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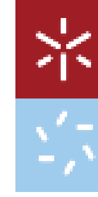
Ana Rita Pereira da Rocha

Use of natural yeast isolates to produce succinic acid: biotechnological exploitation of *Torulaspora delbrueckii*

Outubro 2019



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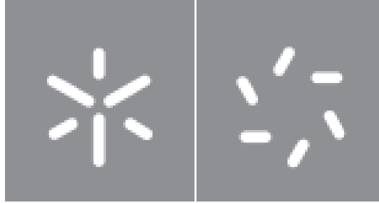

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Use of natural yeast isolates to produce succinic acid: biotechnological exploitation of *Torulaspora delbrueckii*

Dissertação de Mestrado
Mestrado em Genética Molecular

Trabalho efetuado sob a orientação de
Doutor Ricardo Filipe Azevedo Franco Duarte
Professora Doutora Célia do Sacramento Santos Pais

Outubro 2019

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// Agradecimentos

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STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

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Utilização de isolados naturais de leveduras para a produção de ácido succínico: exploração biotecnológica de *Torulaspora delbrueckii*

Resumo

O ácido succínico (AS) é um químico de plataforma, atualmente produzido pela conversão química do anidrido maleico através de processos petroquímicos. Este ácido tem um importante papel como precursor para a síntese de poliésteres biodegradáveis, resinas, corantes, fármacos, como aditivo na indústria alimentar e muito mais. No entanto, o processo petroquímico implica altos custos e vários problemas ambientais, por isso outras alternativas precisam de ser encontradas para produzir AS. Nos últimos anos, o uso de leveduras para a produção do AS começou a ser um dos maiores desafios para os investigadores. O principal objetivo deste trabalho é avaliar a produção de AS por isolados de *Torulaspora delbrueckii*. Adicionalmente, testamos também a capacidade desta levedura crescer tendo como fonte de carbono o AS de forma a determinar se tem transportadores de membrana para esse ácido.

Inicialmente, dez espécies de leveduras foram exploradas para identificar a melhor espécie produtora de AS. Os dois isolados com maior produção foram estripes de *T. delbrueckii*, o que se encontra em concordância com vários estudos que afirmam que *T. delbrueckii* é promissora para a produção de AS. Seguidamente, uma vez que o objetivo é produzir AS a nível industrial é necessário arranjar uma maneira de obter altos rendimentos. A fim de alcançar isto, nove estirpes de *T. delbrueckii* foram testadas em diferentes condições experimentais, para otimizar a concentração de AS produzido. O valor mais alto obtido foi de 1,5 g/L. Apesar deste valor ser baixo, já é considerado promissor sendo que foi obtido usando isolados naturais. De seguida, com o objetivo de tentar aumentar a produção de AS, uma estirpe de *T. delbrueckii* (T12) foi submetida a condições de stresse, em particular à presença de altas concentrações de etanol. A levedura selecionada quando exposta a etanol não produziu AS, concluindo que a presença de etanol no meio leva a uma condição de stresse que interrompe a produção de AS pela levedura, no entanto quando se transfere as células adaptadas a etanol para um meio sem etanol há um aumento na concentração do succinato. Mais tarde, quatro estirpes de *T. delbrueckii* foram testadas considerando o crescimento num meio sólido e líquido com AS como fonte de carbono, de modo a perceber se esta levedura consegue crescer bem na presença deste ácido, o que pode indicar que possuía um sistema de transporte capaz de transportar o ácido para dentro da célula. Os resultados mostram que uma estirpe *T. delbrueckii* foi capaz de utilizar o AS como fonte de carbono, com um crescimento considerável, indicando que o sistema de transporte pode estar presente. No entanto, a sua presença deve ser dependente da estirpe porque apenas uma estirpe das quatro testadas é que foi capaz de crescer de modo semelhante ao controlo positivo. Este é o primeiro estudo que mostra que as células

de *T. delbrueckii* podem apresentar transportadores para o AS e trabalhos posteriores serão necessários para confirmar e identificar que tipo de transportadores poderão estar presentes.

Palavras-chave: Ácido succínico, levedura, *Torulaspora delbrueckii*, transportadores

Use of natural yeast isolates to produce succinic acid: biotechnological exploitation of *Torulaspora delbrueckii*

Abstract

Succinic acid (SA) is a platform chemical currently produced by chemical conversion of maleic anhydride through petrochemical processes. This acid plays an important role as a precursor for the synthesis of biodegradable polyesters, resins, dyestuff, pharmaceuticals, as food industry additive and many more. However, petrochemical process entails high costs and several environment problems, therefore other alternatives need to be found to produce SA. In recent years, the use of yeasts for SA production has become one of the main challenges of researchers. The main aim of this work was the evaluation of isolates of *Torulaspora delbrueckii* for SA production. In addition, we tested the capacity of this yeast to grow having SA as carbon source in order to understand if has membrane transporters for this acid.

Initially, a screening comprising ten yeast species was performed to identify the best species for SA production. The two isolates with the highest production were *T. delbrueckii* strains, which is in agreement with several studies stating *T. delbrueckii* as a promising yeast for succinate production. Further on, once the goal is to produce SA at industrial level it was necessary to find a way to obtain high yields. In order to achieve that, nine *T. delbrueckii* strains were tested in different experimental conditions, to optimize the concentration of SA produced. The higher value obtained was 1.5 g/L of SA. Although this value is low, it is already considered as promiser since it was obtained using natural isolates. With the objective of trying to increase SA yields, *T. delbrueckii* strain (T12) was subjected to conditions of stress, in particular by the presence of high ethanol concentrations. The selected yeast when exposed to ethanol, failed to produce SA concluding that the presence of ethanol in the medium leads to a stressful condition that halts the production of SA by the yeast, however when transferring cells adapted to ethanol to a medium without ethanol there is an increase in the concentration of succinate. Afterwards, four *T. delbrueckii* strains were tested considering growth in a solid and aqueous media containing SA as sole carbon source, in order to understand if this yeast could grow well in the presence of this acid, which could be an indication of some transport system that could lead the acid inside the cell. Results showed that one *T. delbrueckii* strain was able to efficiently use SA as carbon source, with considerable growth, indicating that a transport system could be present. However, its presence must be strain dependent because only one of the four tested strains was able to grow similarly to the positive control. This is the first study showing that *T. delbrueckii* cells could display transporters for the uptake of SA, and further work is mandatory to confirm and identify which kind of transporters are present.

