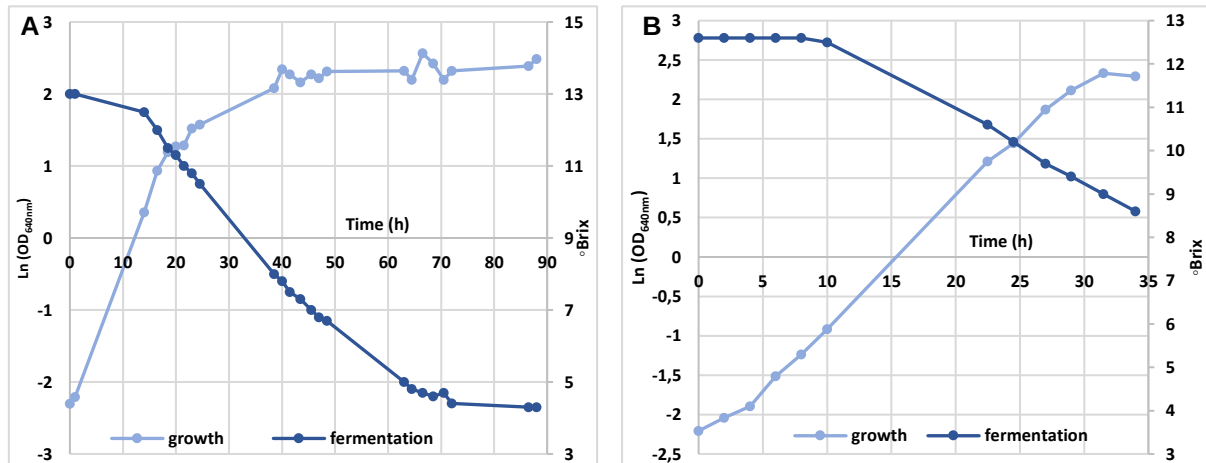


Figure 3.3- Fermentation profiles of *Sch. pombe* in apple juice. (A): Fermentation profile of *Sch. pombe* in apple juice during 88 hours. **(B):** Profile of the first 34 hours of the fermentation process with *Sch. pombe* in apple juice. Each figure represents an independent biological assay. In the main axis is represented the $OD_{640 nm}$ of the culture during the fermentation process and in the secondary axis the corresponding Brix degree.

This outcome made us wonder how cell growth proceeded during the delay in the onset of “fresh” orange juice fermentation. Hence, during the first stage of juice fermentation, YPD plates were made for subsequent CFUs counting, from the moment of cell inoculation to the fermentation onset. The growth profile obtained is presented in Figure 3.4. The fermentation dynamics in orange juice was different from the apple juice fermentation. While in apple juice no growth inhibitor appears to be on substrate (since cells started to grow immediately), in orange juice the cells remained in the lag phase and then, after they reached a sufficient number of cells, the fermentative process started (Figure 3.4 and Figure 3.3-(B)). This outcome proved to be very important, since not only allowed us to conclude that in fact the delayed fermentation of orange juice was due to a deferral in cell growth, but also allowed us to outline experimental evolution approaches for *Sch. pombe*.

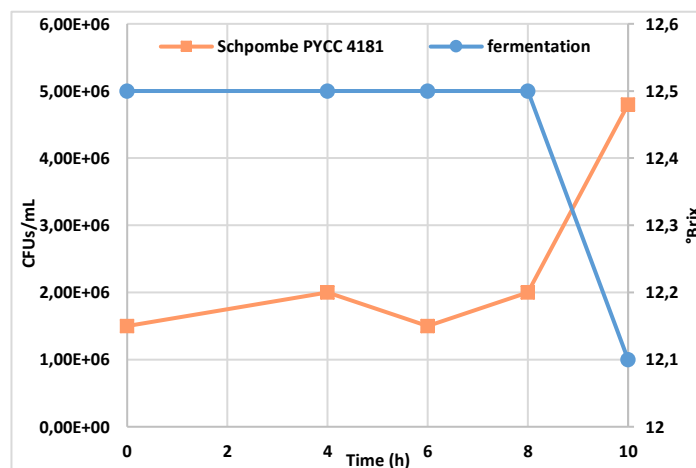


Figure 3.4- *Sch. pombe* growth (CFUs/mL) in “fresh” orange juice from the moment of cell inoculation to fermentation onset. The results of the calculated CFUs/mL are represented in the main axis and in the secondary axis the corresponding Brix degree.

Also, the initial and final cell concentration in the apple juice fermentation was measured, and the fermentation and growth rates were calculated (Table 3.5). According to the results described in

Table 3.5, it was possible to conclude that *Sch. pombe* grew about two orders of magnitude from the moment of inoculation, as observed in the fermentations with orange juice using the same yeast. On the other hand, the apple juice fermentation rate was more than 3 times slower than that of orange juices when fermented by *Sch. pombe* (Table 3.5 and Table 3.3).

Table 3.5- Values of initial and final cell concentrations and growth/fermentation rates measured for apple juice fermented by *Sch. pombe*.

<i>Sch. pombe</i> Fermentation	Initial cell concentration (OD _{640nm})	Final cell concentration (OD _{640nm})	Fermentation rate (°Brix/h)	Growth rate (h ⁻¹)
apple juice	0,1	12	-0,17	0,17

Apple juice pH was measured before and after fermentation. The measured pH values are shown in Table 3.6. The fermentation of apple juice by *Sch. pombe* promoted a decrease of approximately 0,41 pH units. This result was very similar to that obtained at the end of orange juice fermentation by the same yeast (Table 3.4 and Table 3.6). Apple juice contains large amounts of malic acid, which in turn may be metabolized by *Sch. pombe*. Since pH monitoring was not performed, it is not known whether during the fermentative process the pH value increased due to malic acid consumption. However, since this species is reported to produce high levels of acetic acid, the decrease in final pH may be due to accumulation of acetic acid in the apple juice (Herrero *et al.*, 1999; Loira *et al.*, 2015; Minnaar *et al.*, 2017).

Table 3.6- pH values of apple juice before and after fermentation by *Sch. pombe*

<i>Sch. pombe</i> Fermentations	pH value
fermentation beginning	3,60
fermentation end	3,19

In challenging environments, namely industrial environments, long fermentations onsets are crucial drawbacks, and as such, should be prevented. In addition to being more prone to microbial contamination, they may also lead to large economic losses (El-Mansi, 2012). With that in mind, the main purpose of this study was to devise an experimental evolution approach to shorten the fermentation lag time, by improving the resistance of *Sch. pombe* and *T. delbrueckii* to the orange EO present in the juice.

3.2. Influence of orange essential oils in yeast growth

Since it was previously concluded that yeast growth during orange juice fermentations was affected by the presence of orange EO, *Sch. pombe* and *T. delbrueckii* were cultured in YPD/YNB liquid and solid medium supplemented with increasing orange EO concentrations. The microbial growth was monitored to elucidate in what way orange EO disrupted cell growth.

3.2.1. YPD medium

The growth curves in YPD liquid medium in the presence of different orange EO concentrations are depicted in Figure 3.5. Table 3.7 shows the effects of various orange EO concentrations on the duration of the lag phase, and growth rate of *T. delbrueckii* and *Sch. pombe* in YPD liquid medium.

The results depicted in Figure 3.5-(A) and (B) show that, in both species, lag phases were lengthened by increasing orange EO concentrations in the medium, particularly from concentrations higher than 200 mg/L. It was also possible to note that in YPD liquid medium, *Sch. pombe* was the species most sensitive to orange EO, not only because it had longer lag phases compared to *T. delbrueckii* in the presence of the same orange EO concentration (e.g., for the concentration of 200 mg/L, *Sch. pombe* exhibited a lag phase of approximately ≈12,6 hours, whereas *T. delbrueckii* in the same concentration only took ≈ 5 hours to start the exponential phase), but also because it achieved a lower number of cells, more evident for concentrations higher than 200 mg/L (Figure 3.5-(B) and Table 3.7).

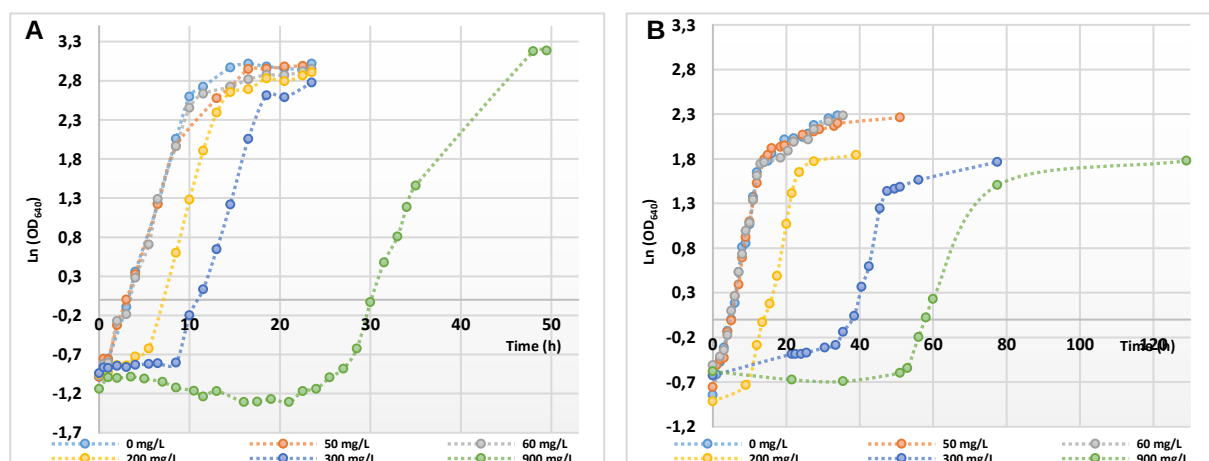


Figure 3.5- Growth assays of *T. delbrueckii* and *Sch. pombe* in YPD liquid medium in the presence of increasing concentrations of orange EO. The appropriate volumes of orange EO were added immediately after the inoculation of (A) *T. delbrueckii* and (B) *Sch. pombe* in the YPD liquid medium.

According to Figure 3.5-(A), it was observed that *T. delbrueckii* growth was practically undisturbed in the presence of orange EO concentrations of up to 60 mg/L. Moreover, with increasing orange EO concentrations, *T. delbrueckii* growth rates were similar to the growth rate in orange EO-free medium, except at the highest tested concentration (900 mg/L), which showed a decrease, reaching a growth rate of ≈ 0,3h⁻¹ (Table 3.7). Concerning *Sch. pombe* growth, it was not only observed that the cells grew in the orange EO-free YPD medium at half the rate compared to *T. delbrueckii* in the same medium, but also that they remained undisturbed with increasing EO concentrations, except at the two highest concentrations (300 and 900 mg/L) which showed a growth rate disturbance represented by a 0,1h⁻¹ decrease. In contrast to *T. delbrueckii* performance, *Sch. pombe* growth was impaired at the lowest concentrations (50 and 60 mg/L) with an increase of approximately 1,5 hours in the length of the lag phase, compared to cell growth in YPD medium without orange EO (Table 3.7).

Table 3.7- Overview of lag phases and growth rates associated to each *T. delbrueckii* and *Sch. pombe* growth, in YPD liquid medium in the presence of increasing concentrations of orange EO. The microbial growth profiles corresponding to each result are illustrated in Figure 3.5-(A) and (B).

Strain	EO concentrations (mg/L)	μ (h ⁻¹)	<i>t</i> lag (h)
<i>T. delbrueckii</i>	0	0,4	0,8
	50	0,4	1,0
	60	0,4	1,0
	200	0,4	5,0
	300	0,4	8,6
	900	0,3	27,0
<i>Sch. pombe</i>	0	0,2	1,4
	50	0,2	3,0
	60	0,2	2,9
	200	0,2	12,6
	300	0,1	33,2
	900	0,1	52,1

Additionally, cell performance was also assessed in YPD solid medium for both yeasts. Therefore, *T. delbrueckii* and *Sch. pombe* cells were diluted and spotted in YPD solid medium with the same orange EO concentrations used to perform the YPD liquid assays. The results obtained are illustrated in the Figures 3.6-(A) and (B), respectively. Apparently, contrary to what was expected, yeast growth in YPD solid medium was not affected in the presence of the various concentrations of orange EO. Moreover, comparing to *Sch. pombe*, it was possible to verify that *T. delbrueckii* have a more vigorous growth, corroborating a detail observed in the Figures 3.5-(A) and (B), which is that in rich medium *T. delbrueckii* reaches higher ODs in comparison with *Sch. pombe*.

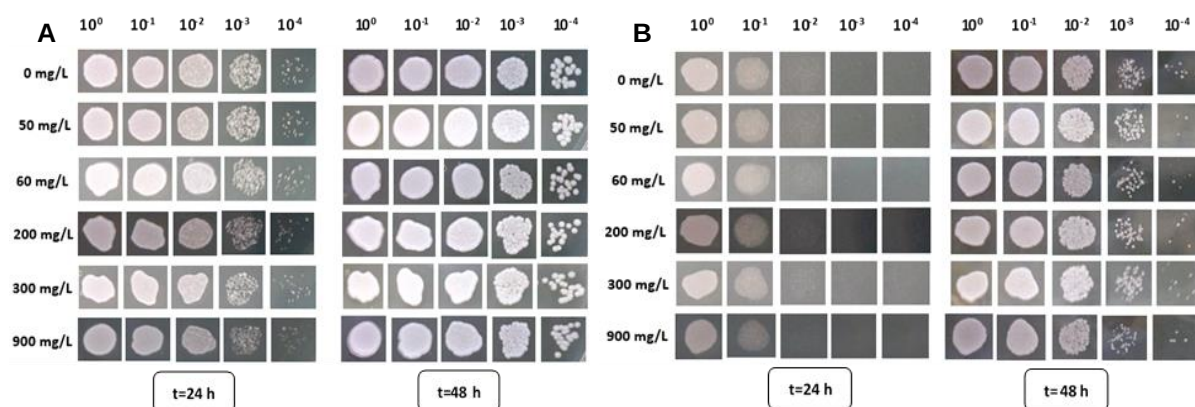


Figure 3.6- Spotting test on YPD agar plates with increasing amounts of orange EO. Growth of *T. delbrueckii* (A) and *Sch. pombe* (B) determined by the spotting test. The growth assays were inspected after 24 and 48 hours.

3.2.2. YNB medium

Since it was intended to proceed the experimental evolution in YNB liquid medium, the antimicrobial effects of orange EO in YNB medium were also analyzed. The growth curves performed by both yeasts are shown in Figure 3.7-(A) and (B). Additionally, the main results from the growth experiments were grouped in Table 3.8.

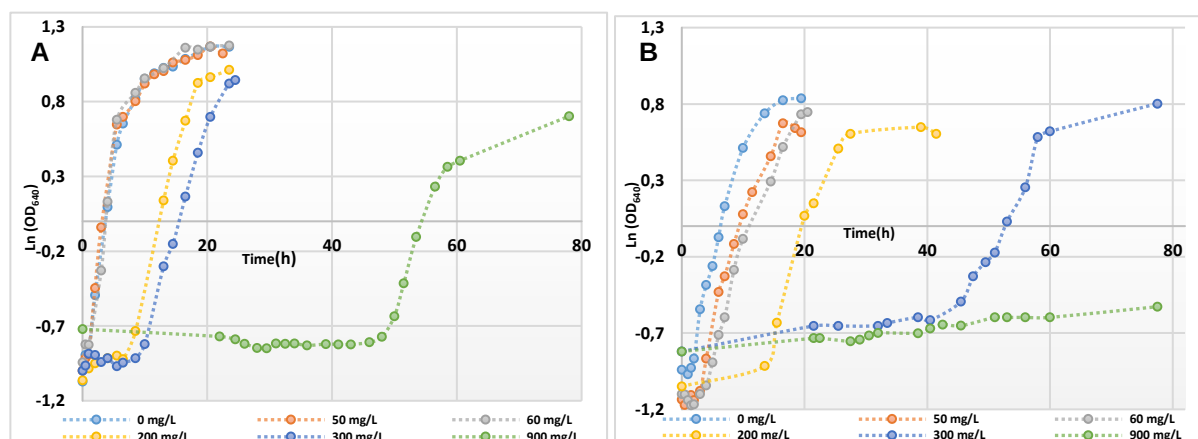


Figure 3.7-Growth assays of *T. delbrueckii* and *Sch. Pombe*, in YNB liquid medium in the presence of increasing concentrations of orange EO. The appropriate volumes of orange EO were added immediately after the inoculation of (A) *T. delbrueckii* and (B) *Sch. pombe* in the YNB liquid medium.

As in YPD liquid medium, *T. delbrueckii* growth in the presence of concentrations of up to 60 mg/L was practically undisturbed, since the growth rates and lag times occurred in the presence of these concentrations were very identical to those obtained during growth in YNB liquid medium without EO addition (Table 3.8). At the same time, it was observed not only that, *T. delbrueckii* growth rates were lower compared to the rates obtained in YPD liquid medium (Table 3.7 and 3.8) but also, that these same rates decreased after exposure to the highest orange EO concentrations (200-900 mg/L). The increase in lag times was slightly higher to that observed in YPD liquid medium, though at the highest orange EO concentration (900 mg/L) this increase was more pronounced, compared to that occurred in YPD liquid medium (Table 3.8 and Table 3.7). With these results, it was possible to conclude that orange EO were more deleterious for *T. delbrueckii* growth in YNB liquid medium, than in YPD liquid medium.

Table 3.8- Overview of lag phases and growth rates associated to each *T. delbrueckii* and *Sch. pombe* growth in YNB liquid medium in the presence of increasing concentrations of orange EO corresponding to each result are illustrated in Figure 3.7- (A) and (B).