Keywords: Succinic acid, yeast, *Torulaspora delbrueckii,* transporters

Table of Contents

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS ii

Agradecimentos iii

STATEMENT OF INTEGRITY v

Resumo vi

Abstract viii

List of abbreviations and acronyms xii

List of Figures xiii

List of tables xiv

List of equations xiv

Chapter 1: INTRODUCTION 1

1. Drivers for biobased chemicals 2

1.1. Succinic acid (SA) 2

2. Microbial succinate production 4

2.1. *Saccharomyces cerevisiae* yeast 5

2.2. Non-*Saccharomyces* yeasts 5

2.1.1. *Torulaspora delbrueckii* 8

3. Metabolic engineering 9

3.1. Succinate formation pathway 9

3.1.1. Oxidative pathway of TCA cycle 10

3.1.2. Reductive branch of TCA cycle 11

3.1.3. Oxidative pathway of the glyoxylate cycle 11

3.2. SA production by genetically engineered strains 12

3.2.1. Metabolic engineering strategies in *Saccharomyces cerevisiae* 15

3.2.2. Metabolic engineering strategies in non-*Saccharomyces* yeasts 16

4. Analytical methods for metabolite quantification 18

4.1. High performance liquid chromatography (HPLC) ... 18
4.1.1. HPLC Advantages ... 19
4.1.2. HPLC Instrumentation ... 20
Chapter 2: AIMS ... 21
Chapter 3: MATERIALS AND METHODS ... 23
1. Yeast strains ... 24
2. Culture media ... 26
3. Yeast growth conditions ... 28
3.1. Metabolite quantifications ... 28
3.1.1. Screening from different yeast species for SA production ... 28
3.1.2. Optimization of experimental conditions for SA production ... 28
3.1.3. Production of SA by yeast under optimized conditions and under stress conditions ... 28
3.1.4. Chromatography analysis of SA production ... 29
3.1.5. Data analysis ... 30
3.2. Growth of yeasts in a medium that contains SA as a sole carbon source ... 30
4. The concentration of SA in metabolic engineered strains ... 31
Chapter 4: RESULTS ... 33
1. Screening of different yeast species for SA production ... 34
1.1. Evaluating of SA production ... 34
1.2. Evaluation of other compounds of interest ... 37
2. Optimization of experimental conditions for SA production ... 38
2.1. Optimization of the medium ... 38
2.2. Optimization of the agitation rates ... 40
3. Production of SA by the selected yeast ... 43
3.1. Selected yeast under optimized conditions ... 43
3.2. Effects of ethanol concentration in SA production ... 44

4. Growth of yeasts in medium that contains SA as sole carbon source 47
4.1. Solid medium 47
4.2. Aqueous medium 49
5. SA production by genetically modified yeasts 50
Chapter 5: DISCUSSION 52
Chapter 6: REFERENCES 61
Chapter 7: APPENDIX 70

List of abbreviations and acronyms

ANOVA	Analysis of variance
ATP	Adenosine triphosphate
CoA	Coenzyme A
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
DOE	Department of energy
EUROSCARF	European *Saccharomyces cerevisiae* archive for functional analysis
GC	Gas chromatography
GHG	Greenhouse gases
GRAS	Generally recognized as safe
HCi	Citric acid
HMa	Malic acid
HPLC	High performance liquid chromatography
HTa	Tartaric acid
ITS	Internal transcribed spacer
kt	Kiloton
Mb	Megabase
SM	Synthetic medium
NADH	Nicotinamide adenine dinucleotide
OD	Optical density
ORF	Open reading frames
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDC^-	pyruvate-decarboxylase-negative
RI	Refractive index
Rpm	Rotations per minute
SA	Succinic acid
SD	Standard deviation
sSM	Simplified synthetic medium
TAE	Tris-acetate-EDTA
TCA	Tricarboxylic acid
USD	United states dollar
YNB	Yeast nitrogen base
YPD	Yeast extract peptone dextrose
YPG	Yeast extract peptone glycerol

List of Figures

Figure 1. Succinic acid production by petrochemical production via maleic anhydride and through alternative production from renewable resources via glucose 3

Figure 2. Major metabolic pathways for succinate formation 10

Figure 3. Method of separation (HPLC) used in this work............ 19

Figure 4. Schematic diagram of the components of a HPLC system 20

Figure 5. Illustration of T12 stress experiment. 29

Figure 6. Concentration of succinic acid obtained by HPLC analysis using 13 distinct yeast isolates, in two media (M1- glucose as carbon source, and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h). 36

Figure 7. Concentration of succinic acid obtained by HPLC analysis using two *T. delbrueckii* strains, in the four tested media (YPD, M3, and M4- glucose as carbon source and M2- sucrose as carbon source) in two time-points of growth (120h and 192h). 39

Figure 8. Concentration of succinic acid obtained by HPLC analysis using seven *T. delbrueckii* strains in two media (YPG- glycerol as carbon source and M3- glucose as carbon source) in two time-points of growth (120 h and 192 h) at two agitation rates: 75 rpm and 180 rpm. 41

Figure 9. Concentration of succinic acid obtained by HPLC analysis using *T. delbrueckii* (T12) strain in M3 medium, in 8 time-points of growth (24,48,72,96,120,192 h) 43

Figure 10. Concentration of succinic acid obtained by HPLC analysis using *T. delbrueckii* (T12) strain, in three media (M3- the control medium and M3+4 %- medium with 4 % of ethanol and M3+8 %- medium with 8 % of ethanol), in four time-points of growth (24 h,48 h,72 h,96 h) 45

Figure 11. Growth curve of the *T. delbrueckii* (T12) strain, in the control medium (M3) and in medium with 4 % and 8 % of ethanol 45

Figure 12. Concentration of succinic acid obtained by HPLC analysis using *T. delbrueckii* (T12) yeast strain, in M3 medium but with three different pre-inoculum: cells grown of YPD (M3), as control; cells grown of medium with 4 % of ethanol - M3(4 %); cells of medium with 8% of ethanol - M3(8 %)............ 46

Figure 13. Drop tests at 30 °C and 18 °C, of four *Torulaspora delbrueckii* strains (T11, T12, T13, T14) and three other species: *Schizosaccharomyces pombe* (DB387) and *Debaryomyces hansenii* as positive control (DB600) *and Saccharomyces cerevisiae* as negative control (DB34)............ 48

Figure 14. Cell growth of 7 yeast strains in YNB supplemented with 1 % of succinic acid as carbon source, with a pH of 5.0 49

Figure 15. Concentration of succinic acid obtained by enzymatic kit in nine *S. cerevisiae* strains; eight strains with mutated genes and one *S. cerevisiae* under normal conditions, as control....................... 51

List of tables

Table 1. SA production using natural non-*Saccharomyces* yeasts (*Yarrowia lipolytica*, *Candida stellata*, and *Candida cantarelli*) and the conditions of each experiment, the carbon source used, incubation temperature and agitation rates, and the pH of the medium, as revised from literature. 7

Table 2. Main metabolic engineering strategies to improve succinate production in *Saccharomyces cerevisiae* and in non-*Saccharomyces* species (*Yarrowia lipolytica* and *Issatchenkia orientalis*). 14

Table 3. List of the 21 yeast strains used in this work... 25

Table 4. Composition of culture media used throughout experiments... 27

Table 5. Composition of complex culture media used throughout experiments.................................. 27

Table 6. Composition of the 6 bottles presented in the enzymatic kit. 31

Table 7. List of the eight mutated genes.. 50

List of equations

Equation 1. Calculation to obtain succinic acid concentration. 32

Chapter 1: INTRODUCTION

1. Drivers for biobased chemicals

The concern about the environment, including climate change, sustainability, and energy security, has been increasing in our days. One of the most important environmental challenges is to reduce the production of chemical processes through fossil fuel resources and to start using more sustainable procedures to produce bio-based chemicals and materials from renewable resources. In fact, this high interest is mainly due to the rapid increase in greenhouse gases (GHG) concentration in the atmosphere, the main cause of global warming and climate change (Ong, *et al.*, 2019). Approximately 65% of these GHG are released into the atmosphere, as carbon dioxide (CO_2), sulfur dioxide (SO_2), nitrous dioxide (N_20), among others due to the combustion process of fossil fuels (Tanha Ziyarati, Bahramifar, Baghmisheh, & Younesi, 2019).