Strain	EO concentrations (mg/L)	μ (h ⁻¹)	t lag (h)
<i>T. delbrueckii</i>	0	0,3	0,9
	50	0,3	0,9
	60	0,3	1,1
	200	0,2	6,5
	300	0,2	9,4
	900	0,1	47,8
<i>Sch. pombe</i>	0	0,2	1,0
	50	0,2	2,5
	60	0,2	3,8
	200	0,1	13,4
	300	0,1	45,0
	900	0,0	> 77,5

Sch. pombe was the species most sensitive to orange EO in YNB liquid medium, reflecting a more pronounced increase in lag times with progressive increase in orange EO concentrations, when compared to the growth in YPD liquid medium and *T. delbrueckii* (Table 3.8 and 3.7). Regarding the estimated growth rates, it was observed that the *Sch. pombe* grew in YNB liquid medium at a slower rate, compared to *T. delbrueckii* growth in the same medium. In the presence of concentrations of up to 60 mg/L, *Sch. pombe* growth rates were similar to those observed in the medium without orange EO, however, as orange EO concentrations increases, growth rates decreased by half. At the highest orange EO concentration (900 mg/L), *Sch. pombe* did not grow during the investigation time, revealing that the latter was a highly toxic concentration in YNB liquid medium.

Regarding the results attained during the assays in both liquid media, it was possible to elucidate that orange EO have more pronounced effects on the growth lag phases, than on the growth rates (Table 3.8 and 3.7). Other researchers, namely (Tserennadmid *et al.*, 2011), have observed similar results using malt extract medium with increasing concentrations of various EO, in particular from lemon, juniper, clary sage, and marjoram. These authors reported that antimicrobial activity of EO was reflected by extremely lengthened lag phases, although without disturbance in growth rates. Moreover, of all the yeasts tested, among them *Sch. pombe* MB-89 and *S. cerevisiae* MB-21, the *Sch. pombe* MB-89 growth was the most impaired in the presence of all EO tested (Tserennadmid *et al.*, 2011). Similarly, in bacteria, in particular *Escherichia coli* and *Staphylococcus aureus*, an increase in lag times was observed in the presence of increasing amounts of EO from *Thymus vulgaris*, and any disturbance in exponential phase growth rates was exhibited (Janssen, Scheffer and Svendsen, 1987).

Cell growth was also assessed in YNB solid medium with different orange EO concentrations. To this end, *T. delbrueckii* and *Sch. pombe* cells were diluted and spotted in YNB solid medium with the same orange EO concentrations to which cells were subjected in YNB liquid medium. The results are shown in Figures 3.8 and 3.9, respectively. As observed in the YPD solid medium assays, *T. delbrueckii* growth in YNB solid medium did not appear to be impaired in the presence of the various orange EO concentrations (Figure 3.8). Although *Sch. pombe* growth in the presence of orange EO concentrations from 50 mg/L to 300 mg/L was also not affected, at 900 mg/L its growth was impaired, since after 48 hours it was clearly visible that at the highest dilutions there was no growth, which was observable only after 108 hours (Figure 3.9).

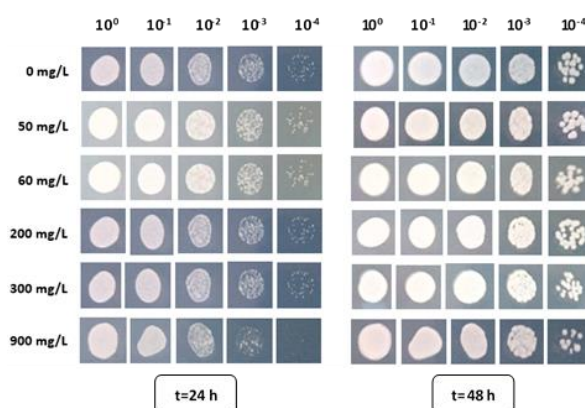


Figure 3.8- Growth of *T. delbrueckii* determined by the spotting test on YNB agar plates with increasing amounts of orange EO. The growth was inspected after 24 and 48 hours.

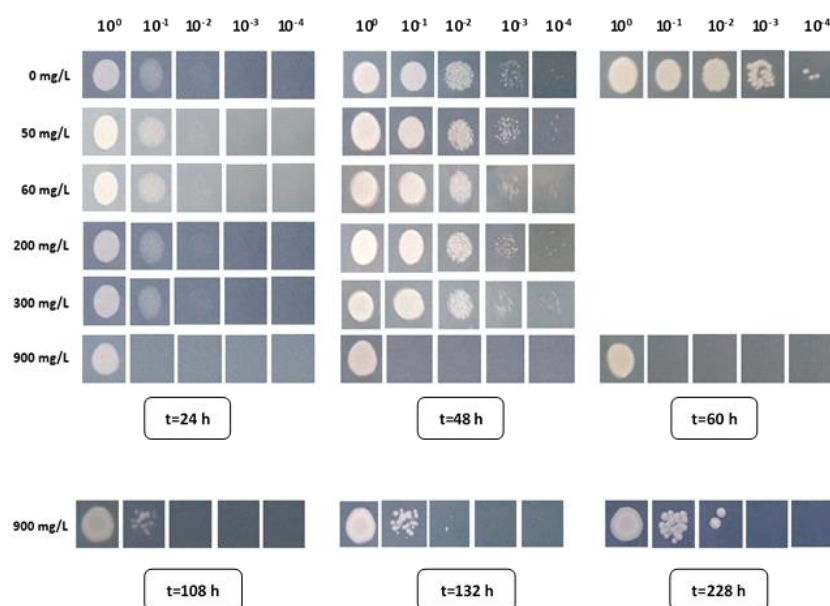


Figure 3.9- Growth of *Sch. pombe* determined by the spotting test on YNB agar plates with increasing amounts of orange EO. The growth was inspected after 24, 48, 60, 108, 132 and 228 hours.

Before conducting the evolution experiments, it was necessary to establish robust screening methods to distinguish presumably evolved strains. The spotting test performed in this study had as main objective select a range of orange EO concentrations suitable to be employed in an effective screening method, based on growth alterations in the presence of orange EO. It was possible to infer from the results obtained in both solid media that the yeasts did not present any alterations in their growth in the presence of several concentrations of orange EO (Figures 3.6-(A) and (B), 3.8 and 3.9). One possible explanation for this unexpected growth is that the spotted cells in the solid medium are placed both close to and on top of each other, thereby reducing the accessibility of the yeast to the orange EO, and thus allowing protected growth from the toxic effects conferred by them (Kwolek-Mirek and Zadrag-Tecza, 2014). Also, water (in-)solubility and orange EO evaporation are difficult to monitor, limiting orange EO diffusion through the agar medium and possibly leading to erroneous results. Therefore, under these conditions, the spotting test did not add useful information for the development of a good screening method to be applied after experimental evolutions. To overcome this cellular overlay, for both yeasts, spreading plate assays were performed in media with orange EO. These assays aimed to investigate possible differences in colony size when cells are exposed to orange EO, assuming that colonies with a larger diameter were derived from cells that could grow better in medium with a certain concentration of orange EO (Brennan *et al.*, 2015). The screening method applied in this study is described in chapter “2.6 Screening of evolved mutants”, and the results after its application are discussed in chapter “3.6. Experimental evolutions, screening, and evaluation of the fermentative performance of mutants”.

Another growth assay was performed in YNB liquid medium, to evaluate if the timing of the orange EO addition had a different impact on cell growth. Hence, unlike the previous growth assays in which EO were added at the moment of cell inoculation, in this assay orange EO were added in mid-exponential growth. The results are depicted in Figure 3.10. After the addition of 50 and 60mg/L of

orange EO, *T. delbrueckii* growth progressed without any disturbances, accompanying the growth of the culture to which no orange EO was added. On the other hand, at 200-900mg/L, *T. delbrueckii* growth was inhibited (Figure 3.10). These results corroborated those previously obtained when the orange EO were added at the moment of cell inoculation.

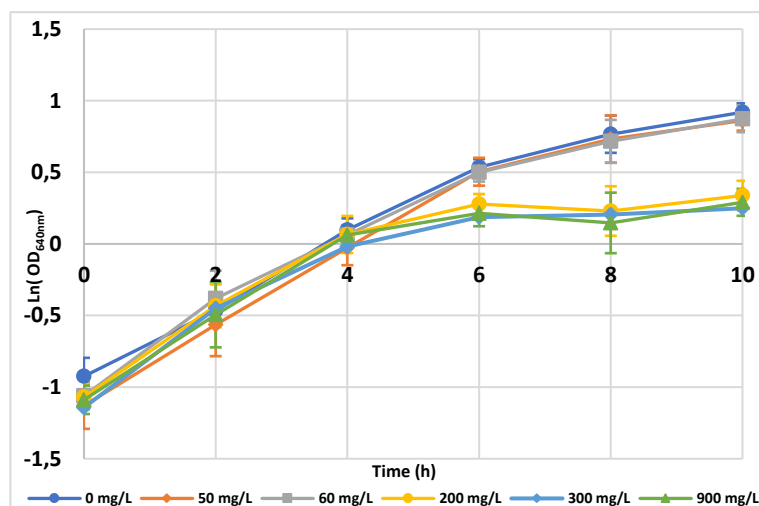


Figure 3.10- Growth assay of *T. delbrueckii* in YNB liquid medium after addition of orange EO in mid-exponential growth. Results are from three independent experiments (n=3), and therefore respective standard deviations, as well as the mean of the measurements are represented in the graph.

EO are composed by a variety of natural compounds present in different concentrations. Hence, their biological properties are significantly determined by the components that are present at high concentrations (Bakkali *et al.*, 2008). Since the volatile orange EO present in orange fruits usually contain large amounts of D-Limonene (between 70-96%), our growth results are in agreement with those of (Subba, Soumithri and Rao, 1967; Grohmann, Baldwin and Buslig, 1994; Wilkins, Widmer and Grohmann, 2007; Chee, Kim and Lee, 2009; Settanni *et al.*, 2012a; Ünal *et al.*, 2012) who reported a growth impairment in the presence of D-Limonene and orange EO.

The following step was to elucidate cell viability during the lag phases, in order to understand if the lengthened lag phases are due to the possible occurrence of cell death in the presence of orange EO.

3.3. *T. delbrueckii* cell viability

Cell viability may be determined by several methods based on different cellular parameters. They are usually grouped into distinct categories between them, methods based on the ability of cells to reproduce on liquid and solid culture medium, such as CFUs, measurement of growth inhibition zone, spotting test and OD measurement in liquid media; methods based on microscopic examination of stained cells, for instance methylene blue, propidium iodide, rhodamine, among others; and lastly, methods based on observations of several aspects of the physiological state of cells, more directed for measurements of cell vitality (Bapat *et al.*, 2006; Kwolek-Mirek and Zadrag-Tecza, 2014).

In this study, cell viability during *T. delbrueckii* growth in the presence of various orange EO concentrations was evaluated, using two distinct methods from two different categories, namely the

analysis of the CFUs and the methylene blue staining. Before using these methods, it was necessary to define the viable cell concept in both procedures. Therefore, regarding the CFUs method, it was assumed that the development of one colony represent the presence of one viable cell in the sample, since it was able to multiply and give rise to daughter cells (assuming that one colony originates from only one cell). Regarding staining with the methylene blue, the colorless cells were considered viable as they could reduce the dye. Firstly, was evaluated the occurrence of cell death during *T. delbrueckii* growth in YNB liquid media in the presence of various orange EO concentrations, added immediately after cell inoculation. The viability results of growth assays are depicted in the Figure 3.11.

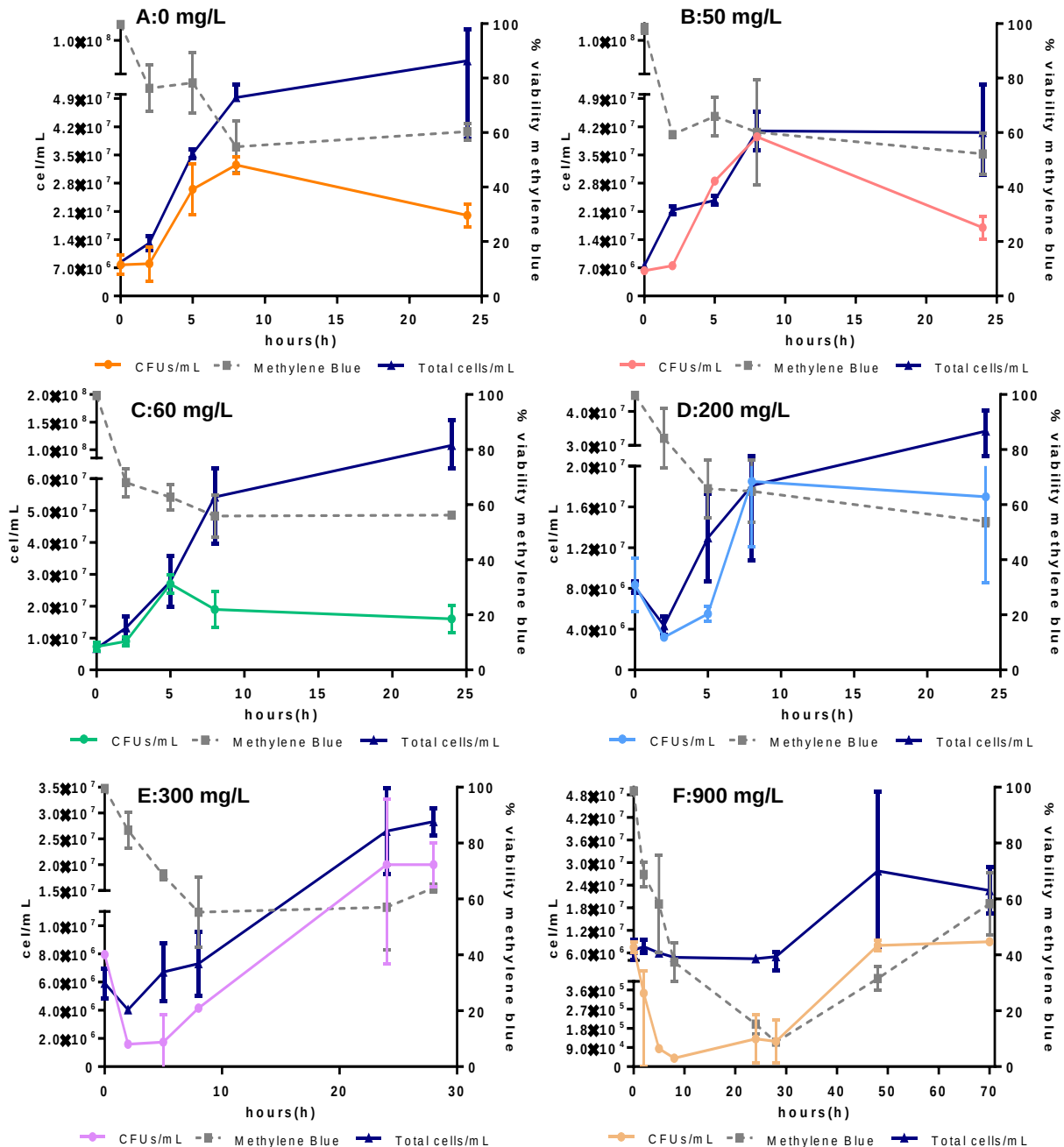


Figure 3.11- *T. delbrueckii* cell viability estimated by methylene blue staining and CFUs method during growth in YNB liquid media in the presence of various orange EO concentrations, added immediately after cell inoculation. In the main axis are represented the CFUs and total cells over time (t=0,2,5,8,24,28,48 and 70) and in the secondary axis the corresponding percentage of viability by staining with methylene blue. The results are from three independent experiments (n=3), and therefore respective standard deviations, as well as the mean of the measurements are represented in the graphs.

In the culture to which no orange EO was added, it was observed that until up to 8 hours of growth, the number of total cells increased exponentially, subsequently reaching a stationary phase where the number of total cells remained stable (Figure 3.11-(A)). However, considering the number of CFUs over the same time, it was possible to conclude that, not only the number of viable cells was smaller than the number of total cells counted, but also that occurred a lag phase followed by an exponential phase with the duration of at least 5h, and finally an stationary phase. This difference between the counted CFUs and the number of total cells indicated that there were unviable cells in the culture. The viability percentage obtained by microscopic readings with the methylene blue staining confirmed that loss of cell viability. For instance, at time $t=5h$ approximately $2,65 \times 10^7$ CFUs/mL and $3,59 \times 10^7$ total cells/mL were counted. This difference indicates that roughly 73% of the culture was viable, which was in accordance with the result of viability percentage obtained by methylene blue staining ($\approx 78\%$). Therefore, although there was some loss of cell viability over time, the growth of *T. delbrueckii* in YNB liquid medium without orange EO was very similar to what was portrayed in the previous growth assays monitored by OD_{640nm} measurements, shown in Figure 3.7-(A).

However, the good correlation between the two methods that had been observed in the orange EO-free culture was interrupted. It was noticed that cell viability estimated by methylene blue staining was often higher, than that observed by the analysis of the number of CFUs (Figure 3.11). For instance, with 900 mg/L of orange EO at $t=5$ (Figure 3.11-(F)), the number of counted CFUs was $\approx 8,5 \times 10^4$ CFU/mL, and the estimated number of total cells remained similar to that counted at the beginning of the assay ($\approx 7,92 \times 10^6$ CFU/mL). This estimate means that approximately only 1% of cells were viable at that moment, in contrast with the $\approx 58\%$ obtained by staining with methylene blue.

The mechanism of action of methylene blue depends on the properties of the plasma membrane. The compounds present in the dye are able to enter in living and dead cells. Subsequently, living cells may reduce the dye by transmembrane reductases, and remain colorless. In this work, during microscopic observations, living and dead cells were often difficult to differentiate. Considering this fact and similar difficulties reported by other authors, this method proved to be error prone, which may have led to an overestimation of cell viability and uncertain results (Vaija, Ghommidh and Navarro, 1993; Kwolek-Mirek *et al.*, 2011).