Due to the above exposed reasons, in 2004 the US Department of Energy (DOE) and the European Commission tried to find a solution to control this specific environmental problem (Ahn, Jang, & Lee, 2016). After analyzing several substances based on biomass precursors, the DOE drafted a list of 12 potential platform chemicals that can be biochemically produced and economically viable (Beauprez, Mey, & Soetaert, 2010). Platform chemicals are compounds that serve as building blocks that can be produced from sugars biologically or chemically and subsequently converted into a variety of high value biology-based chemicals or materials using biorefinery approaches.

1.1. Succinic acid (SA)

Succinic acid is a four-carbon dicarboxylic acid with the molecular formula $C_4H_60_4$ and is one of the most useful chemicals of the aforementioned 12 building blocks, currently produced by chemical conversion of maleic anhydride (Ahn, Jang, & Lee, 2016). This acid plays an important role as a precursor for the synthesis of biodegradable polyesters (clothing fibers) as polybutyrate succinate and polyamides, in resins, dyestuff, paints, pharmaceutical products, vehicle water cooling systems, as ion chelator to prevent corrosion, in chemical industries, surfactants and detergents, green solvents and biodegradable plastics, in food industry as flavoring agent, a pH modified and an anti-microbial agent (Kamzolova, Yusupova, Vinokurova, & Morgunov, 2009; Yan, *et al.*, 2014; Zeikus, Jain, & Elankovan, 1999; Morales, *et al.*, 2016; Sauer, Porro, Mattanovich, & Branduardi, 2008; Franco-Duarte, *et al.*, 2017). In medicine, besides being a precursor to pharmaceutical products, SA is also used for therapeutic purposes as anti-

stress, as active immunological agent and as an antihypoxic (Kamzolova, Yusupova, Vinokurova, & Morgunov, 2009). This dicarboxylic acid can be converted into useful chemicals, including 1,4-butanediol, adipic acid, tetrahydrofuran, N-methylpyrrolidone, butyrolactone, and other value-added chemical products (Ito, Hirasawa, & Shimizu, 2014). As a result of its far-reaching applications, in 2015, SA presented an overall market size of about 60 kt and highly optimistic projections believe that in 2020 this value will increase to more than 600 kt (Liebal, Blank, & Ebert, 2018).

However, SA is currently produced petrochemically, which implies high costs and severe environmental problems. The best way to overcome these problems is to find a solution that is highly efficient, environmentally friendly and with cost-effectiveness (Li, Gao, Yang, & Lin, 2017), for example recurring to the use of renewable resources (Figure 1). The present market price of SA lies between 2500 and 3000 united states dollar (USD) per ton, however the price of raw material for maleic anhydride is 1300 USD per ton of produced SA, whereas the raw material cost for sugars like glucose or fructose is way lower, about 500 USD per ton of produced bio-SA (Morales, *et al.*, 2016). The production of SA through fermentation brings benefits to the environment since during this process one mole of CO_2 is fixed per one mole of SA (Ahn, Jang, & Lee, 2016). Consequently, in recent years, microbial production of SA has received increased attention, since besides using sugars from cheap waste streams it also beneficially contributes to the environment (Babaei, *et al.*, 2019).

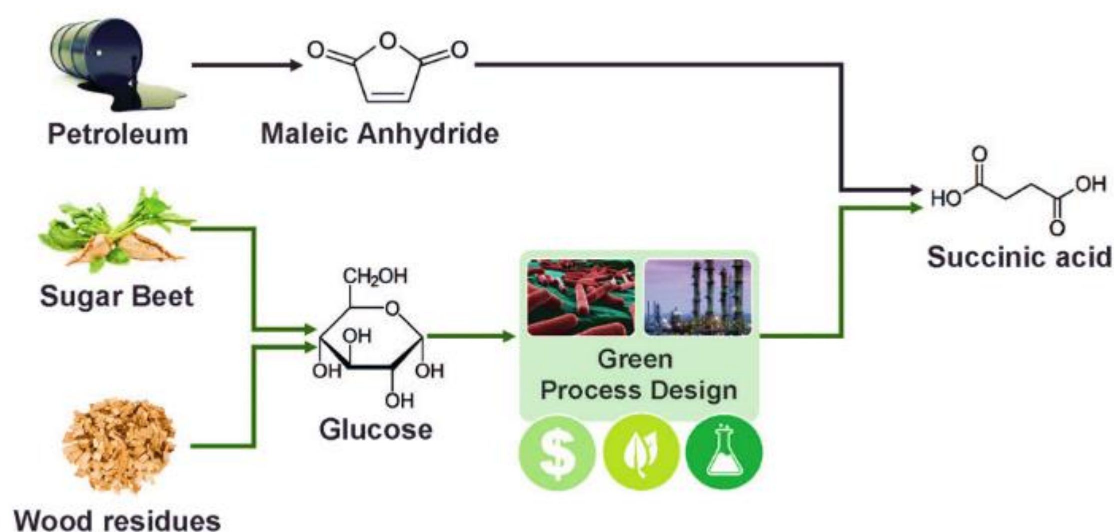

Figure 1. Succinic acid production by petrochemical production via maleic anhydride and through alternative production from renewable resources via glucose. Adapted from (Morales, *et al.*, 2016).

2. Microbial succinate production

Several studies have used many different microorganisms as an intermediate of the central metabolism or as a fermentation end-product to produce SA from various carbon sources. Among them, bacteria have been most intensively studied due to their ability to produce, naturally, a relatively large amount of SA. The bacteria *Actinobacillus succinogenes*, *Mannheimia succiniproducens*, isolated from bovine rumen, the recombinant *Escherichia coli*, *Corynebacterium glutamicum*, *Anaerobiospirillum succiniproducens*, *Prevotella ruminocola*, *Succinivibrio dextrinosolvens* and *Basfia succiniciproducens* have been used in many studies for succinate production (Zheng, Dong, Sun, Ni, & Fang, 2009; Lee, Lee, Chang, & Hong, 2002; Vemuri, Eiteman, & Altman, 2002; Okino, Inui, & Yukawa, 2005; Lee, Cheon, Lee, & Chang, 2008; Ahn, Jang, & Lee, 2016; Agren, Otero, & Nielsen, 2013). Since bacteria naturally produce SA through fermentation, large yields are already expected. In a recent study (Lee, Song, & Lee, 2006), the authors tested the production of SA using *M. succiniproducens* and a production of about 14 g/L of SA was obtained. When using *A. succinogenes*, much higher production of 53.2 g/L was reached. Better results were achieved by Vemuri, Eiteman, & Altman. (2002) with *E. coli* and by Guettler, Rumler, & Jainf, (1999) with *A. succinogenes* that obtained succinate titers of up to 106 g/L. Despite the great interest in producing SA through microorganisms, there is not an economically advantageous process that can compete with petroleum-derived chemical synthesis. The SA purification stage (60-70 % of product cost) is the main limitation of industrial applications through the biotechnological process. Since the obtained product during the fermentation is succinate salt, there is the need to convert this salt into free acid. The solution to this problem is to find a way where the free SA is the main product. However, bacteria strains do not tolerate low pH values, so it is necessary to use neutralizing salts to make the pH of the solution more neutral (Yuzbashev, *et al.*, 2010). In addition, the bacteria are obligate anaerobes, potentially pathogenic, or do not tolerate osmotic stress and high glucose levels (Song & Lee, 2006). These factors limit their industrial applications. Nevertheless, some advantages can be obtained from the use of yeasts to produce SA as the fact of not being necessary the use of salts because yeasts tolerate high acidity values, they are not obligate anaerobes nor potentially pathogenic and they are robust and osmo-tolerant (Raab & Lang, 2011; Pentjuss, *et al.*, 2017).

2.1. *Saccharomyces cerevisiae* yeast

Saccharomyces cerevisiae is undoubtedly the most studied yeast. One key characteristic that has been very well studied lately is their capacity to produce organic acids, specifically to produce SA. This species presents good growth characteristics, such as the capacity to utilize several carbon sources, it is generally recognized as safe (GRAS) which facilitates waste disposal and product approval, and is called robust and scalable to environmental stresses conditions such as low pH (Cui, *et al.*, 2017; Agren, Otero, & Nielsen, 2013). In addition to these characteristics, the physiology and genetics of this yeast are extensively documented. It has a 12 Mb genome divided into 16 chromosomes, that encodes more than 5400 genes with a well-developed metabolic engineering toolbox available and the CRISPR–Cas9 system enables efficient genomic insertion of DNA fragments as well as mutations or deletions (Tondini, Lang, Chen, Herderich, & Jiranek, 2019; Zahoor, Küttner, Blank, & Ebert, 2019). Another reason why some researchers started to study yeasts instead of bacteria for SA production was the availability of the EUROSCARF (European *S. cerevisiae* archive for functional analysis) collection center. This center was created in 1994 for the purpose of delivering biological materials generated in genome analysis networks and has already about 5100 genetically mutated strains, covering 82% of the ~6200 annotated yeast ORFs (open reading frames). These mutations consist of genes deletion from the yeast genome by reverse genetics and it is already possible to easily access each mutant of *S. cerevisiae* or even, when the deletion cassette is also available, to introduce such deletions into any strain of *S. cerevisiae* (Entian & Kötter, 1998).

2.2. Non-*Saccharomyces* yeasts

Even though non-*Saccharomyces* yeasts continue to be termed as unconventional yeasts and although there is little information on their genetic and biological characterization, there are already some studies comparing non-*Saccharomyces* yeasts to *S. cerevisiae* and stating that these unconventional yeasts have certain advantages that *S. cerevisiae* does not have. As for example, *S. cerevisiae* yeast is characterized by growing in high sugar concentrations, most of which transformed in ethanol. However, in natural habitats, there is not so much availability of carbon source and non-*Saccharomyces* yeasts use other alternative routes for substrate utilization and product formation, deflecting carbon into other metabolites, avoiding the ethanol formation (Pais, *et al.*, 2016; Contreras, *et al.*, 2015).

As mentioned before, the attention to the use of non-*Saccharomyces* yeasts is increasing, for example, *Yarrowia lipolytica* has been one of the most studied non-*Saccharomyces* yeasts for the SA production, for several reasons. Firstly, although it is an unconventional and strictly aerobic yeast, it accumulates a large amount of SA with a good yield (Cui, *et al.*, 2017). Secondly, it can carry out the entire production process using various types of carbon sources such as glucose, ethanol, glycerol, acetate as well as lipids and fatty acids. Besides, it is very tolerant to environmental stress, can withstand low temperatures and acidic and alkaline pH (Gao, *et al.*, 2016). Finally, this species is already well studied in the literature and therefore it is considered as a safe species because the sequence of the genomic DNA of several strains is known, as well as its genetic tools like CRISPR-Cas9 mediated gene editing, DNA Assembler and Golden Gate Assembly system (Cui, *et al.*, 2017; Yuzbashev, *et al.*, 2010).

There are other non-*Saccharomyces* yeasts of interest for SA production such as *Pichia pastoris*, *Hansenula polymorpha*, *Pichia stipitis*, *Kluyveromyces marxianus*, *Candida stellata*, *Candida cantarelli* and *Torulaspora delbrueckii* (Pais, *et al.*, 2016; Ferraro, Fatichenti, & Ciani, 2000). In fact, there are already studies with some of these yeasts. In Table 1, some of the most relevant studies accomplished to date to produce SA are revised, demonstrating the producing of relevant quantities of this acid without any metabolic engineering, regardless of the type of carbon source and experimental conditions used.

Table 1. SA production using natural non-*Saccharomyces* yeasts (*Yarrowia lipolytica, Candida stellata*, and *Candida cantarelli*) and the conditions of each experiment, the carbon source used, incubation temperature and agitation rates, and the pH of the medium, as revised from literature.

Yeast	Strain	Carbon source	Incubation T°	Agitation rates	SA production	pH	Reference
Y. lipolytica	VKM Y-2412	Ethanol	28°C	130 rpm	63.4 g/L after 8 days	4.5-5.5	[1]
	VKM Y-2412	Rapeseed oil	30°C	800 rpm	69.0 g/L after 156 h	3.5-4.5	[2]
	VKPM Y3753	Glucose	30°C	250 rpm	47.1 g/L after 68h	NA	[3]
C. stellata	DBVPG 3827	Glucose and Fructose	25°C	NA	1.2 g/L after 14 days	3.5	[4]
C. cantarelli	BCc479	Glucose	28°C	250 rpm	0.79 g/l after 696 h	NA	[5]

[1] - (Kamzolova, Yusupova, Vinokurova, & Morgunov, 2009)

(2) - (Kamzolova, *et al.*, 2014)

[3] - (Bondarenko, Fedorov, & Sineoky, 2017)

[4] - (Ciani & Ferraro, 1998)

[5] - (Toro & Vazquez, 2002)

NA: Not available

2.1.1. *Torulaspora delbrueckii*

As previously mentioned, another yeast of major interest for the SA production is *T. delbrueckii* that is considered as spoilage in food like salads, vegetables, meats, and dairy products. Besides *T. delbrueckii*, five more species belong to this genus: *T. franciscae*, *T. pretoriensis*, *T. microellipsoides*, *T. globose* and *T. maleeae*. The genus *Torulaspora* reproduces both: asexually and sexually; asexually by cell division in multilateral budding, and sexually across asci that have one to four spherical ascospores. The cells of this species are spherical to ellipsoidal, with dimensions of approximately 2-4 x 3-5 μm (Benito, 2018).

T. delbrueckii is the most popular non-*Saccharomyces* yeast in winemaking because it has good oenological characteristics such as a high concentration of esters, higher alcohols, phenolic aldehydes, terpenes, 2-phenylethanol, linalool and methylvanilin (Sanoppa, Huang, & Wu, 2019). In fact, *T. delbrueckii* was one of the first yeasts industrially marketed to be released, but only five strains are accessible: Prelude™, Biodiva™, Zymaflore® Alpha, Vinifer NS TD and Primaflora® VB BIO (Benito, 2018). Wine fermentation results in a very large set of metabolites, including SA, that it is one of the most important by-products affecting the quality of the wine (Andorrà, *et al.*, 2019). Some studies claim that this acid contributes positively to the analytical profile of wines, increasing their acidity and as being responsible for antibacterial activities (Robles, Fabjanowicz, Chmiel, & Płotka-wasylka, 2019; Jolly, Varela, & Pretorius, 2014). Some authors also considered this species as a promiser yeast for SA production due to its reduced production of undesirable by-products mainly acetic acid and ethanol (Belda, *et al.*, 2017; Puertas, Jiménez, Cantos-Villar, Cantoral, & Rodríguez, 2016). These two compounds are part of the pathways that lead to SA production so the lower the concentration of these metabolites, the higher the SA concentration. Recent studies investigated different strains of *T. delbrueckii* concluding that despite being the same species, there are many differences in their fermentation ability and in the concentrations of metabolites produced (Benito, 2018).