Occasionally, it has been reported that the toxic effects of some compound or stress factor may not manifest themselves in cell death, but in morphological and/or metabolic alterations that impair cellular vitality and may result in reproductive disability. (Kwolek-Mirek *et al.*, 2011) reported one of those cases, where cells exposed to acrylamide compounds were unable to reproduce, however, remained viable (Kwolek-Mirek *et al.*, 2011). Although the term “viable cell” is controversial and underwent some evolution, in this work a viable cell was considered to be a cell that could give rise to daughter cells. Therefore, all the final conclusions inferred from the viability assays were based mostly on the CFUs method, since it is a robust test and in fact allows to count the cells capable of reproducing.

Throughout the viability assay, to perform the plates for CFUs counting, serial dilutions were carried out. Hence, in order to complement the information in Figure 3.11, 5 μ L of each dilution were reused and spotted on YPD plates. The results are depicted in Figure 3.12.

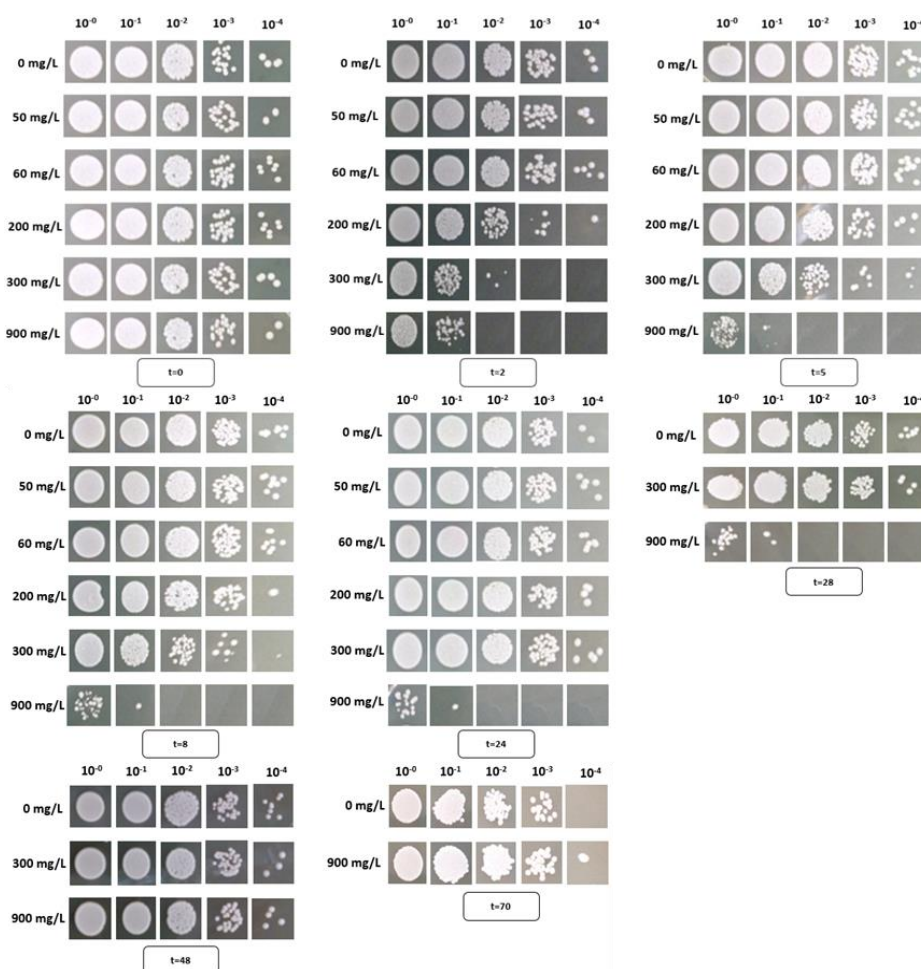


Figure 3.12- *T. delbrueckii* viability determined by the spotting test. Cells were incubated with several orange EO concentrations in liquid medium and subsequently grown on YPD plates. The results shown correspond to 48 hours of growth.

Succinctly, it was possible to conclude that, when *T. delbrueckii* were exposed to the two lowest orange EO concentrations (50 mg/L and 60 mg/L), no growth impairment occurred, since no decrease in CFU/mL compared to the initial cell concentration was observed (Figure 3.11-(B)-(C) and Figure 3.12). On the other hand, from 200 mg/L to 900 mg/L, losses in cell viability during the lag phase were observed (Figure 3.11-(D) to (F) and Figure 3.12). Moreover, at the highest orange EO concentration (900mg/L), cellular concentrations decreased more sharply during the lag phase, reaching values of two orders of magnitude lower than the initial cell density of the inoculum. This fact is clearly illustrated in Figure 3.12, since at t=5 to t=28 no growth was observed at the highest dilutions. These results confirmed that the lengthened lag phases observed in culture media after addition of orange EO were due to a loss of cell viability. In line with this, in orange juice fermentations with higher orange EO concentrations, the viability of the inoculum was compromised, and lengthy fermentation onsets occurred.

Additionally, cell viability was also assessed when the cells were exposed to the various orange EO concentrations, at the mid-exponential phase of growth. The results are depicted in Figure 3.13. For the two lowest concentrations (50 and 60 mg/L), an increase in CFUs/mL was observed, accompanying the growth of cells that were not exposed to orange EO (Figure 3.13-(A) to (C)). On the other hand, for the highest concentrations (200 to 900 mg/L), a decrease in CFUs/mL was observed (Figure 3.12-(D) to (E)). These results suggest that the loss of cell viability in the presence of high concentrations of

3. RESULTS AND DISCUSSION

orange EO, namely 200, 300 and 900 mg/L, was responsible for the growth arrest observed after the addition of orange EO in mid-exponential phase (Figure 3.10).

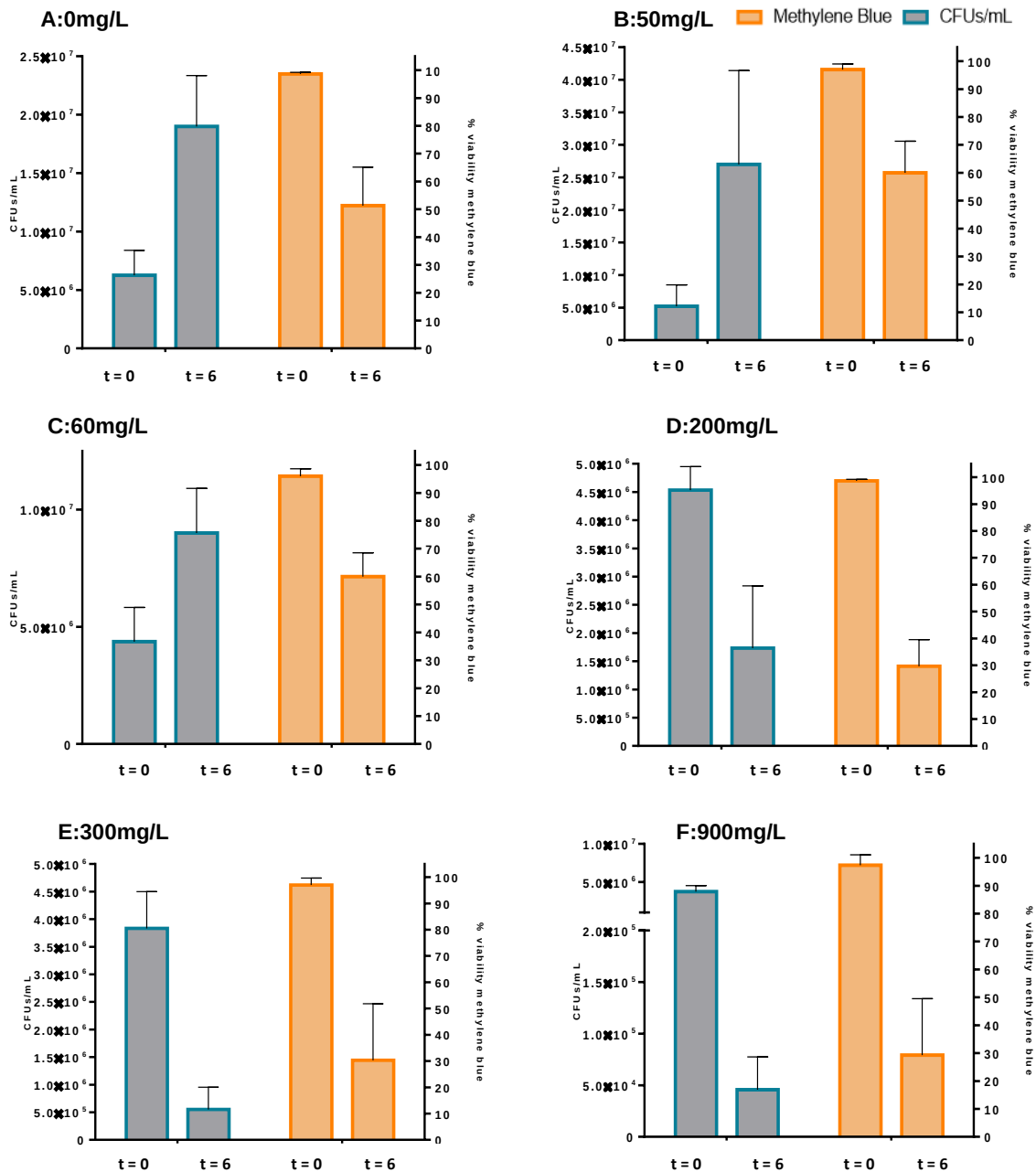


Figure 3.13- *T. delbrueckii* cell viability estimated by methylene blue staining and CFUs in YNB liquid medium after 2 hours of exposure to various orange EO concentrations, added in mid-exponential phase of growth. In the main axis are represented the CFUs/mL and in the secondary axis the corresponding percentage of viability by staining with methylene. Results are from three independent experiments (n=3), and therefore respective standard deviations, as well as the mean of the measurements are represented in the graphs.

It was also possible to observe that the cells in the mid-exponential phase were more sensitive to orange EO, since lower CFUs/mL were measured, in comparison with the cell concentrations obtained after exposure to orange EO immediately after cell inoculation (Figure 3.12-(D) to (E) and Figure 3.13-(D) to (E)). This sensitivity in the exponential phase has been reported, for example in the case of heat shock, when compared to the stationary phase. In the lag phase, yeast metabolism and protein synthesis are activated for the cells to adapt to their surroundings. When the cells are in the

exponential phase, their metabolism is directed for rapid growth, and thus resistance mechanisms are sacrificed. In addition, during the exponential phase, the cell wall in the budding sites is much less thick. Therefore, these vulnerabilities allow the orange EO compounds to enter in the cells more efficiently, possibly leading to greater cell damages (Elliott and Fletcher, 1993; Bakkali *et al.*, 2008)

Previous studies by other researchers, using various methodologies, have demonstrated a significant relation between orange EO or limonene addition, and antimicrobial activity. Similar to the results obtained in this study by CFUs analysis, after addition of 200 mg/L of limonene in the mid-exponential phase of *S. cerevisiae* growth, (J Liu *et al.*, 2013) reported a loss of cell viability of approximately 50%. Likewise, (Subba, Soumithri and Rao, 1967) demonstrated a total loss of cell viability during the growth of *S. cerevisiae*, *Zygosaccharomyces mellis* and *Torula utilis*, on solid media containing 500 and 1000 ppm of orange EO. Other researchers, using propidium iodide, performed a viability assessment of *S. cerevisiae* cells after an exposure for two hours to limonene, and contrary to what is reported by other authors, no loss of cell viability was observed. In this case, the highest limonene concentration tested was 0,6 mM, which corresponds to approximately 80 mg/L. The most similar concentration tested in our work (60 mg/L) also showed no loss of cell viability (Brennan *et al.*, 2012).

The literature reveals a diversity of opinions about the causes of loss of cell viability in the presence of EO. Due to the variety of chemical structures present in the natural extracts, antimicrobial effects are not attributed exclusively to a mechanism, but to a progressive cascade of cellular events occurring throughout the cell. Orange EO are mostly composed by limonene monoterpenes, and due to their lipidic character it is assumed that the main antimicrobial action of these compounds occurs at the level of the microbial plasma membranes and cell walls, since they may pass through them causing damage and disturbance to their native properties (Bakkali *et al.*, 2008; Nazzaro *et al.*, 2013).

Most studies support the premise that limonene cytotoxicity occurs at the plasma membrane, a structure that encloses the cells with crucial functions in cell viability maintenance. Several authors have observed in yeast and bacteria that the interaction of orange EO and limonene compounds with the lipidic environment of the plasma membrane destabilizes the profile of fatty acids (ratio between saturated and unsaturated fatty acids), increasing its permeability (Prashar *et al.*, 2003; Di Pasqua *et al.*, 2006, 2007). This permeabilization is associated with subsequent events of leakage of cell content and dissipation of the proton motive force, thus affecting various energy processes including solute transport, and ATP synthesis and hydrolysis (Sikkema *et al.*, 1994). Moreover, in other studies, were reported damage in the proteins embedded in the membranes, and also cytoplasm coagulation phenomena, due to the mismatch between permeability changes and poor transport of ions and other molecules (Sikkema *et al.*, 1994). In a detailed study, conducted by (Sikkema, de Bont and Poolman, 1994), it was concluded that the hydrocarbons that constitute the EO, may accumulate in the membranes leading to disturbances in the phospholipids. Damage in the membranes of *S. cerevisiae* organelles has also been reported, as a result of increased production of reactive oxygen species (ROS) (Jidong Liu *et al.*, 2013), and inhibition of oxygen consumption, in the case of mitochondrial membranes (Uribe and Pena, 1990).

Although not often reported, the cell wall also appears to be mentioned as being a sensitive target to the antimicrobial action of orange EO. Authors that report this fact observed morphological changes by scanning electron microscopy, due to cell wall deterioration in bacterial cells, in particular in *E. coli* O157:H7, *Salmonella enterica* serovar *Typhimurium*, *Pseudomonas fluorescens*, *Brochothrix thermosphacta* and *Staphylococcus aureus*, after exposure to 0,2M of limonene, eugenol, carvacrol and thymol (Di Pasqua et al., 2007). (Brennan, Krömer and Nielsen, 2013) also observed, using a dye that preferentially binds to chitin, an impairment in the *S. cerevisiae* cell wall integrity after two hours of exposure to 107 mg/L of limonene, indicating that this essential component of the cell wall may be a primary target of limonene action.

Thereby, the loss of cell viability observed in *T. delbrueckii* growth after exposure to orange EO, may be due to membrane and/or cell wall damage, which consequently may lead to physiological changes and compromise microbial metabolism, resulting in cell death. Since *T. delbrueckii* cells have been subjected to a mixture of various natural compounds, synergistic effects may also have occurred.

In the course of the *T. delbrueckii* cell viability assessment assay, in particular during the CFUs counting, interesting morphological differences were observed between the colonies that grew on the YPD plates. The colonies that were inoculated after two hours of exposure to 60 mg/L of orange EO in YNB liquid medium, exhibited a homogeneous morphology (Figure 3.14-(A)-60 mg/L). Nevertheless, after exposure to a concentration of 200 mg/L or higher, the colonies had a heterogeneous appearance, namely a mixture of “large” and “small” colonies, as depicted in Figure 3.14-(A)-200 mg/L. Two hypotheses could be put forward, namely either the different sizes of the colonies reflected different physiological stages of adaptation to orange EO, or that the large colonies harbored mutations conferring resistance to orange EO. To distinguish between these two hypotheses, each different colony was pre-grown overnight, re-inoculated in YPD liquid medium supplemented with 200 mg/L of orange EO and subsequently, after two hours, the cells were spread in YPD solid medium (Figure 3.15-(A)). Figure 3.15-(B) depicts the morphology of the colonies derived from “large” and “small” colonies after 48 hours and after 1 week. An examination of the plates 48 hours after its inoculation revealed that after two hours of exposure to 200 mg/L, the descendants of both “large” and a “small” colonies gave rise to similar numbers of colonies with identical heterogeneity i.e., both distinct colonies after exposure to orange EO gave rise to “large” and “small” colonies in the same proportion (Figure 3.15- (B)). After a week, the colonies that were signed as “small” reached the size of the colonies that are already “large”, and therefore it was possible to conclude that the appearance of “small” colonies was due to the presence of debilitated cells with a slower growth, due to exposure to high concentrations of orange EO.

It has been reported that exposure to an environment characterized by stressful conditions, may cause changes in cell proliferation, namely a transient arrest of the yeast cell cycle, frequently in phase G1 (e.g. as the case of nutrient starvation). This arrest in the cell cycle may prevent damage in the most vulnerable phases of the cell cycle, namely at the S (DNA duplication) and M (mitosis) phase, and consequently allow cells to adapt while in G1 phase. After adaptation to the unfavorable environment, the cells resume proliferation (Hohmann, 2003). If this response mechanism occurred after exposure to orange EO, then it is possible that cells that gave rise to “small” colonies were in the process of adaptation to the imposed stress, while “larger” colonies derive from cells that were already adapted.

Also, in line with this, when cells from colonies with different morphologies are harvested and reinoculated in YPD solid medium, all colonies are large (Figure 3.14-(B)). Taking all these observations into account, it can be ruled out that the cause for the heterogeneity was genetic.