This yeast species was initially known by other designations such as *T. rosei*, *T. fermentati*, *T. vafer,* and *S. rosei*, now considered as synonyms recurring mainly to new techniques of DNA sequencing. This species is therefore considered as taxonomically similar to *Saccharomyces*, however, it turned out that their metabolism is very different, especially under hyperosmotic stress, as well as their genomes. *T. delbrueckii* features a genome with 9.7 Mb, divided into 8 chromosomes and 5000 ORFs (Tondini, Lang, Chen, Herderich, & Jiranek, 2019; Benito, 2018).

Despite the fact that majority of works with *T. delbrueckii* have not focused deeply on SA production, some studies revealed already that this yeast produces SA in higher amounts than *S. cerevisiae*. Ciani & Maccarelli. (1997) reported that *T. delbrueckii* take over *S. cerevisiae* in what regards SA production. Contreras *et al.* (2014) reviewed that this yeast can reach production yields higher than 1 g/L, depending on oxygen availability during fermentation. According to Puertas, Jiménez, Cantos-Villar, Cantoral, & Rodríguez. (2016), *T. delbrueckii* produced values between 0.84 g/L and 1.11 g/L while *S. cerevisiae* values reached a maximum of 0.65 g/L. Liu, Laaksonen, Kortesniemi, Kalpio, & Yang. (2018) reported that *T. delbrueckii* strains compared to *S. cerevisiae* strains, produced the highest values of SA, in pure and sequential fermentations, and a reduction in ethanol and acetic acid.

3. Metabolic engineering

Biotechnology consists of the use of cellular and biomolecular processes in order to develop technologies and products that help to improve our life and our planet. However, this process does not always have the best results, due to their limited yields, concentrations, productivities and difficulties in the product recovery from the broth, or the need for pretreatment of most of the raw substrates (Cheng, Wang, Zeng, & Zhang, 2013). Nevertheless, the metabolic engineering strategy can improve all these limitations, mainly to produce SA in high amounts through microorganisms. With metabolic engineering, it is possible to optimize existing pathways and introduce new genes or delete certain genes into the pathway, with the aim to produce and increase succinate as an end product of fermentation when sugar or glycerol is used as a carbon source (Ito *et al.*, 2014).

3.1. Succinate formation pathway

In yeast, the SA production process begins in the cytosol due to glycolysis, where the glucose molecule will give rise to two molecules of pyruvate. Glycolysis provides yeasts with energy, together with precursor molecules and reducing power for biosynthesis pathways. It is after this process that there will be a variation depending on the path that the yeasts follow. There are three pathways for succinate formation, which are illustrated in Figure 2: oxidative pathway of the TCA cycle, a reductive branch of the TCA cycle and oxidative pathway of the glyoxylate cycle. The TCA cycle can be found in yeast mitochondria and it can be used in energy production (ATP), amino acid and lipid synthesis (Rezaei, Aslankoohi, Verstrepen, & Courtin, 2015).

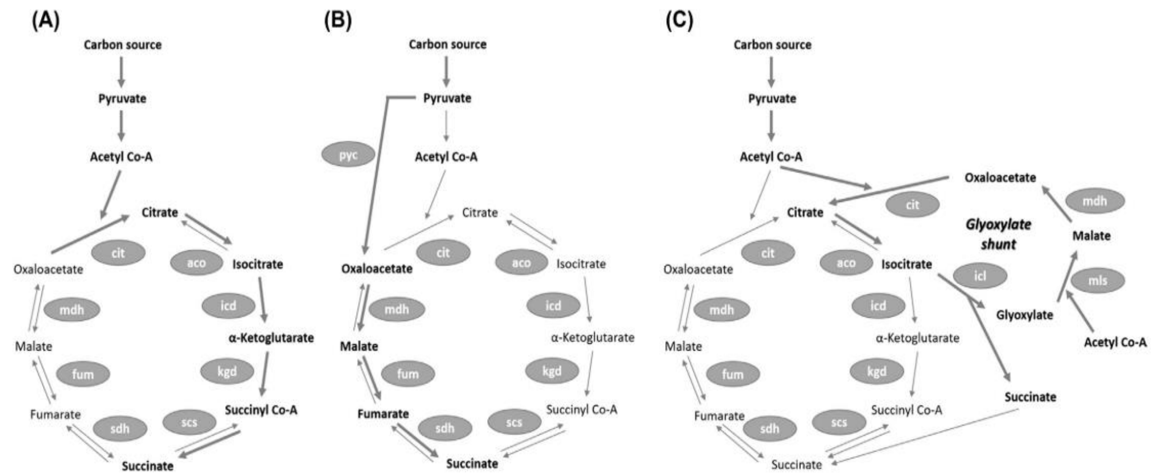

Figure 2. Major metabolic pathways for succinate formation. Arrows indicate major routes for succinate synthesis starting from carbon source; (A) oxidative pathway of the TCA cycle (aerobic conditions) (B) reductive branch of the TCA cycle (anaerobic conditions) (C) oxidative pathway of the glyoxylate cycle (aerobic conditions). Not all enzymatic steps are shown. Abbreviations used for enzymes are: *cit* (citrate synthase); *aco* (aconitase); *icd* (isocitrate dehydrogenase); *kgd* (α-ketoglutarate dehydrogenase); *scs* (succinyl-coenzyme A synthetase); *sdh* (succinate dehydrogenase); *fum* (fumarase); *mdh* (malate dehydrogenase); *pyc* (phosphoenol pyruvate carboxylase); *icl* (isocitrate lyase); *mls* (malate synthase). Adapted from (Pais, *et al*., 2016).

3.1.1. Oxidative pathway of TCA cycle

Succinate can also be produced through the oxidative pathway of the TCA cycle under oxygen-containing conditions, the aerobic metabolism. In this process, pyruvate will give origin to acetyl-CoA, which is converted to citrate (encoded by the *CIT* gene). The cycle begins with the conversion of citrate into isocitrate through an enzyme called aconitase (*ACO1*) and is subsequently transformed into the α-ketoglutarate molecule by isocitrate dehydrogenase which is encoded by the *IDH* and *IDP1* genes. The α-ketoglutarate molecule will then be decarboxylated by α-ketoglutarate dehydrogenase and form succinyl-CoA. Finally, succinyl-CoA is converted into succinate by succinyl-CoA synthetase (encoded by the *LCS1* gene). However, succinate is subsequently converted to fumarate by succinate dehydrogenase because the succinate under aerobic conditions is only an intermediate of the TCA cycle. To solve this problem, it is necessary to deactivate the *SDH* genes, which is a determinant factor in the transformation of succinate into fumarate and therefore the final product becomes succinate (Cheng, Wang, Zeng, & Zhang, 2013).

3.1.2. Reductive branch of TCA cycle

The reductive branch of the TCA cycle is also known as the fermentative pathway under anaerobic conditions when succinate is the H-acceptor instead of oxygen. In this via, SA production occurs after four steps. In the first step, phosphoenolpyruvate, which is the ester derived from the enol of pyruvate and phosphate, is converted to oxaloacetate by the enzyme pyruvate carboxylase that is encoded by the *PYC* gene. In the second step, the enzyme malate dehydrogenase, codified by the gene *MDH*, is activated and thus the oxaloacetate is reduced to malate. In the third step, the malate is transformed into fumarate due to the action of the gene *FUM*. In the fourth and final step, succinate is finally obtained with the reduction of the fumarate, which is encoded by genes *FRDS1* and *OSH1*. This process has the main advantage of setting the CO_2 and not releasing it, which brings enough benefits to reduce global warming, and has a higher theoretical yield than the other pathways, about 1.714 mol per mol of glucose (Cheng, Wang, Zeng, & Zhang, 2013; Yan, *et al.*, 2014). Nevertheless, it is not the best alternative for the production of SA because it entails some obstacles such as: (1) the limitation of NADH since to produce succinate it is necessary 2 moles of NADH, but in the process of glycolysis 1 molecule of glucose provide only 2 molecules of NADH. Thus, in the end, with 1 molecule of glucose, only 2 molecules of succinate are obtained, which is not satisfactory. (2) The genes encoding the reduction of fumarate (*FRDS1* and *OSH1*) can only be expressed under anaerobic conditions (Yan, *et al.*, 2014). (3) The fact that yeast fumarate (*FUM*) is only able to convert the malate into fumarate and not the reversible process (Pines, *et al.*, 1996). (4) Not to mention that this process results in an almost uniform energy balance, not providing any ATP for maintenance and active transport processes (Raab & Lang, 2011).