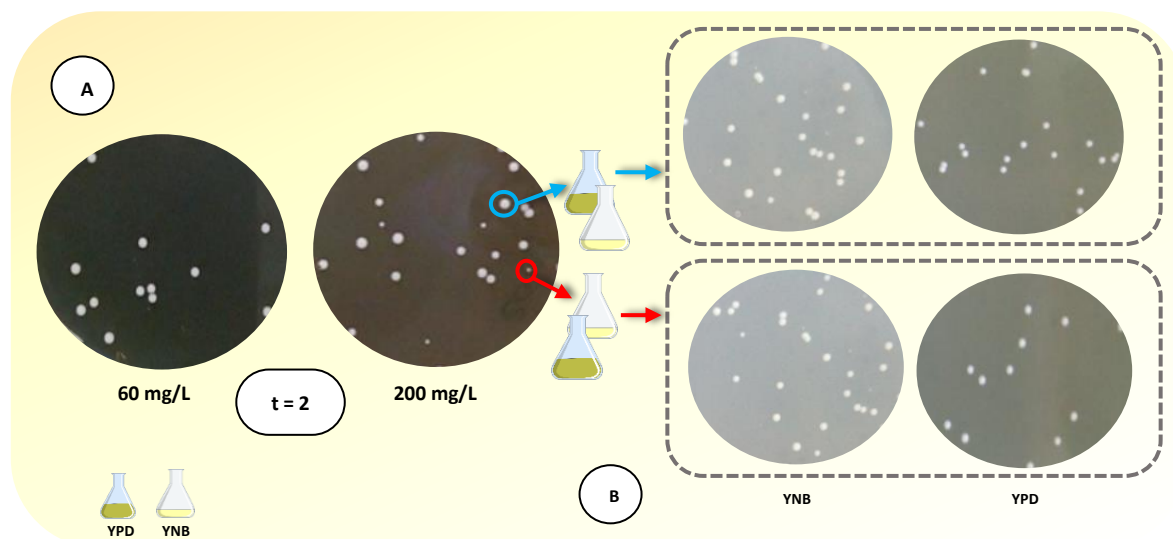


Figure 3.14- Growth of a “large” and “small” colony in YPD and YNB liquid medium without addition of orange EO. A: Visual aspect of the colonies grown on the YPD plates from CFUs viability assay (exposure to 60 and 200 mg /L of orange EO at t=2). Each colony, one "large" represented by the blue circle and arrows and one "small" represented by the red circle and arrows, was inoculated in YNB and YPD liquid medium. **B:** Visual appearance of colonies after each growth in YNB and YPD liquid medium.

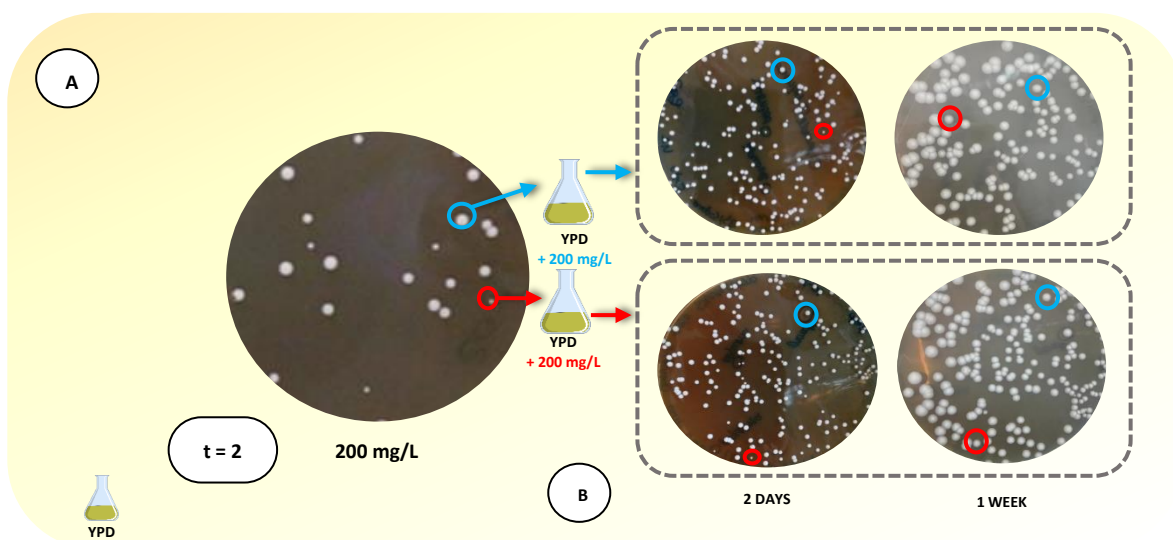


Figure 3.15- Growth of a “large” and “small” colony in YPD liquid medium with 200 mg/L of orange EO. A: Visual aspect of the colonies grown on the YPD plates from CFUs viability assay (exposure to 200 mg /L of orange EO at t=2). Each colony, one "large" represented by the blue circle and arrows and one "small" represented by the red circle and arrows, was inoculated in YPD liquid medium supplemented with 200 mg/L of orange EO. **B:** Visual appearance of colonies after growth in YPD liquid medium with 200 mg/L of orange EO after 2 days and 1 week.

3.4. Transformation of toxicological properties of orange essential oils

During this study, a change in the toxicological properties occurred in the orange EO stock that had been used to conduct the previous growth assays, which consisted in an increase in toxicity. To continue the last experiments, we used another orange EO stock that was stored at 4°C (EO2). The

increase in orange EO toxicity was probably due to limonene oxidation phenomena that may have occurred due to routine handling of the Schott flask. Under aerobic conditions, limonene may oxidize and be transformed into limonene hydroperoxide, cis-and trans-limonene-1,2-oxide, cis-and trans-carveol, and carvone (Nilsson *et al.*, 1996; Chubukov *et al.*, 2015). These oxidized forms of limonene are much more toxic than limonene itself, since they are much more water soluble (e.g., $S_{1,2\text{-epoxide}}=137$ mg/L) and have low volatility.

Figure 3.16 shows the chronological order of the assays, as well as the oils used in each experiment, throughout the year. *T. delbrueckii* growth assays were conducted to assess the toxicity of the different EO samples, as described in Table 3.9. The growth assays are depicted in Figure 3.17.

Table 3.9- Description of the orange EO used in this study.

EO	Description
EO	EO in their initial state used in the previous growth assays
EO2	Stored orange EO used to give continuity to the assays
EO3	EO deteriorated, possible oxidized form of EO. EO were renamed EO3 as soon as a change in the toxicological properties of EO was detected.

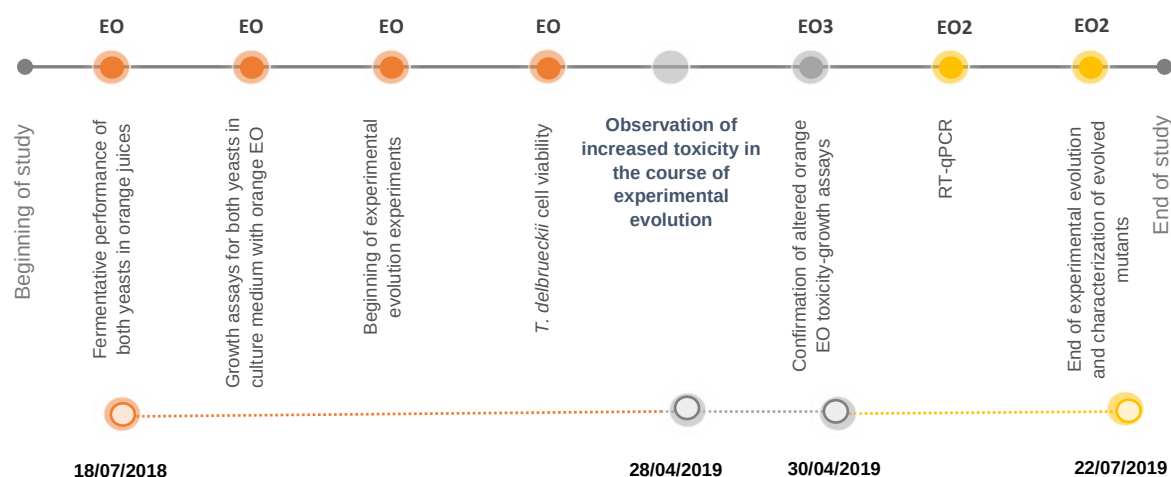


Figure 3.16- Timeline of the assays conducted throughout the year. The dates shown refer to the beginning of the corresponding event illustrated in the timeline. (orange circles: EO; gray circles: EO3; yellow circles: EO2). Each orange EO is described in the Table 3.9.

According Figure 3.17, it was possible to conclude that the oils used to continue the last experiments (EO2) were more toxic than the EO used in the previous growth assays (before a change in toxicological properties was detected). It was also observed that *T. delbrueckii* did not grow in the presence of 100 mg/L of EO3, i.e., of deteriorated EO (Figure 3.17).

It is important to note that, since the oxidation phenomena occur over time, it is possible that some previous results that emerged from exposure to EO (orange zone in the timeline represented in Figure 3.16) may have been influenced due to this increased toxicity. Furthermore, it cannot be guaranteed that the various orange EO exposures have always been under the same toxicity conditions.

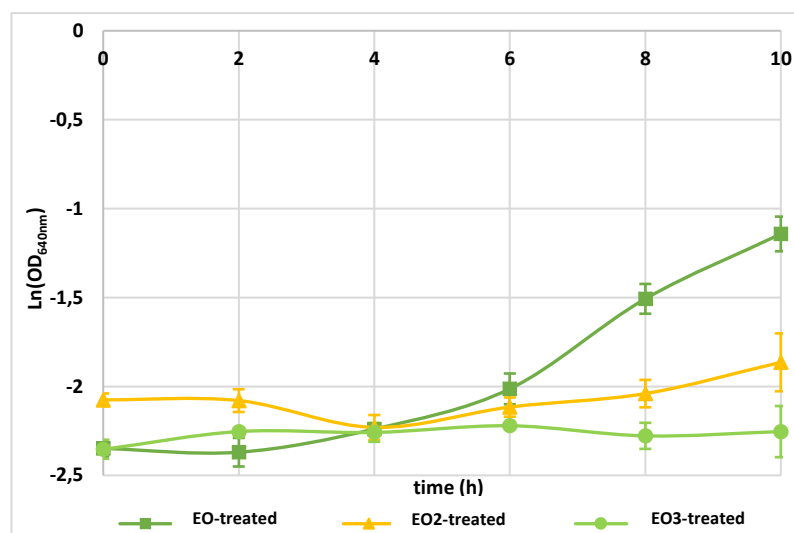


Figure 3.17- Growth of *T. delbrueckii* in acidified YNB liquid medium with 100 mg/L of each orange EO sample, added immediately after cell inoculation. The green squares represent the growth of *T. delbrueckii* in acidified YNB liquid medium complemented with 100 mg/L of EO in their initial state. The triplicates were performed from 19/11/2018 to 22/11/2018); the yellow triangles represent the growth of *T. delbrueckii* in acidified YNB liquid medium supplemented with 100 mg/L of EO2. The triplicates were performed from 01/05/2019 to 03/05/2019); and the green light circles represent the growth *T. delbrueckii* in the same medium supplemented 100 mg/L of the in EO3 i.e. deteriorated EO. The triplicates were performed from 28/04/2019 to 30/04/2019). The growth assays were monitored by OD_{640nm} measurements for 10 hours.

3.5. Analysis of quantitative differential expression of *GPX2*, *PIR3* and *ERG6* orthologous genes in *T. delbrueckii*

Many microorganisms are capable of adapting to growth in a range of stress conditions through the deployment of a series of cell stress responses. Such responses result in changes in gene expression profiles, cellular metabolic activities and cell cycle adaptations, to increase activities with protective functions and to maintain the physiological activities of microorganisms.

Many studies have described the regulation of stress responses in *S. cerevisiae* at the level of transcription, to counteract the harmful effects of limonene. Earlier research findings showed that several genes related to plasma membrane maintenance, particularly of the mevalonate pathway, inositol and lipid biosynthesis, were upregulated after limonene (J Liu *et al.*, 2013) and α -terpinene exposure (Parveen, 2004). Some researchers also argue that the exposure to limonene reshape *S. cerevisiae* cell wall properties, since the expression of genes implicated in the cell wall integrity signaling pathway (CWI), a mechanism that detects and responds in case of stress at the cell wall level, was modulated after limonene exposure (Brennan, Krömer and Nielsen, 2013). In addition, according to (Jidong Liu *et al.*, 2013a), *S. cerevisiae* induced a cell oxidative stress response, after addition of limonene in culture media, since upregulation of genes related to antioxidant enzymes and activity was detected (Jidong Liu *et al.*, 2013a).

After a literature review, to get insight into the *T. delbrueckii* stress response in the presence of orange EO2, orthologs of *S. cerevisiae* *GPX2*, *PIR3*, and *ERG6* genes were selected for a quantitative differential expression study (RT-qPCR). The *ERG6* gene encodes a $\Delta(24)$ -sterol C-methyltransferase, one of the enzymes involved in the ergosterol biosynthesis pathway (Gaber *et al.*, 1989). Ergosterol is

the main sterol of the plasma membrane and has essential functions in the *S. cerevisiae*, especially in maintaining the membrane fluidity and permeability in imbalanced conditions. Deletion mutants ($\Delta erg6$) generated phenotypes with alterations in membrane integrity (Gaber *et al.*, 1989). Besides, in *S. cerevisiae*, during exposure to 0,02%(v/v) of limonene, *ERG6* was upregulated approximately 2,59- fold (J Liu *et al.*, 2013). The other selected gene, *PIR3*, encodes an O-glycosylated covalently-bound cell wall protein, which belongs to a class of cell wall proteins that are directly linked to β -1,3 glucan present in the cell wall (Pir-CWPs) (Boorsma *et al.*, 2004; García *et al.*, 2004). This gene is involved in the Cell Wall Integrity (CWI) signaling pathway, which has as main purpose respond to cell wall stress. Also, is part of a cluster of genes that represent the main transcriptional fingerprint for cell wall stress, particularly involved in cell wall biogenesis (García *et al.*, 2004). In (Brennan, Krömer and Nielsen, 2013) work, *PIR3* gene was one of the genes that was most upregulated in the presence of 107mg/L of limonene, exhibiting an expression fold change of approximately 5. Lastly, the change in expression of the *GPX2* gene was also evaluated. This gene encodes a phospholipid hydroperoxide glutathione peroxidase that catalyzes the reduction of hydrogen peroxide and lipid hydroperoxide to water and corresponding alcohols (Inoue *et al.*, 1999). Gpx2p belongs to a group of antioxidant enzymes (Gpxs) that plays an important role in defense against reactive oxygen species (ROS). Of the Gpxs group (GPX1, GPX2, GPX3), although *GPX2* gene is reported to have a minor contribution to oxidative stress tolerance, it is the one that is most induced in a Yap1p-dependent manner under oxidative stress (Inoue *et al.*, 1999; Herrero *et al.*, 2008). Evidence regarding the *GPX2* expression after limonene exposure was reported by (Jidong Liu *et al.*, 2013a), who observed an expression change of 32,2-fold after treatment of *S. cerevisiae* cells with 0,2 μ L/mL of limonene, and reported the substantial role of the D-limonene-induced oxidative stress in the occurrence of cell death. Therefore, all selected genes were upregulated in *S. cerevisiae* after exposure to limonene.

After the choice of the target genes and the housekeeping gene that would be used as reference (*ACT1*), it was necessary to design primers that amplified the corresponding fragments. The primers are listed in Table 6.1, in “6. Appendix”. To confirm primer specificity, PCR reactions were performed, and PCR products were analyzed by electrophoresis in 1% agarose. Fragment amplification results are shown in Figure 3.18. All primer pairs amplified a single fragment with the expected size ($\approx$ 250 bp) and therefore they could be used in the RT-qPCR experiments.

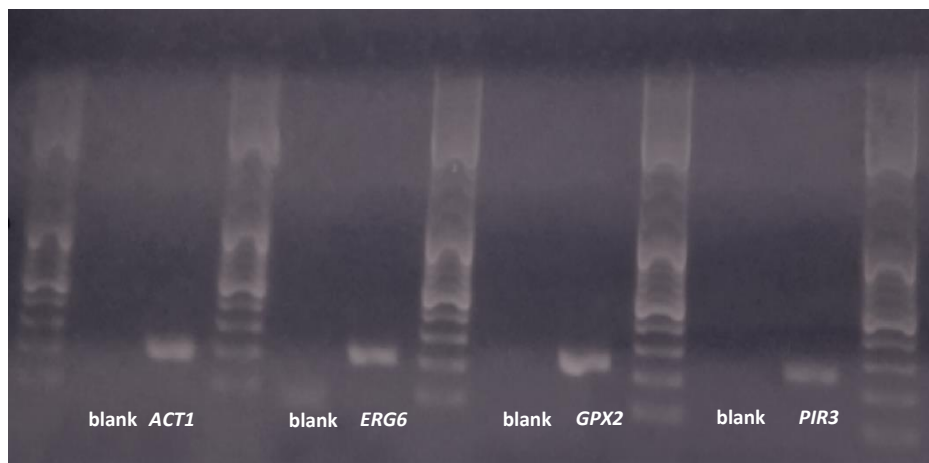


Figure 3.18- Confirmation of amplification of *T. delbrueckii* *ACT1*, *GPX2*, *PIR3* and *ERG6* fragments.

Before RNA extraction, *T. delbrueckii* was cultivated in YPD liquid medium and exposed to 200mg/L of EO2 in mid-exponential phase (Figure 3.19). Since growth inhibition was observed, these conditions were used for RNA extraction.

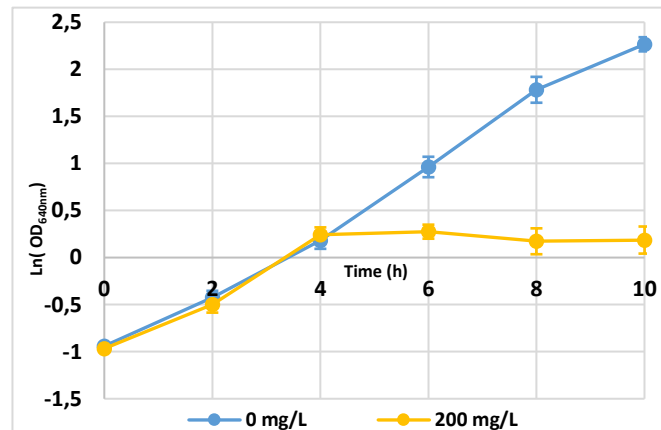


Figure 3.19- *T. delbrueckii* growth in YPD liquid medium after addition of 200 mg/L of orange EO2 in mid-exponential growth. The growth was followed by OD_{640nm} measurements during 6 hours after exposure. Results are from three independent experiments (n=3) and therefore respective standard deviations, as well as the mean of the measurements are represented in the graph.

Figure 3.20 shows the qPCR analysis of *GPX2*, *PIR3*, and *ERG6* gene expression profiles after treatment with 200 mg/L of EO2. There are two distinct methods of RT-qPCR data analysis, namely relative quantification and absolute quantification (Livak and Schmittgen, 2001). In this study a relative quantification approach was used, since it consists in the description of changes in expression of the chosen gene relative to the control (e.g., untreated cells).

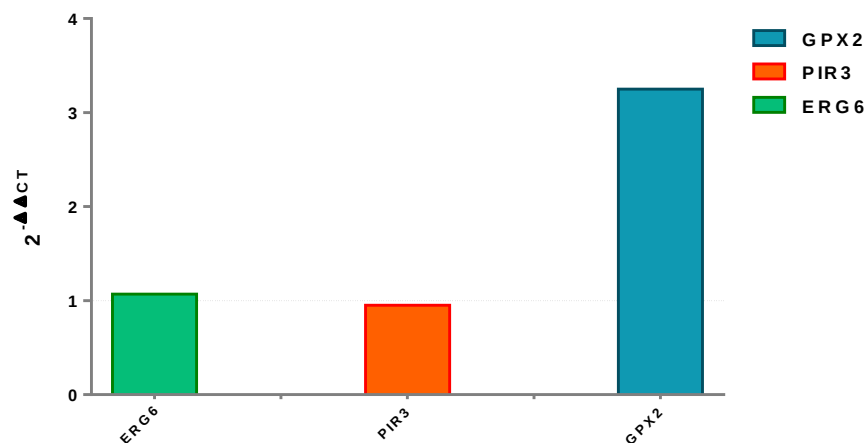


Figure 3.20- qPCR analysis of gene expression profiles after two hours of exposure to 200 mg/L of EO2 in YPD liquid medium. Expression fold changes were normalized in conformity with the housekeeping gene *ACT1*. cDNA *ERG6* (■), cDNA *PIR3* (■) and cDNA *GPX2* (■) templates were obtained from one independent biological sample and were performed three replicates from each sample. Values above 1 indicate that genes are upregulated and below 1 that genes are downregulated, in relation to control (untreated cells).

Results of qPCR showed that in EO2-treated cells, no significant expression changes occurred in the *ERG6* and *PIR3* genes (1,07 and 0,95-fold, respectively), while the *GPX2* gene was upregulated by 3,25-fold, compared to non-EO2 treated cells (Figure 3.20). As such, in contrast to what was reported in limonene-treated *S. cerevisiae* cells, *PIR3* e *ERG6* genes were not upregulated in EO2-treated *T.*

delbrueckii cells. The results point to an induction of oxidative stress after exposure to orange EO2, since an enzyme responsible for scavenging of ROS was upregulated (Figure 3.20). The transcriptional activation of genes coding for antioxidant enzymes has been reported to be the major adaptive response to oxidative stress (Hayes and McLellan, 1999). In line with this, it was possible to conclude that the loss of viability of *T. delbrueckii* in the presence of orange EO may be due to oxidative stress.

Although the antimicrobial action of the limonene or EO remains poorly understood, the following antimicrobial mechanism has been suggested: due to its lipid character, orange EO2 may penetrate and accumulate in the *T. delbrueckii* cell wall and membranes, permeabilizing them and damaging the mitochondrial membranes. Damage at the mitochondrial membrane may disrupt the normal electron flow, hence leading to an accumulation of ROS intracellularly and causing oxidative stress. Under this oxidative stress scenario, biosynthesis of antioxidant enzymes (e.g. *GPX2*) are expected to increase to counteract ROS accumulation (Bakkali *et al.*, 2008; Jidong Liu *et al.*, 2013a; Rahal *et al.*, 2014).

3.6. Experimental evolutions, screening, and evaluation of the fermentative performance of mutants

Various recombinant DNA techniques have been employed to obtain desirable phenotypes for a given function, among which limonene tolerance (Chubukov *et al.*, 2015). However, since the use of genetically modified organisms is not much appreciated by the consumer, and there are legal restrictions on their employment in the food and beverage industry, the applied alternative was an adaptive laboratory evolution approach. According to the literature, this method has been successfully applied to enhance *S. cerevisiae* cells' tolerance to various industrial environments (Çakar *et al.*, 2005; Kuyper *et al.*, 2005; Wisselink *et al.*, 2009), including to limonene exposure. (Brennan *et al.*, 2015) reported positive results after the successive exposure of *S. cerevisiae* to increasing amounts of limonene for roughly 200 generations. As such, a similar approach was performed in this work, with the purpose of increasing *T. delbrueckii* and *Sch. pombe* tolerance to orange EO, assuming that a higher tolerance leads to decreased growth lag times and therefore faster onsets of orange juice fermentations.

The principle of this approach is the mimicry of natural evolutionary mechanisms, followed by successive selections under particular selective pressure to achieve certain phenotype improvements (Dragosits and Mattanovich, 2013). In this study we used the serial batch cultivation strategy, where an initial population (parental cells) was subjected to a certain concentration of orange EO/EO2 (selective pressure), and those cells that had a genetic background that favors their faster growth in the presence of orange EO/EO2 were successively selected by transfers of culture fractions to new media with the same selective pressure for further growth. As such, to collect those that grew faster in the presence of EO/EO2 and funnel evolution in the intended direction, the cultures were allowed to grow approximately 1.5 generations and then 1/3 of the population was successively transferred to new medium with orange EO/EO2.

3.6.1. Adaptive evolution of *T. delbrueckii* in culture medium

The culture medium used to perform the adaptive evolution of *T. delbrueckii* was YNB liquid medium with pH adjustment to 3.5-3.6 (in order to simulate the pH of orange juices), to which 100 mg/L of orange EO was added immediately after cell inoculation. The adaptive laboratory evolution for *T. delbrueckii* was conducted in duplicate from transfer 110 of T.d-A culture, and both profiles are represented in Figure 3.21. For the T.d-A culture, 147 daily sequential transfers were performed (at least 220 generations) and, the T.d-B culture was submitted to 135 transfers during approximately 7 months (at least 200 generations).

From transfer 107, an abrupt decrease in the relative fitness of the T.d-A population was observed. Apparently, this was due to a change in the toxicological properties of the EO orange stock. Therefore, from transfer 109 the cultures were treated with the new EO2, and cell growth resumed without disturbance in relative fitness (Figure 3.21).

Between both cultures, adaptive laboratory evolutions proceeded differently and dynamically, with no progressive increase/decrease in the relative fitness of the population during the evolution experiment, i.e., sometimes, the population was much more adapted and grew 1,5 generations faster than other times (e.g., T.d-A at transfer 134 and 136)(Figure 3.21-(A)).

The nature and dynamics of genetic variations in a population during the natural selection process has been studied with particular focus on large asexual populations. Due to the emergence of spontaneous mutations in the initial population, various phenotypes may appear, be selected by the imposed pressure and possibly compete for their dominance in the population. Thus, several events inherent to asexual reproduction may contribute to the heterogeneity of the population over time. Typically, in large populations, beneficial mutations frequently occur, and as such, mutants may exist simultaneously with different beneficial mutations, which will consequently grow in the population simultaneously. In this scenario, several beneficial phenotypes may exist within the population and interfere with each other. From this, phenomena such as clonal interference and multiple-mutation effect may emerge and promote different dynamics in the course of the evolutionary process (Desai and Fisher, 2007). These two effects have been extensively studied and mathematized during processes of bacterial evolution (Perfeito *et al.*, 2007). Clonal interference may occur when several lineages with benefic mutations emerge and sweep but are successively outcompeted by more-fit mutations. On the other hand, multiple-mutation effect occurs when a second mutation occurs in a microorganism that already has a beneficial mutation before it is fixed. If the combination of the two mutations gives to the cell an increase in fitness, not only the previous single mutants are outcompeted, but also those with less-fit mutations that may arise later in the population. In addition to this, it is necessary to take into account, not only possible interactions between mutations, for instance epistatic effects that may occur and promote oscillations during serial transfers, but also the regular large bottlenecks that were imposed reducing the probability that a beneficial mutation will reach fixation (Crow and Kimura, 1965; Gerrish and Lenski, 1998; Wahl, Gerrish and Saika-Voivod, 2002; Desai and Fisher, 2007; Good *et al.*, 2012; Dragosits and Mattanovich, 2013). All these may have contributed to the dynamics of *T. delbrueckii* evolution over the time.

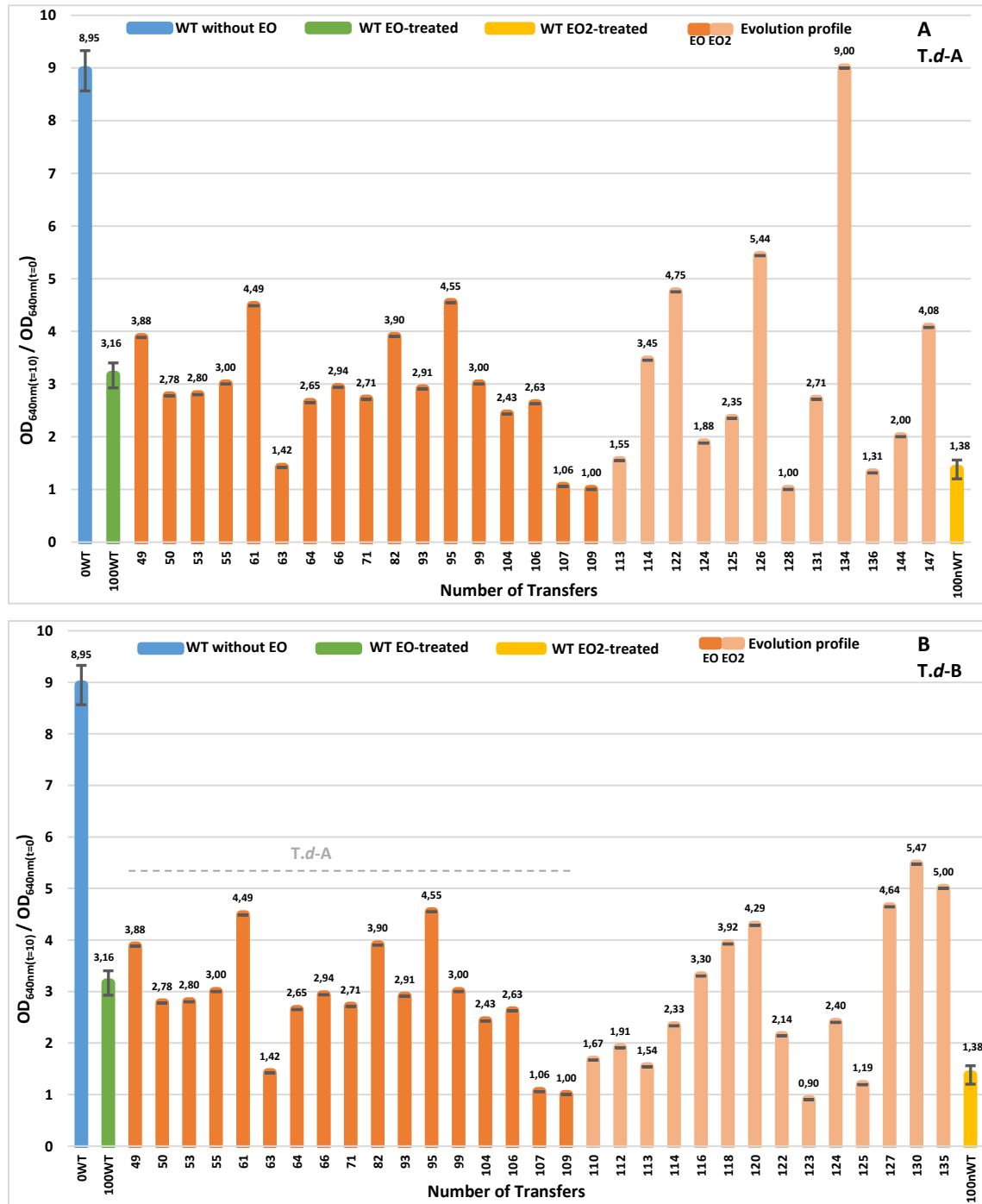


Figure 3.21- Progression of experimental evolution of *T.d-A* (A) and *T.d-B* (B) culture in acidified YNB liquid medium supplemented with 100 mg/L of orange EO/EO2. Each orange bar (—) corresponds to the relative fitness of the population in the various transfer moments during the course of the evolutionary process. The blue bar (—) corresponds to the relative fitness of the WT *T. delbrueckii* in acidified YNB liquid medium without EO addition; the green bar (—) corresponds to the relative fitness of the WT *T. delbrueckii* in acidified YNB liquid medium with 100 mg/L of orange EO and the yellow bar (—) corresponds to the relative fitness of the WT *T. delbrueckii* in acidified YNB liquid medium with 100 mg/L of recent orange EO2. The WT results are from three independent experiments (n=3), and therefore respective standard deviations, as well as the mean of the measurements are represented in the figures.

An increase in a given population phenotype is expected to be consequently determined by an increase in the frequency of the adapted strain in the population (Desai and Fisher, 2007). Since the relative fitness at the end of the experimental evolution was higher than that displayed by the WT *T. delbrueckii* (parental cells) in the evolution medium, this may indicate that there were adaptive mutations

in the population and therefore possible presence of *T. delbrueckii* mutants with increased EO2 tolerance.

3.6.1.1. Screening of evolved mutants in the T.d-B population

The T.d-B population exhibited the best results at the end of evolution and was, therefore, the selected population for the subsequent screening process. The results of the screening assays are depicted in Figure 3.22.

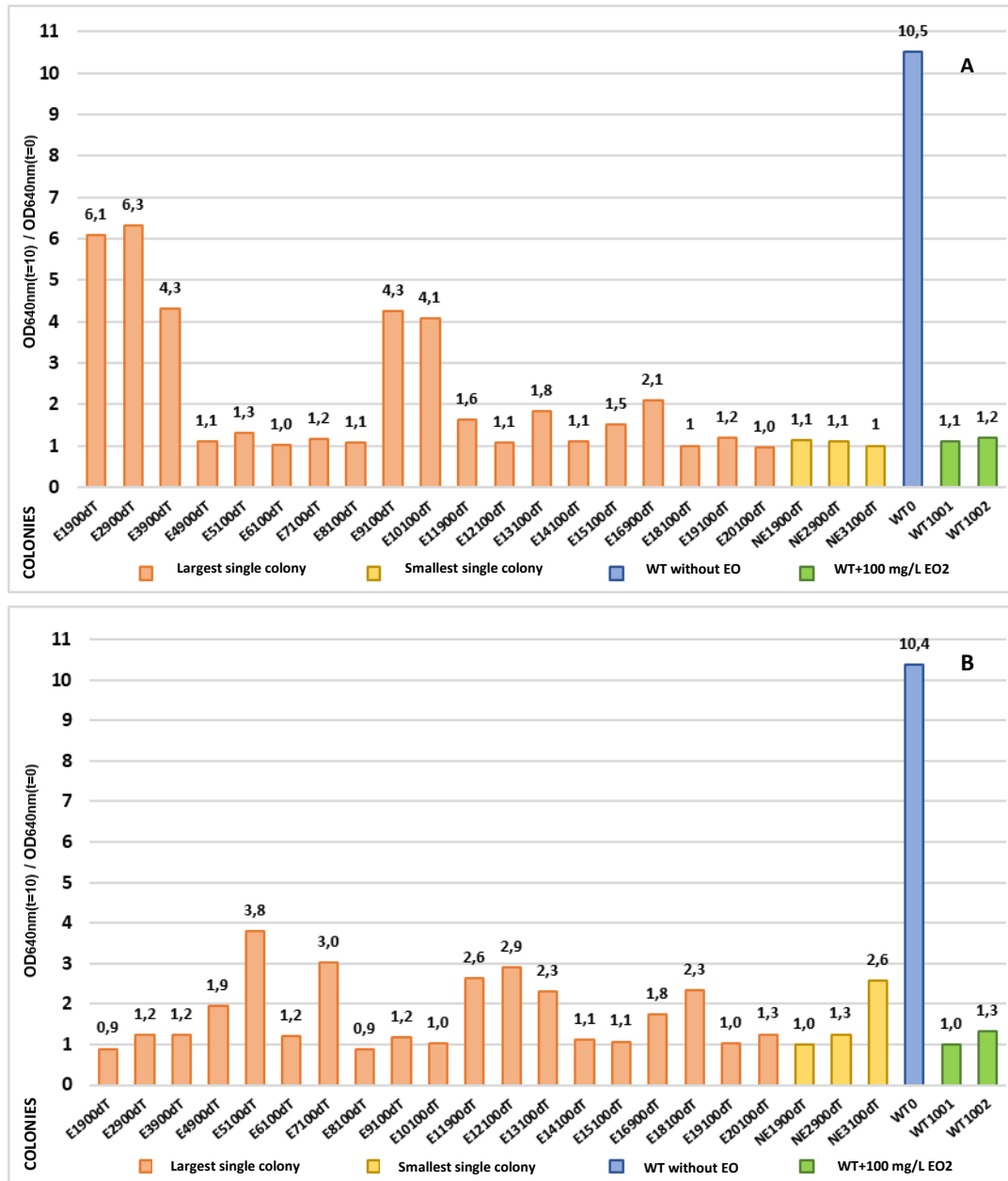


Figure 3.22- Screening of evolved mutants in the population T.d-B. (A): Relative fitness of the *T.d-B* clones after 3 passages through medium without selective pressure (2 in solid agar medium and 1 in liquid medium) and **(B):** after 4 passages through medium without selective pressure (3 in solid agar medium and 1 in liquid medium).