3.1.3. Oxidative pathway of the glyoxylate cycle

The glyoxylate shunt is also a possible route to produce SA under aerobic conditions, but the main source of carbon is acetate rather than glucose because this sugar is absent in this via (Beauprez, Mey, & Soetaert, 2010). The glyoxylate cycle is very similar to the TCA cycle, has almost all the intermediate steps, sharing three enzymes out of the five. However, the whole process is in the yeast cytosol, while the TCA cycle is in the mitochondria (Raab & Lang, 2011). This pathway converts 2 mol acetyl CoA per mole of succinate production (Cheng, Wang, Zeng, & Zhang, 2013). When cells grow at

high glucose concentrations, isocitrate lyase and malate synthase enzymes are suppressed. The activation of isocitrate lyase (encoded by gene *ICL1*) converts isocitrate into glyoxylate and succinate. Then activation of malate synthase converts glyoxylate into malate (Rezaei, Aslankoohi, Verstrepen, & Courtin, 2015).

An efficient alternative for SA production is the use of both the TCA reductive cycle and the glyoxylate shunt, linking the oxidative and reducing routes, providing a positive energy balance and an even redox balance at the same time (Franco-Duarte, *et al.*, 2017). In this way, a succinate value of 1.71 mol per mol of glucose is reached. Nevertheless, its implementation will be a challenge in the subsequent years because it is complicated to achieve since it requires an oxidative and fermentative metabolism to occur at the same time, although this strategy has already been successful in *E. coli* strains (Raab & Lang, 2011).

3.2. SA production by genetically engineered strains

When succinate synthesis is obtained through the TCA reductive cycle, that is, under anaerobic conditions, it sometimes results in by-products accumulation. This accumulation of by-products results in substrate loss, but there are already different strategies for manipulating yeast strains in order to selectively eliminate these by-product pathways and thereby enhance SA production (Cheng, Wang, Zeng, & Zhang, 2013). To obtain an efficient SA production, it is necessary to achieve 3 levels: (1) elimination of alcoholic fermentation, to reduce ethanol production; (2) engineering of metabolic pathways that link glycolytic pathway to the TCA cycle, taking into account NADH and ATP constraints; (3) engineering of product export. Since the goal is to obtain SA and not ethanol the first step is to avoid the path that converts pyruvate into ethanol, which involves two reactions: pyruvate decarboxylase and alcohol dehydrogenase. In the first reaction, pyruvate is decarboxylated to acetaldehyde. In the second reaction, acetaldehyde is reduced to ethanol. Therefore, to reduce or even eliminate ethanol production it is necessary to remove the genes that encode these reactions through metabolic engineering. Some prior works already tested the deletion of the structural genes of alcohol dehydrogenase (*ADH1* to *ADH4*) in *S. cerevisiae*. However, these studies concluded that although the genes were deleted, it did not result in the total elimination of ethanol. It also caused a buildup of large amounts of toxic glycerol and acetaldehyde (Drewke, Thielen, & Ciriacy, 1990). On the other hand, when the genes of pyruvate decarboxylase reaction are deleted, it already results in the total elimination of ethanol. In *S. cerevisiae*

three pyruvate decarboxylase structural genes (*PDC1*, *PDC5,* and *PDC6*) were identified. With the deletion of these genes there is a complete elimination of ethanol because the entire *PDC* complex has been eliminated (Hohmann, 1991; van Maris, *et al.*, 2004). However, pyruvate-decarboxylase-negative (Pdc-) strains grow poorly in complex media and fail to grow in a medium containing only glucose as a carbon source. This process occurs because of the biosynthetic role of pyruvate decarboxylase in the synthesis of cytosolic acetyl-CoA, essential for the synthesis of lysine and lipids. On account of that, with the elimination of *PDC* complex, mitochondrial acetyl-CoA cannot be transported to the cytosol in *S. cerevisiae*. Therefore, the requirement for C2 compounds and a high glucose sensitivity represented major impediments for the use of Pdc– strains for SA production. By applying this two-stage evolutionary engineering process, these limitations can be overcome, and Pdc- *S. cerevisiae* isolates are available showing high concentrations of pyruvate, up to 135 g/L, with a total yield of 0.54 g/g (glucose) (van Maris, *et al.*, 2004). On the other hand, we can always appeal to the use of non-*Saccharomyces* yeasts, since there are already studies that reviewed the low ethanol production in these strains (Jolly, Varela, & Pretorius, 2014).

As mentioned earlier, with metabolic engineering it is possible to increase SA production. There are already many studies that involve the deletion of genes related to the TCA cycle and the glyoxylate shunt (Rezaei, Aslankoohi, Verstrepen, & Courtin, 2015). Table 2 summarizes the main metabolic engineering strategies to improve succinate production in *S. cerevisiae* yeasts and non-*Saccharomyces* yeasts.

Table 2. Main metabolic engineering strategies to improve succinate production in *Saccharomyces cerevisiae* and in non-*Saccharomyces* species (*Yarrowia lipolytica* and *Issatchenkia orientalis*).

Yeast	Strain	Genotype	SA production	References
S. cerevisiae	Kura	*ΔSDH1 ΔFUM1*	2.32 g/L	[1]
	AH22ura3	*ΔSDH1 ΔSDH2 ΔIDH1 ΔIDP1*	3.62 g/L, 0.11 mol/mol glucose	[2]
	8D	*ΔSDH ΔSER3/ SER33*, overexpression of native *ICL1*	0.9 g/L	[3]
	BY4741	*ΔDIC1*	0.23 g/L	[4]
	PMCFf	*ΔFUM1 ΔPDC ΔGPD1*	8.09 g/L	[5]
	S149sdh12	*ΔSDH1 ΔSHD2*, expression of *MAE1* gene	2.36 C-mol yield	[6]
	TAM-6	*ΔGPD1 ΔFUM1*	2.2 g/L	[7]
Y. lipolytica	Y-3312	*ΔSDH1 ΔSDH2*	45 g/L	[8]
	PGC01003	*ΔSDH5*	160.2 g/L	[9]
	PGC202	*ΔYlach*	110.7 g/L	[10]
I. orientalis	SD108	*ΔPYC ΔMDH ΔFUMR ΔFRD*	11.63 g/L	[11]

[1] - (Arikawa, *et al.*, 1999)
[2] - (Raab, Gebhardt, Bolotina, Weuster-botz, & Lang, 2010)
[3] - (Otero, *et al.*, 2013)
[4] - (Agren, Otero, & Nielsen, 2013)
[5] - (Yan, *et al.*, 2014)
[6] - (Ito, Hirasawa, & Shimizu, 2014)
[7] - (Zahoor, Küttner, Blank, & Ebert, 2019)
[8] - (Yuzbashev, *et al.*, 2010)
[9] - (Gao, *et al.*, 2016)
[10] - (Cui, *et al.*, 2017)
[11] - (Xiao, Shao, Jiang, Dole, & Zhao, 2014)

3.2.1. Metabolic engineering strategies in *Saccharomyces cerevisiae*

In *S. cerevisiae* there are already at least 6 studies, with different strains, of metabolic engineering, with the objective to increase SA production yields. The first one was reported by Arikawa *et al*. (1999), using sake yeast strains, where they deleted genes such as *SDH1* and *FUM1* that are part of the TCA cycle. In this study, the authors obtained a production of 2.32 g/L of SA when compared to the wild type, which obtained about 2.7 times more. This only happens if genes are deleted simultaneously since the deletion of the *SDH1* gene only increases the value by about 1.6 times.