After 3 passages through medium without selective pressure, it was possible to discern a wide variability of phenotypes in the *T.d-B* population. The results demonstrated that roughly half of the selected clones had a relative fitness improvement in the presence of EO₂, and that the remaining had a growth similar to WT *T. delbrueckii* in the presence of 100 mg/L of EO₂ (Figure 3.22-(A)). These results suggested that, after 220 generations, either the final *T.d-B* population remained heterogeneous or exhibit unstable spontaneous mutations. To clarify this topic, the same clones were restreaked again in YNB solid medium without selective pressure and cultured again in the presence of 100 mg/L of EO₂. The achieved results are shown in the Figure 3.22-(B). The values of growth rates and lag times of each clone, in each screening assay are listed in Tables 6.3 and 6.4, in “Appendix.” After the additional passage in medium without selective pressure (Figure 3.22-(B)), the results revealed a completely different growth profile from the previous assay. It was observed that some clones that apparently had improved EO₂ tolerance did not grow in the second screening assay (e.g., strain E2900dT, which previously had the highest fitness value (Figure 3.22- (A)). On the other hand, other clones that were not able to grow in the first screening assay (Figure 3.22- (A)), grew after 4 passages to medium without selective pressure (e.g., colony E7100dT (Figure 3.22-(B)).

These puzzling results suggest that there has been an accumulation of beneficial unstable mutations throughout evolution that may cause stochastic instability, reflecting an unpredictable increase in relative fitness.

3.6.1.2. Fermentative performance of *T.d-B* mutants in orange juice substrate

Although the vast majority of clones exhibited unstable fitness in the presence of EO₂, the strain E16900dT, through passages in medium without selective pressure behaved consistently and showed an improvement in EO₂ tolerance. Therefore, 4 microfermentations in “fresh” orange juice with the strain E16900dT were performed and monitored in order to elucidate if a decrease at fermentation lag time occurred. Figure 3.23 shows the elapsed time until the onset fermentation by WT *T. delbrueckii* (parental cells) and evolved strain.

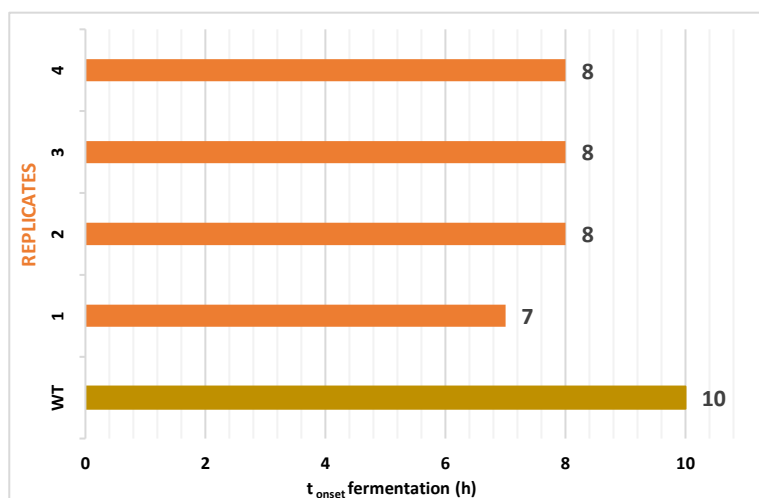


Figure 3.23- Fermentative performance of the WT *T. delbrueckii* as well the mutant E16900dT in “fresh” orange juice. Each orange bar corresponds to an independent microfermentation by the E16900dT mutant in “fresh” orange juice. The dark yellow bar corresponds to a microfermentation conducted by WT *T. delbrueckii* on the same substrate

The evolved strain E16900dT proceeded the fermentation approximately 8 hours after its inoculation in “fresh” orange juice, showing a decrease of roughly 2 hours compared to the onset of fermentation by the WT *T. delbrueckii* (Figure 3.23).

These results indicate that the application of adaptive evolution towards *T. delbrueckii* EO tolerance yielded an evolved stable strain capable of withstanding higher orange EO concentrations.

3.6.2. Adaptive evolution of *Sch. pombe* in culture medium

The adaptive evolution for *Sch. pombe* was conducted in two different media, namely YPD liquid medium containing 60 mg/L of orange EO/EO2, and in YNB liquid medium initially supplemented with 35 mg/L of orange EO. To the YPD liquid medium was even added an antibiotic (clopharphenicol) to reduce possible bacterial contaminations during the serial batch passaging.

The adaptive laboratory evolution for *Sch. pombe* in YPD liquid medium was performed in duplicate from transfer 63 of S.p-A culture, and both profiles are represented in Figure 3.24-(A) and (B). For the S.p-A culture, 97 daily sequential transfers were performed (at least 145 generations) and the S.p-B culture was submitted to 98 serial batch passages (at least 147 generations).

Similarly, as depicted in the evolution conducted with *T. delbrueckii*, in transfer 52 a decrease in the relative fitness of the population was observed, due to the transformation of orange EO properties. From transfer 60, the cultures were treated with the new orange EO2 and growth continued without impairment in S.p-A population fitness (Figure 3.24- (A)).

Furthermore, as observed in T.d-A and T.d-B populations, the adaptive laboratory evolutions of both cultures proceeded differently and dynamically with no progressive increases or decreases in the relative fitness of the populations, although almost always demonstrated relative fitness well above the controls (WT cells treated with orange EO/EO2). As previously mentioned for the evolutions carried out by *T. delbrueckii*, the mutations generate variations in the genetic background of the parental population. In line with this, the dynamic accumulation of beneficial mutations is what may positively alter the fitness of the population in a given environment. Since many effects may occur during the evolution process (especially in large asexual populations), natural selection may act differently between the populations (Crow and Kimura, 1965; Gerrish and Lenski, 1998; Wahl, Gerrish and Saika-Voivod, 2002; Desai and Fisher, 2007; Good *et al.*, 2012; Dragosits and Mattanovich, 2013).

After adaptive evolution, both final populations showed significant improvements in the relative fitness compared to WT *Sch. pombe* in the presence of both orange EO. As such, the results suggested that possibly in both final cultures (S.p-A and S.p-B) there were mutants with greater tolerance to orange EO (Figure 3.24-(A) and (B)).

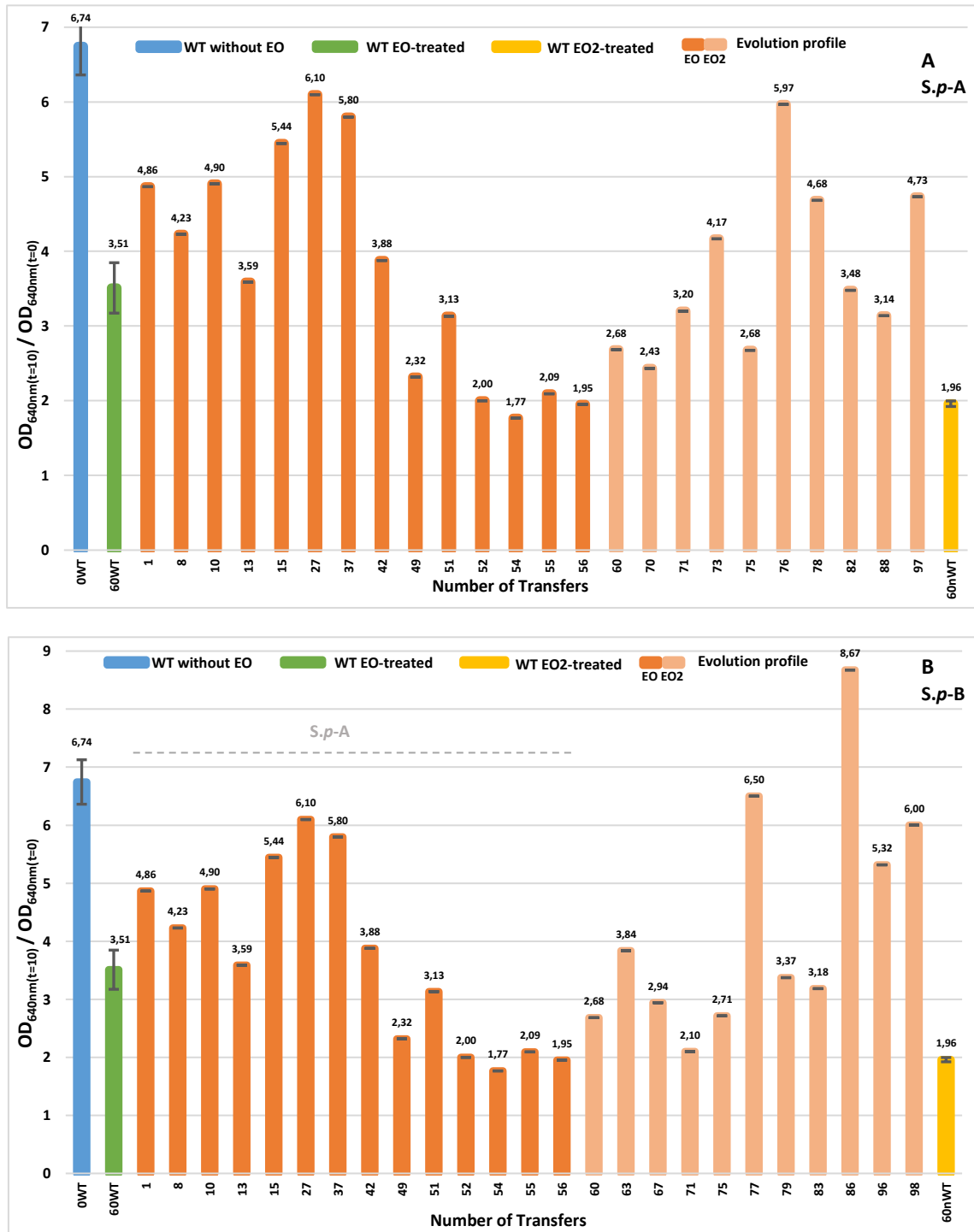


Figure 3.24- Progression of experimental evolution of *S.p-A* (A) and *S.p-B* (B) culture in YPD+clorphanphenicol medium supplemented with 60 mg/L of orange EO/EO2. Each orange bar (■) corresponds to the relative fitness of the population in the various transfer moments during the course of the evolutionary process. The blue bar (■) corresponds to the relative fitness of the WT *Sch. pombe* in YPD+clorphanphenicol without orange EO addition; The green bar (■) corresponds to the relative fitness of the WT *Sch. pombe* in YPD+clorphanphenicol with 60 mg/L of orange EO and the yellow bar (■) corresponds to the relative fitness of the WT *Sch. pombe* in YPD+clorphanphenicol with 60 mg/L of orange EO2. The WT results are from three independent experiments (n=3), and therefore respective standard deviations, as well as the mean of the measurements are represented in the graph.

Also, another evolution experiment was performed with *Sch. pombe* in YNB liquid medium, initially containing 35 mg/L of orange EO. The evolution profile of *Sch. pombe* in YNB liquid medium (S.p-C culture) is depicted in Figure 3.25.

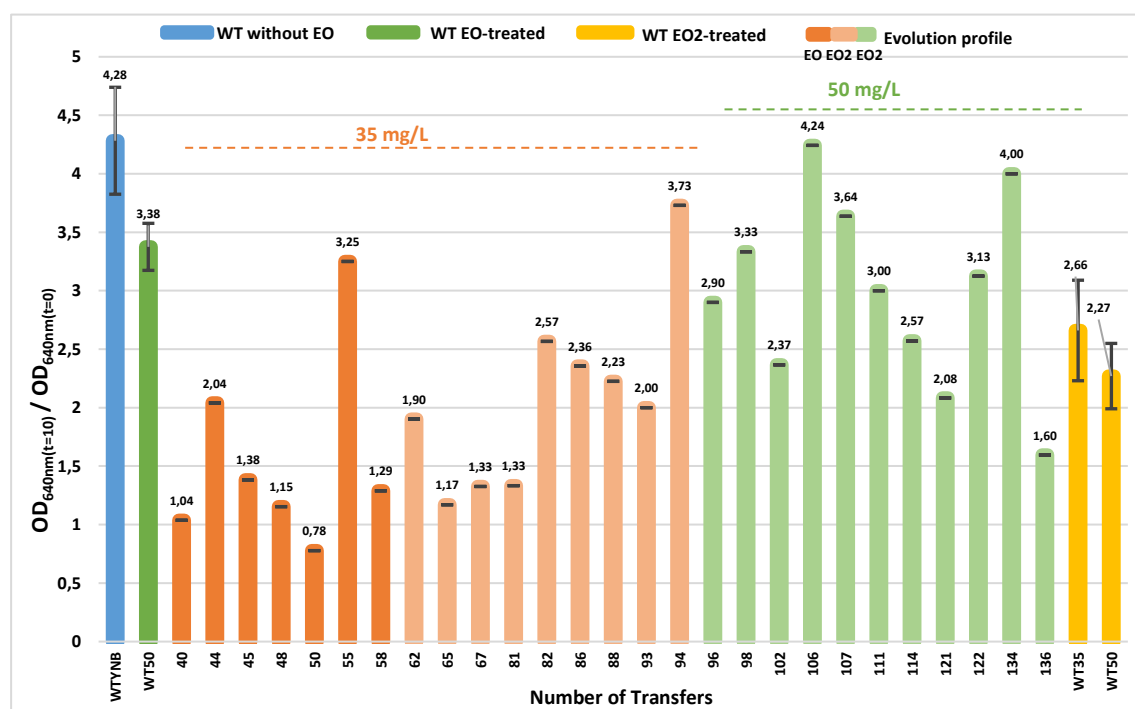


Figure 3.25- Progression of experimental evolution of S.p-C culture in YNB liquid medium. Each orange/green bar () corresponds to the relative fitness of the population in the various transfer moments during the course of the evolutionary process. The blue bar () corresponds to the relative fitness of the WT *Sch. pombe* in YNB liquid medium without orange EO addition; The green bar () corresponds to the relative fitness of the WT *Sch. pombe* in YNB liquid medium with 50 mg/L of orange EO, the yellow bars () correspond to the relative fitness of the WT *Sch. pombe* in YNB liquid medium with 35/50 mg/L of orange EO2. The WT results are from three independent experiments (n=3), and therefore respective standard deviations, as well as the mean of the measurements are represented in the graph.

During the serial selection under the selective pressure of 35 mg/L of EO, the relative fitness of the population was again variable and often lower than wild type. After 140 generations, there was a considerable increase in the relative fitness of the population, and the selective pressure was increased. Several oscillations in relative fitness were observed, but with more frequent fitness increases. After 136 passages, the relative fitness of the population was calculated, and a frozen stock was prepared (Figure 3.25).

Throughout the selection process, the cells became increasingly sensitive to the presence of EO, even at the lowest concentrations. This result suggests that possibly the medium used for the adaptive laboratory evolution might not have been the most appropriate, leading to the constant permanence of an injurious environment to the cells, further delaying the evolutive process (Figure 3.25). Contrary to what the previous adaptive evolutions suggested, the cells that were present in the last transfer of culture S.p-C (after at least 220 generations) do not seem to have an increased resistance to orange EO, since when the adaptive evolution was terminated, the population had a lower fitness value, compared to the parental strain with and without EO. Due to this, no screening for clones with the desired characteristics was conducted in this population.

3.6.2.1. Screening of evolved mutants in *S.p-B* population

The *S.p-B* population exhibited the best results at the end of evolution and was, and therefore, it was the selected population for the subsequent screening process. The results of the screening assays are depicted in Figure 3.26.

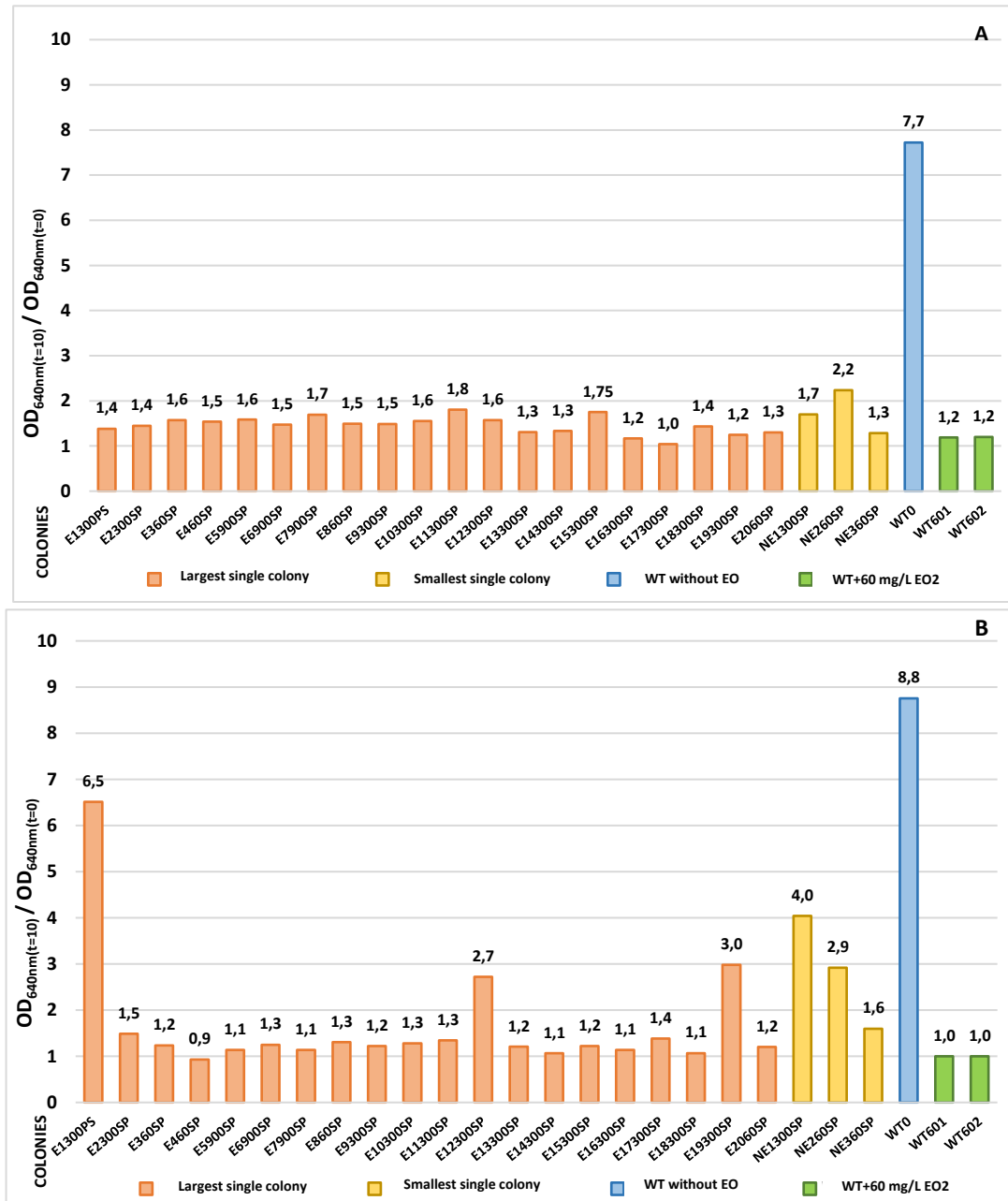


Figure 3.26- Screening of evolved mutants in the population *S.p-B*. (A): Relative fitness of the *S.p-B* clones after 3 passages through medium without selective pressure (2 in solid agar medium and 1 in liquid medium) and (B): after 4 passages through medium without selective pressure (3 in solid agar medium and 1 in liquid medium).