Raab, Gebhardt, Bolotina, Weuster-botz, & Lang. (2010), reported that with the deletion procedure of 4 genes (*SDH1*, *SDH2*, *IPH1*, *IDP1*), relevant to oxidative succinate production, the modified strain produced 3.62 g/L of SA when compared to wild type, which had a 4.8 times higher yield. In this second study, there has already been an increase in production compared to the values reported by Arikawa *et al*. (1999), yet there is still a generation of many by-products such as ethanol, glycerol, and others.

Another metabolic engineering strategy to produce SA was carried out by Otero *et al*. (2013). To increase SA production, they decided to eliminate the *SDH3* gene, together with the exclusion of 3-phosphoglycerate dehydrogenase (*SER3* / *SER33*), which consists of glycolysis-derived serine, since serine has an association with succinate. In addition to the elimination of these genes, Otero and colleagues also overexpressed native *ICL1*. The mutant strain obtained a yield of 0.9 g/L of SA compared to the reference strain which had a SA production about 43 times higher.

Another attempt to examine SA production is described by Agren, Otero, & Nielsen. (2013). Initially, they deleted 3 genes (*MDH1*, *OAC1*, *DIC1*) and concluded that only the elimination of the *DIC1* gene did allow an increase in SA production, obtaining a value of 0.23 g/L.

S. cerevisiae has also been studied by Yan *et al*. (2014) in order to obtain a higher SA production. In the report, they deleted 3 genes (*FUM1*, *PDC*, *GPD1*), obtaining a value of 8.09 g/L. After obtaining this value, further attempts were made to improve production. After several tests, it was concluded that, by regulating urea and biotin levels, it is possible to reach a value of 9.98 g/L of SA and by using a bioreactor capable of obtaining optimal CO_2 conditions, higher values can still be obtained (12.97 g/L).

Subsequently, Ito, Hirasawa, & Shimizu. (2014) tested the deletion of *SDH1* and *SDH2* genes with the expression of the *MAE1* malic acid transporter from *Schizosaccharomyces pombe*. With this carrier it was possible to transport the acid out of the cell, increasing the levels of SA in the extracellular part, thus achieving a yield of 2.36 C-mol yield.

Finally, Zahoor, Ebert, & Blank. (2019) explored the potential of the TAM-6 strain that carries the six-step path (*PYC2-PCKA-MDH3-FUMC-FRDS1-MAE*), eliminating two genes: glycerol phosphate dehydrogenase encoding (*GPD1*) and fumarase encoding (*FUM1*). They obtained a maximal titer of 2.2 g/L of SA, almost 2.5 times higher compared to the TAM-6 strain.

3.2.2. Metabolic engineering strategies in non-*Saccharomyces* yeasts

As mentioned before, there are also metabolic engineering strategy studies in non-*Saccharomyces* yeasts. The first was made in *Y. lipolytica* in which the *SDH1* and *SDH2* genes were deleted (Yuzbashev, *et al.*, 2010). The aim of this study was to obtain strains that have temperature-sensitive mutations in the *SDH1* gene through in vitro mutagenesis. These mutants were used to optimize the compound of the media for a selection of transformant strains with the deletion of the gene *SDH2* in *Y. lipolytica*. Although the effects of each succinate dehydrogenase subunit avert the growth on glucose, the mutant strains grew on glycerol and produced succinate in the presence of the buffering agent $CaCO_3$. It was concluded that the strain was capable of accumulating SA with levels above 45 g/L from glycerol in shaking balloon and over 17 g/L without buffering agent.

Another study conducted on *Y. lipolytica* has been described by Gao, *et al.* (2016). The authors designed a PGC01003 succinate production strain by deleting the gene encoding an *SDH5* subunit of succinate dehydrogenase in the Po1f wild type strain and found that, as expected, *SDH* activity decreased. This strain was evaluated for growth and substrate consumption in 7 different media, being the YPG medium the one allowing higher levels of SA production. Meanwhile, under conditions with 200 g/L crude glycerol, PGC01003 produced 5.5 g/L of SA, which was 13 times more than Po1g. The obvious accumulation of SA indicated that the SA pathway leading to fumaric acid production was blocked by the deletion of Ylsdh5. Then, to achieve high SA yield, fed-batch fermentation was carried out. After 400 h of cultivation with six times the feed, the final production of SA reached was 160.2 g/L, which is the largest fermentative production of SA achieved until the date. This result confirmed that the genetically modified strain *Y. lipolytica* PGC01003 can tolerate a very high concentration of SA and have great potential in the fermentative production of it. However, when the strain was grown under low pH conditions, the resultant SA titer (5.2 g/L) was relatively low.

Y. lipolytica was also studied by Cui *et al.* (2017), where the strain PGC01003 used in Gao *et al.* (2016), was metabolically engineered through the elimination of the gene that caused the acetic acid

overflow and through the overexpressing of the genes that are capable to increase the production of SA through the reductive pathway of the TCA cycle and oxidative pathway of the TCA cycle. After studying the genes, they concluded that the responsible for acetic acid overflow is the CoA-transferase encoded by *Ylach* in *Y. lipolytica* mutant during fermentation. Thus, when the *Ylach* gene was silenced, it caused a decrease in acetic acid production. In this report, the authors obtained a production of 110.7 g/L of SA when they used glycerol as a carbon source and without pH control, leading to the highest production once achieved by fermentation with low pH levels.

Another non-*Saccharomyces* used for SA production was *I. orientalis* manipulated by Xiao, Shao, Jiang, Dole, & Zhao. (2014). The authors found that this species has enough tolerance to produce organic acids at a low pH. They studied their genome in order to insert 4 genes (*PYC*, *MDH*, *FUMR*, and *FRD*) that are part of the reductive pathway of the TCA cycle. Since high transcription was required, they used strong promoters in order to prevent the SA from being exported from the cytoplasm through the inner membranes. They tested 2 strains (*IoΔura3* + SA and *IoΔura3* + *ura3*) in the same time period. The strain *IoΔura3* + SA was able to consume all the glucose, obtaining a SA production of 11.63 g/L. On the other hand, the strain *IoΔura3* + *ura3* consumed 99.29 ± 0.08 g/L of glucose and produced 1.43 ± 0.04 g/L of SA.

4. Analytical methods for metabolite quantification

Chromatographic methods consist of quantification, separation, detection, and characterization of the metabolites in biological samples. Two chromatographic methods can be pointed out, as being used routinely in laboratorial practices to quantify metabolites produced by yeasts, such as gas chromatography (GC) and high performance liquid chromatography (HPLC) (Robles, Fabjanowicz, Chmiel, & Płotka-wasylka, 2019).