After 3 passages through medium without selective pressure, it was possible to discern a wide variability of phenotypes in the *S.p-B* population. The results showed that 19 clones derived from large colonies had a slightly higher relative fitness, than WT *Sch. pombe* in the presence of 60 mg/L of EO2.

Besides, one strain that formed a smaller colony (NE260SP) had the best improvement in the relative fitness compared to all other selected clones (Figure 3.26-(A)). After 4 passages through medium without selective pressure, the results revealed a completely different growth profile from the previous assay illustrated in Figure 3.26-(A). Some clones, namely E1300PS, E12300SP, E19300SP, NE1300SP, exhibited a much higher fitness compared to the observations from the first assay (Figure 3.26-(B)). As the results of *T. delbrueckii* suggested, possibly an accumulation of unstable beneficial mutations occurred throughout selective passages, resulting in unstable phenotypes due to stochastic effects.

To elucidate if the phenotypic instability was observed due to successive passages through medium without selective pressure, a biological replicate of the assay of Figure 3.26-(B) was performed. The results of these growth assays are depicted in Figure 3.27. The values of growth rates and lag times of each clone, in each screening assay are listed in Tables 6.5 to 6.7, in “Appendix”.

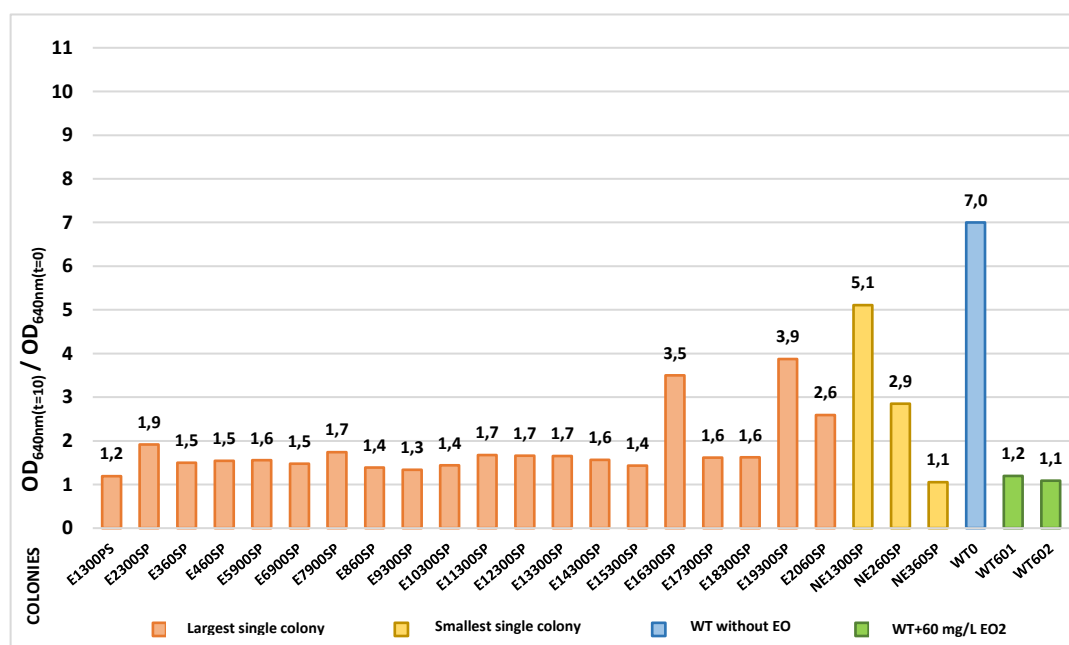


Figure 3.27- Biological replicate of screening assay illustrated in Figure 3.26-(B).

According to Figure 3.27 and compared to the results of Figure 3.26-(B), it was observed a different phenotypic profile. Adopting the E1300PS strain as an example, it was observed that after four passages through medium without selective pressure, its fitness seemed to have increased significantly, however, when a biological replicate was performed, this tolerance was not reproduced. As such, it was possible to conclude that the strains behave inconsistently between the assays. These results indicate that this phenotypic instability of the isolates was not due to successive passage to medium without selective pressure, but due to the acquisition of unstable phenotypes. Besides, it was possible to observe that there was no correlation between the size of the colony and its ability to tolerate orange EO, since two isolated strains that formed small colonies presented significantly higher values of relative fitness compared to the strains that formed larger colonies (Figure 3.26-(A) and Figure 3.27).

In order to deepen the suspicion of stochastic instability and rule out errors during the assays, two different assays were performed with a frozen E1300PS strain (assuming that the congelation

preserves all its features), since it had the highest fitness variance between passages through medium without selective pressure. In the first assay was performed a pre-inoculation of the strain E1300PS into two different Erlenmeyer flasks containing YPD liquid medium, one without addition of orange EO2 and another supplemented with 60mg/L of orange EO2. After overnight growth, five Erlenmeyer flasks containing YPD liquid medium supplemented with 60 mg/L of EO2 were inoculated with the same cell concentration ($OD_{640nm} \approx 0,4$). The fitness results of these growth assays are illustrated in Figure 3.28-(A).

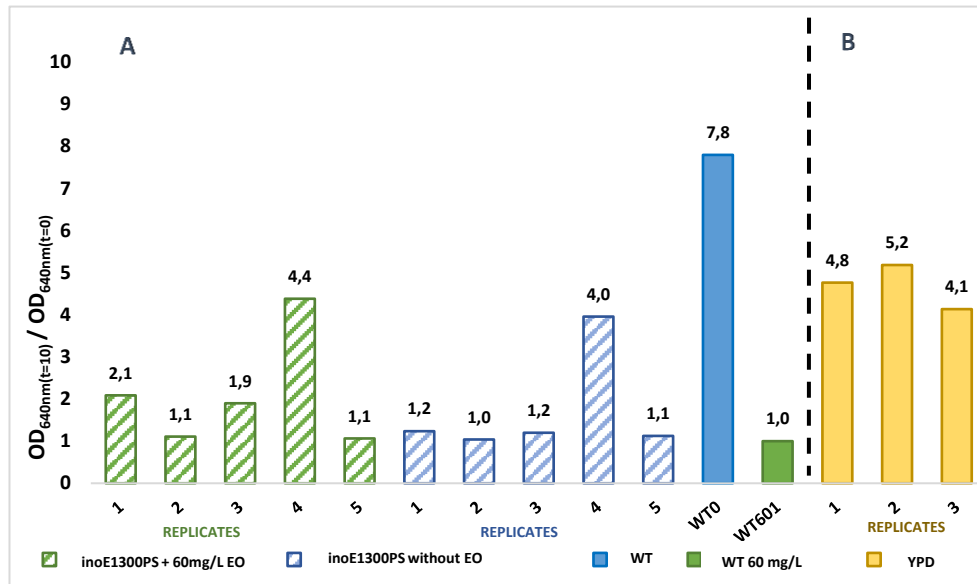


Figure 3.28- Performance evaluation of mutant E1300PS under different inoculation conditions. **A:** Fitness results corresponding to the growth assays in YPD liquid medium supplemented with 60 mg/L of EO2, conducted by a inoculum previously grown in same medium (■) and in YPD liquid medium without EO addition (■). The fitness represented by the green bar (■) corresponds to the WT *Sch. pombe* growth in YPD liquid medium supplemented with 60 mg/L of EO2, and the blue bar (■) in YPD liquid medium without EO supplementation. **B:** The results displayed by the yellow bars (■) correspond to the growth assays of E1300PS in YPD liquid medium without EO addition. All results are demonstrated using relative fitness as metrics that is defined as the ratio of the cell density, expressed as the OD_{640nm} , at 10 h to the cell density at 0 h.

Under both pre-inoculum conditions, the various biological replicates showed different fitness values after exposure to EO2 (Figure 3.28-(A)). With these assays, it was clarified the presence of stochastic instability (possibly acquired tolerance is not stable) in this strain, conclusion that may be extended to strains that exhibit a variable behavior in the *S.p-B* and *T.d-B* population.

In addition, growth of E1300PS was evaluated in YPD liquid medium without addition of EO2. The fitness results of these growth assays are illustrated in Figure 3.28-(B). Compared to the growth of cells that have not undergone evolution (WT *Sch. pombe*), the strain E1300PS showed a consistent growth impairment in YPD liquid medium. Some mutations acquired during the adaptive evolution may frequently involve trade-offs. In the presence of this scenario, the strain E1300PS was “adapted” to a certain selective pressure (60 mg/L of EO2) in a way that inhibits its growth on the substrate, in this case YPD liquid medium, where the WT cells grow adequately. To assess whether trade-offs occur in these assays, gene expression analysis would be required to discover which pathway was modified and to showing that there is a trade-off taking place (Wenger *et al.*, 2011; Çakar *et al.*, 2012; Kvitek and Sherlock, 2013; Mans, Daran and Pronk, 2018). An example of trade-off after adaptive evolution was

reported by (Wenger *et al.*, 2011), who demonstrated that the clones selected after evolution, consistently outperformed the parental strain under carbon-limiting conditions, however in some cases they underperformed the WT in aerobic and carbon-rich environments, concluding that trade-offs may appear when resources are not limiting.

For industrial uses it is convenient that a phenotype-enhanced microorganism should show not only improved fitness, but also lower trade-offs in other environments where it may be placed (Dragosits and Mattanovich, 2013).

3.6.2.2. Fermentative performance of *S.p-B* mutants in orange juice substrate

Although the results showed a high phenotypic instability in several strains, two clones, namely NE1300SP and NE260SP, through the passages in medium without selective pressure, showed an improvement in EO₂ tolerance with consistent values of relative fitness. In line with this, microfermentations in “fresh” orange juice with both strains were performed and monitored in order to elucidate if a decrease occurred in the delay on the fermentation onset. Figure 3.29 shows the elapsed time until the onset fermentation by WT *Sch. pombe* (parental cells) and evolved strains.

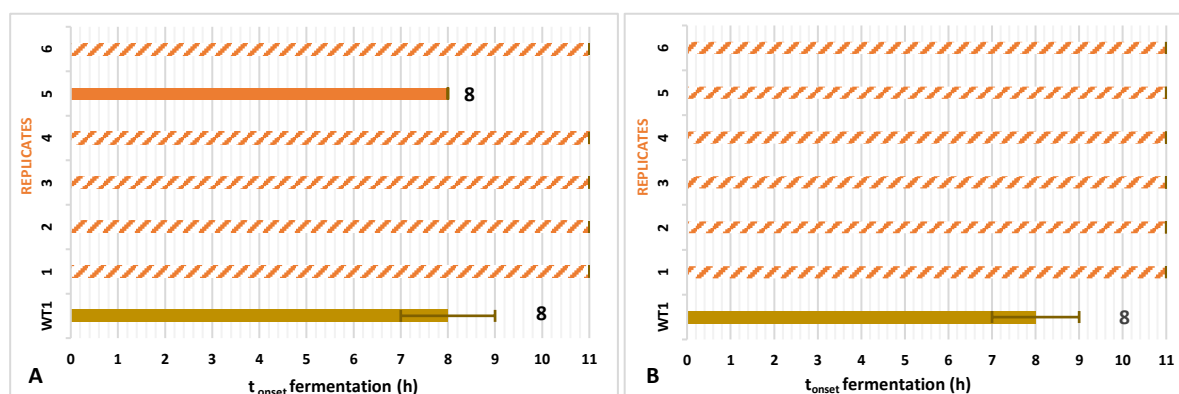


Figure 3.29- Fermentative performance of the WT *Sch. Pombe* as well the mutants NE1300SP and NE260SP in “fresh” orange juice. Each orange bar corresponds to an independent microfermentation by (A) NE1300SP and (B) NE260SP mutants in “fresh” orange juice. The dark yellow bar corresponds to microfermentation conducted by WT *Sch. pombe* on the same substrate. The bars listed indicate that during the investigation time, the fermentation did not start.

Five fermentations carried out by NE1300SP did not initiate the fermentative process during the time of the assay, and one began 8 hours after its inoculation in “fresh” orange juice, exhibiting a behavior similar to that of the parental cells in the same substrate (Figure 3.29-(A)). Additionally, no biological replicate of NE260SP triggered the fermentative process during the investigation time (Figure 3.29-(B)).

When the strains were faced with a different environment, the acquired mutations may imply trade-offs (characterized for presenting increased fitness under one certain trait obtained by selection, causing decreased fitness under another) possibly due to the accumulation of mutations in a pathway that impair the fermentative capacity in this complex substrate. These results suggest the presence of trade-offs. The results obtained from this experimental evolution conducted by WT *Sch. pombe* did not show satisfactory results in obtaining evolved strains capable of fermenting “fresh” orange juice faster than parental cells.

3.6.3. Adaptive Evolution of *Sch. pombe* in orange juice substrate

In addition, a different serial batch passing approach was conducted with *Sch. pombe* using as selective pressure the substrate where it is intended to grow better (“fresh” orange juice). The cells were subjected to sequential incomplete fermentations, monitored to determine the moment of fermentation initiation. The evolution of fermentation onset time throughout the transfers is depicted in Figure 3.30. Cell growth was also evaluated before each transfer and is illustrated in Figure 3.31.

After roughly 49 generations, no consistent improvement (decreasing the delay in the onset of fermentation) was observed, except between the transfers 2 and 5, where a progressive decrease in fermentation onset was observed, having then reverted for the usual values (Figure 3.30).

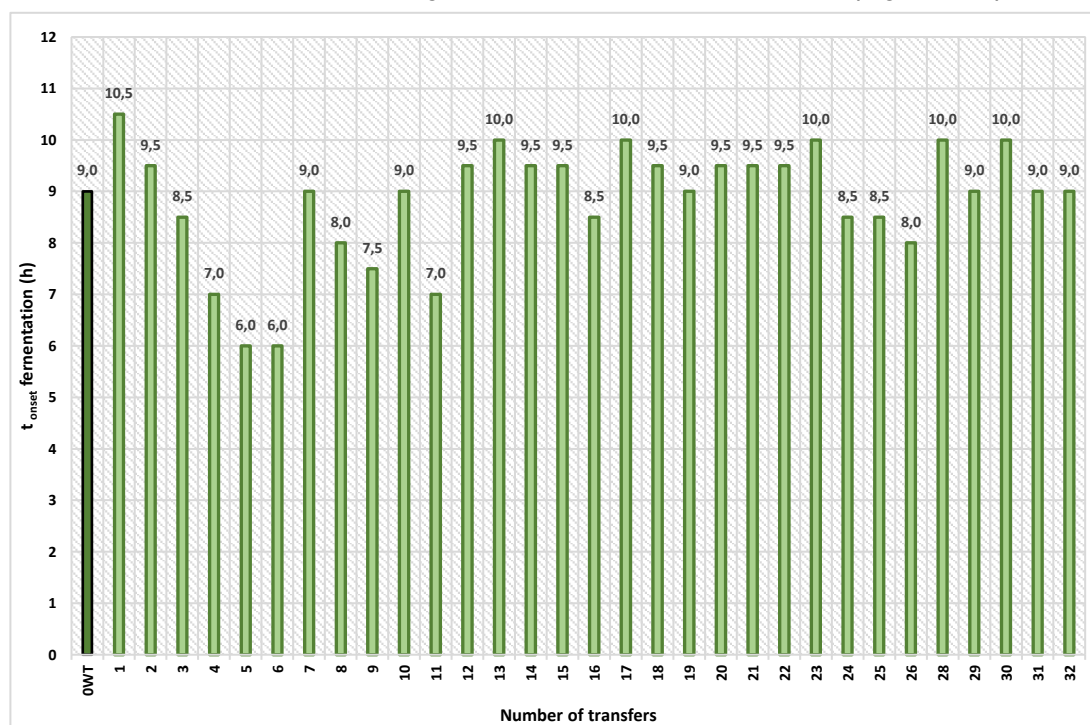


Figure 3.30- Panorama of the first stage of “fresh” orange juice fermentation during the evolutionary process with *Sch. pombe*. Each bar corresponds to the onset time of the “fresh” orange juice fermentation throughout the evolution.

As depicted in Figure 3.31, WT *Sch. pombe* took about 10 hours to obtain enough cells to initiate “fresh” orange juice fermentation. Likewise, the same behavior was observed at transfer 17. However, in the subsequent fermentations, it appears that the population sometimes was able to reach a sufficient number of cells to start fermentation (between $> 5 \times 10^6$ cell/mL and 10^7 cell/mL), more rapidly, namely between 6 and 8h. In spite of that, no decrease in fermentation onset time was observed. Since WT *Sch. pombe* growth on this substrate lacks more biological replicates, it is not possible to draw assertive conclusions about this inconsistent reduction in the time required to reach enough cells to initiate the fermentation process and EO tolerance.

3. RESULTS AND DISCUSSION

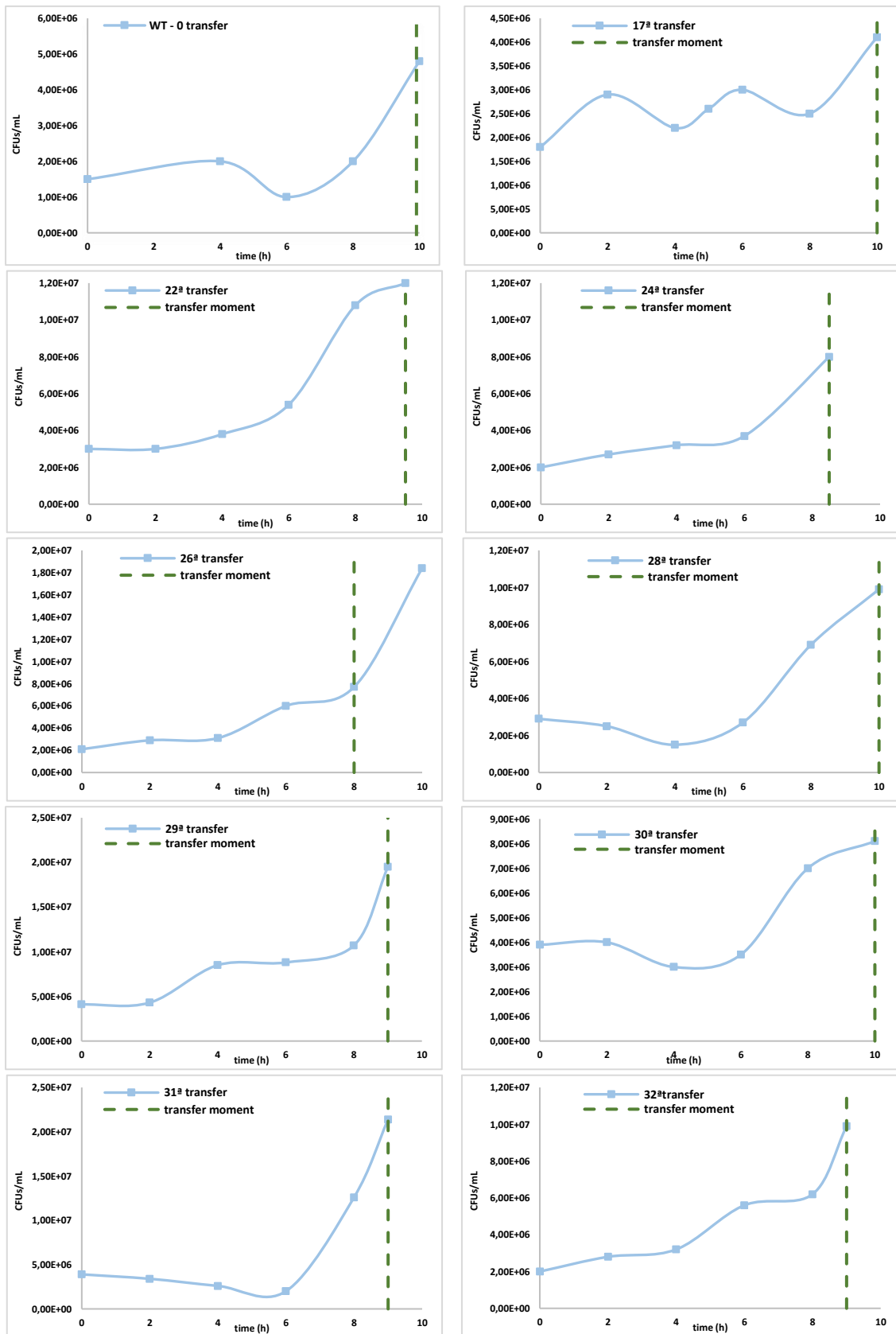


Figure 3.31- *Sch. pombe* growth (CFUs/mL) before each transfer during the adaptive evolution in "fresh" orange juice. Aliquots were withdrawn and appropriate dilutions were performed. The light blue line corresponds to growth profile of the *Sch. pombe* cells until the plating (green dashed line).

3.6.3.1. Screening and fermentative performance of mutants from adaptive evolution in “fresh” orange juice.

Finally, although the evolution in orange juice did not show promising results regarding the presence of evolved *Sch. pombe* cells, a screening assay was conducted aiming at the isolation of individual clones with an improved phenotype. Microfermentations in “fresh” orange juice with the selected strains were performed and monitored in order to elucidate if a decrease occurred in the delay on the fermentation onset. Figure 3.32 shows the elapsed time until the onset fermentation by WT *Sch. pombe* (parental cells) and evolved strains.

As the results of the adaptive evolution predicted, the selected strains did not show an improvement in their fermentative capacity, since no reduction in the time needed for the onset of fermentation was observed. Of the 9 strains tested, 4 did not initiate the fermentation process during the time of the assay (NE1, SE8, SE5 and SE1), SE6 and SE2 started with a longer delay relative to that exhibited by the WT *Sch. pombe*, and the remaining three resembled the wild type phenotype (SE7, SE6 and SE3).

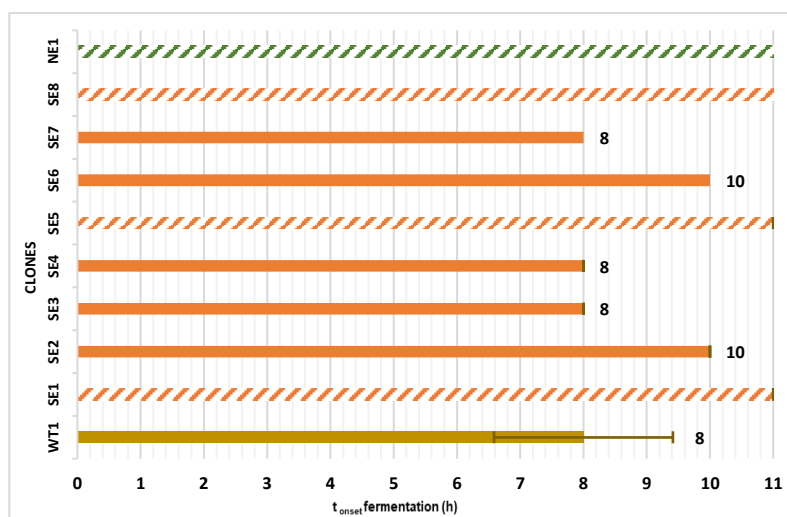


Figure 3.32- Fermentative performance of the WT *Sch. pombe* as well the mutants obtained after screening in “fresh” orange juice. Each orange bar corresponds to one independent microfermentation by each “large” clone in “fresh” orange juice. The green bar corresponds to one independent microfermentation by the “small” clone in “fresh” orange juice. The dark yellow bar corresponds to the microfermentation conducted by WT *Sch. pombe* on the same substrate. The bars listed indicate that during the investigation time, the fermentation did not start.

As observed before, the relationship between large/small colonies and evolved/unevolved clones were not illustrated in this assay, as both large and the small colonies had poor fitness compared to that exhibited by the WT cells.

The data obtained from experimental evolution of *Sch. pombe* carried out in “fresh” orange juice did not show satisfactory results. This may be due to the reduced number of generations occurred.

4. CONCLUSIONS AND FUTURE PERSPECTIVES

The first assay of this work aimed to evaluate the fermentative performance of *Sch. pombe* and *T. delbrueckii* in two orange juices with different concentrations of orange essential oils (EO), often reported to have inhibitory effects on microbial growth. For both species, an increase in fermentation onset time due to an increase in yeast growth lag phase was observed in “*fresh*” orange juice. Consistent with this being due to the higher concentrations of orange EO, growth assays in liquid culture media supplemented with increasing orange EO concentrations displayed longer lag phases, especially from the concentration of 200 mg/L.

The adaptive evolution conducted with WT *T. delbrueckii* in culture medium, resulted in mutants with unstable phenotypes, possibly due to stochastic effects. However, after several passages through medium without selective pressure, clone E16900dT showed an improved stable phenotype and fermented “*fresh*” orange juice more rapidly than WT *T. delbrueckii*. Although this clone is probably more tolerant to the orange EO present in orange juices in the laboratory environment, it is still necessary to evaluate its performance in juices under industrial conditions. Future work on this matter could involve more physiological and genetic characterization. It could be interesting to conduct a genetic study regarding the target mutations that were harbored during the evolution process, which had led to an increase in resistance to orange EO. This study can be performed using molecular biology techniques, namely whole-genome sequencing.

In what concerns the adaptive evolution of *Sch. pombe*, three different media were used, and none of them showed promising results. To clarify the existence of fitness costs due to the evolution of a given trait, the mutants obtained must undergo extensive genetic and physiological characterization.

In the course of this work, we were able to give an insight into the cell viability, as well as the cellular response of *T. delbrueckii* in the presence of the orange EO. The results suggest that after exposure to high orange EO concentrations (namely, from 200 to 900 mg/L), there was a loss of *T. delbrueckii* viability, possibly due to the occurrence of oxidative stress. Evidence regarding the occurrence of oxidative stress in *T. delbrueckii* requires additional biological replicates, as well as a study with a broader set of genes. During the assays, it was observed that *Sch. pombe* had a strong tendency to form aggregates in culture medium. Therefore, an assessment of *Sch. pombe* cell viability in the presence of orange EO was not performed, since this characteristic would lead to significant errors during the counting of cells microscopically and by CFUs. It would also be interesting to conduct a quantitative differential expression study with *Sch. pombe* genes, in order to understand how *Sch. pombe* respond in the presence of orange EO.

5. REFERENCES

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6. APPENDIX

Table 6.1- List of primers used in this study.

Primer	Sequence (5' - 3')	Description
ACT1Fw	TCCATCATGAAGTGTGATGTCGATG	Amplification of <i>ACT1</i> gene of <i>Torulaspora delbrueckii</i> strain PYCC 4639
ACT1Rv	AGAACCACCGATCCAGACGGA	
ERG6Fw	GCGCCAAGTCTGGAAGGTG	Amplification of <i>ERG6</i> gene of <i>Torulaspora delbrueckii</i> strain PYCC 4639
ERG6Rv	AGCGCTCTTGAGTGCCTCTT	
GPX2Fw	GCTCAGTTCTGTAAGACTAAGTATGG	Amplification of <i>GPX2</i> gene of <i>Torulaspora delbrueckii</i> strain PYCC 4639
GPX2Rv	CAATTCAGGCTCCAAAGTGTGTG	
PIR3Fw	TCGGTTCCATTGTCGCTAACAG	Amplification of <i>PIR3</i> gene of <i>Torulaspora delbrueckii</i> strain PYCC 4639
PIR3Rv	TTAACAGCTAACAAGGTCGATGAC	

Table 6.2- RT-qPCR program performed for amplification of genes *ACT1*, *ERG6*, *PIR3* and *GPX2*.

RT-qPCR Program	
95°C- 2 min	
95°C- 5seg	} X 40 cycles
58°C- 10seg	
72°C- 20seg	

Table 6.3- Growth parameters of each clone selected from culture T.d-B after 3 passage to medium without selective pressure. The growth rate (μ) was obtained by calculating the slope in the microbial exponential phase. The value of latency time was calculated by the intercept of the line developed in the exponential phase of growth and the horizontal line associated with lag time (lag phase was the time that the culture remained at the initial absorbance).

STRAINS	E1900dT	E2900dT	E3900dT	E4900dT	E5100dT	E6100dT	E7100dT	E8100dT	E9100dT	E10100dT	E11900dT	E12100dT	E13100dT	E14100dT	E15100dT	E16900dT	E18100dT	E19100dT	E20100dT	NE1900dT	NE2900dT	NE3100dT	WT0	WT1001	WT1002
μ (h ⁻¹)	0,22	0,24	0,23	0,00	0,08	0,00	0,05	0,00	0,24	0,20	0,11	0,00	0,11	0,00	0,10	0,14	0,00	0,00	0,00	0,00	0,00	0,00	0,25	0,01	0,03
t _{lag} (h)	2,20	2,51	3,66	>10	6,17	>10	6,23	>10	3,80	4,11	5,76	>10	5,88	>10	6,29	5,29	>10	>10	>10	>10	>10	>10	0,00	>10	>10

Table 6.4- Growth parameters of each clone selected from culture T.d-B after 4 passage to medium without selective pressure. The growth rate (μ) was obtained by calculating the slope in the microbial exponential phase. The value of latency time was calculated by the intercept of the line developed in the exponential phase of growth and the horizontal line associated with lag time (lag phase was the time that the culture remained at the initial absorbance).

STRAINS	E1900dT	E2900dT	E3900dT	E4900dT	E5100dT	E6100dT	E7100dT	E8100dT	E9100dT	E10100dT	E11900dT	E12100dT	E13100dT	E14100dT	E15100dT	E16900dT	E18100dT	E19100dT	E20100dT	NE1900dT	NE2900dT	NE3100dT	WT0	WT1001	WT1002
μ (h ⁻¹)	0,00	0,04	0,05	0,12	0,19	0,00	0,16	0,01	0,00	0,00	0,16	0,15	0,14	0,01	0,00	0,07	0,11	0,00	0,00	0,01	0,01	0,17	0,24	0,01	0,03
t _{lag} (h)	>10	5,75	5,12	6,52	4,39	>10	3,85	>10	>10	>10	5,21	5,39	5,43	>10	>10	4,04	3,86	>10	>10	>10	>10	4,91	0,00	>10	>10

Table 6.5- Growth parameters of each clone selected from culture S.p-B after 3 passages to medium without selective pressure. The growth rate (μ) was obtained by calculating the slope in the microbial exponential phase. The value of latency time was calculated by the intercept of the line developed in the exponential phase of growth and the horizontal line associated with lag time (lag phase was the time that the culture remained at the initial absorbance).

STRAINS	μ (h ⁻¹)	t _{lag} (h)
E1300PS	0,09	2,11
E2300SP	0,03	2,00
E360SP	0,05	0,00
E460SP	0,05	0,00
E5900SP	0,03	0,00
E6900SP	0,05	0,00
E7900SP	0,06	0,00
E860SP	0,04	>10
E9300SP	0,06	3,18
E10300SP	0,05	2,69
E11300SP	0,14	3,38
E12300SP	0,04	0,00
E13300SP	0,05	0,00
E14300SP	0,03	10
E15300SP	0,06	0,00
E16300SP	0,02	>10
E17300SP	0,00	>10
E18300SP	0,04	0,00
E19300SP	0,03	0,00
E2060SP	0,06	0,00
NE1300SP	0,05	0,00
NE260SP	0,09	0,00
NE360SP	0,07	2,00
WT0	0,20	0,00
WT601	0,02	>10
WT601	0,02	>10

Table 6.6- Growth parameters of each clone selected from culture S.p-B after 4 passages to medium without selective pressure. The growth rate (μ) was obtained by calculating the slope in the microbial exponential phase. The value of latency time was calculated by the intercept of the line developed in the exponential phase of growth and the horizontal line associated with lag time (lag phase was the time that the culture remained at the initial absorbance).

STRAINS	μ (h ⁻¹)	t _{lag} (h)
E1300PS	0,20	0,00
E2300SP	0,04	0,00
E360SP	0,05	>10
E460SP	0,00	>10
E5900SP	0,00	>10
E6900SP	0,00	>10
E7900SP	0,00	>10
E860SP	0,06	6,67
E9300SP	0,00	>10
E10300SP	0,00	>10
E11300SP	0,00	>10
E12300SP	0,20	6,01
E13300SP	0,00	>10
E14300SP	0,00	>10
E15300SP	0,03	4,65
E16300SP	0,00	>10
E17300SP	0,12	6,33
E18300SP	0,00	>10
E19300SP	0,24	6,16
E2060SP	0,00	>10
NE1300SP	0,19	4,79
NE260SP	0,25	6,09
NE360SP	0,07	7,34
WT0	0,22	0,00
WT601	0,00	>10
WT601	0,00	>10

Table 6.7- Growth parameters of each clone selected from culture *S.p-B* after 4 passages to medium without selective pressure (replicate). Repetition of screening from *S.p-B* culture after 4 passages to medium without selective pressure. The growth rate (μ) was obtained by calculating the slope in the microbial exponential phase. The value of latency time was calculated by the intercept of the line developed in the exponential phase of growth and the horizontal line associated with lag time (lag phase was the time that the culture remained at the initial absorbance).

STRAINS	E1300PS	E2300SP	E360SP	E460SP	E5900SP	E6900SP	E7900SP	E860SP	E9300SP	E10300SP	E11300SP	E12300SP	E13300SP	E14300SP	E15300SP	E16300SP	E17300SP	E18300SP	E19300SP	E2060SP	NE1300SP	NE260SP	NE360SP	WT0	WT601	WT601
μ (h ⁻¹)	0,00	0,10	0,10	0,05	0,05	0,08	0,08	0,09	0,12	0,15	0,00	0,09	0,06	0,20	0,11	0,14	0,07	0,18	0,21	0,13	0,22	0,09	0,00	0,20	0,00	0,00
t _{lag} (h)	>10	3,84	5,78	2,12	0,00	3,28	3,39	6,17	7,76	7,79	>10	6,09	2,18	8	6,84	0,20	4,97	7,92	3,28	1,12	3,42	0,00	>10	0,00	>10	>10