To evaluate which method is the best to use, there are innumerable criteria to be considered, such as accuracy, specificity, selectivity, the limit of detection, the limit of quantification, linearity, range, ruggedness, robustness, sensitivity, among others (Taverniers, De Loose, & Van Bockstaele, 2004). In the present work, we used HPLC due to the numerous advantages that this method presents and because 50% of researchers applied this method in their published cases and compared to the other methods is the one that performs better, relatively to the criteria mentioned above (Robles, Fabjanowicz, Chmiel, & Płotka-wasylka, 2019).

4.1. High performance liquid chromatography (HPLC)

HPLC is a versatile form of chromatography that was developed in early 1960. It can be used for the separation of a wide range of chemicals, including heavy industrials and biochemicals, pharmaceuticals and foods. This technique consists in the separation (partition) of the mobile phase (the solvent) from the stationary phase (the column packing). There are four separation mechanisms: adsorption chromatography, partition chromatography, ion-exchange chromatography and size exclusion chromatography (Bélanger, Jocelyn Paré, & Sigouin, 1997). In this work, it was used an HPLC ion-exchanged (Figure 3) in order to determine the existence and the percentage of organic acids, sugars, and ethanol.

There are two types of a solvent delivery system that depends directly on the type of separation to be performed: isocratic separation and gradient elution separation. The isocratic separation consists of a single solvent (or solvent mixture) used throughout the analysis. In the gradient elution separation, the composition of the mobile phase is altered using a microprocessor-controlled gradient programmer, which mixes appropriate amounts of two different substances to produce the required gradient (Bélanger, Jocelyn Paré, & Sigouin, 1997).

Figure 3. Method of separation (HPLC) used in this work. The system is composed by a UV detector, a column oven, an auto sampler, and a pump.

4.1.1. HPLC Advantages

The speed, simplicity, sensitivity, stability, and versatility of HPLC make this the method of choice for the separation of many small molecules of biological interested (Lian, Mao, Ye, & Miao, 1999). This technique is very accurate and precise, usually non-destructive and can avoid derivatization procedures as it can handle compounds with limited thermal stability or volatility. HPLC columns can have a long life and the resolution achieved with them is much higher than with other older methods. HPLC is also easy to operate and has basic systems that are very affordable (Bélanger, Jocelyn Paré, & Sigouin, 1997).

4.1.2. HPLC Instrumentation

The major components of the HPLC system are a sample injector loop, a column, a pump, a detector, a solvent reservoir, a recorder, a sample collector and a data system (Figure 4).

To inject the sample into the column, an injector valve is used, in which the sample is pumped into a holding circuit. Loops are designed so that a specific volume is injected into the column, usually in the order of 10 to 20 µL.

HPLC columns are usually made of stainless steel, and all components valves are manufactured from materials which can withstand the high pressures involved (Jones, Reed, & Weyers, 2007). There are three types of columns based on the type of packing and particle size, namely rigid solids, hard gels porous and pellicular layer beads but usually the columns of smaller particles (3-10 µL) are preferred because they offer high efficiency. The internal diameter of columns is usually between 4.5 to 5 mm and is 10 to 25 cm in length (Bélanger, Jocelyn Paré, & Sigouin, 1997).

The pumping system is very important because it controlled the pressure and flow, keeping the solvent flow constant during the analysis. If the solvent flow is not controlled, it will influence the retention time. Separation efficiency increases as flow decreases and pumps are capable to pump flow rates from 1 to 10 µL/min up to 5 to 10 mL/min (Bélanger, Jocelyn Paré, & Sigouin, 1997).

HPLC systems are linked to a detector of high sensibility. There are two different types of detection used in HPLC methods: selective detectors or universal detectors. The selective detectors give different responses depending on the molecular structure of the sample to be analyzed, such as absorbance and fluorescence detectors. On the other hand, for universal detectors, their response is similar for all samples as the refractive index (RI) detector (Bélanger, Jocelyn Paré, & Sigouin, 1997).

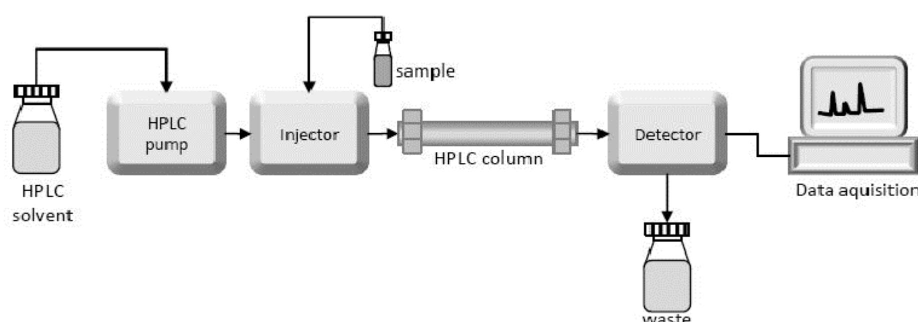

Figure 4. Schematic diagram of the components of a HPLC system. The heart of the system is the column, where the separation occurs. Adapted from (Czaplicki, 2013).

Chapter 2: AIMS

Succinic acid (SA) production recurring to renewable resources, namely the use of microorganisms, has been increasing in importance over the last years. Although bacteria have been the most intensively studied microorganism for SA production, it was concluded that yeasts have some advantages for succinate production that bacteria do not have. Some researchers consider *Torulaspora delbrueckii* as a promiser yeast for succinate production.

The main aim of this work was the evaluation of isolates of *T. delbrueckii* for SA production. In addition, we aimed to understand if this same yeast could transport SA throughout the plasma membrane by testing its growth in a medium containing SA as sole carbon and energy source. In particular, the specific objectives delineated for this thesis are defined as follows:

i) Identify promising yeasts with capacity for an improved production of succinate, recurring to a screening comprising different yeast species, and testing their SA production;

ii) Increase the production yield of SA in *T. delbrueckii* by optimizing some experimental conditions, namely the medium, the carbon sources and the agitation rates;

iii) Analyze the behavior and the SA production of *T. delbrueckii* when subjected to stress conditions;

iv) Understand if *T. delbrueckii* could transport SA through the membrane to the inside of cell, considering the yeast growth in a solid and aqueous medium with SA as sole carbon source;

v) Use the EUROSCARF yeast collection, to understand whether a deletion of a certain gene could influence the SA production.

The present work was developed at the Centre of Molecular and Environmental Biology (CBMA), in the Department of Biology of the University of Minho and was supported by the project "TODOMICS-Omics approaches towards the industrial exploitation of *Torulaspora delbrueckii*: Elucidation of the molecular basis underlying complex cellular traits"-(POCI-01-0145-FEDER-030488).

Chapter 3: MATERIALS AND METHODS

1. Yeast strains

A total of 21 yeast isolates were chosen from different collections belonging to the Department of Biology, in the University of Minho, as referred in Table 3. To each yeast, a code was attributed: "DB" is from the yeast collection of the Department of Biology, "TB" is from collections systemized from research projects in the field of Microbiology, namely the EU project TRANSBIO-Biotransformation of by-products from fruit and vegetables processing industry into valuable bioproducts (FP7/n°289603), and "T" is from the collection of *Torulaspora delbrueckii* available at CBMA. In order to maintain the membrane integrity, all yeasts were preserved in cryotubes at -80 °C with 1 mL glycerol (30 % v/v).

Before using the mentioned isolates in the subsequent experiments, yeast species were identified using PCR amplification of the internal transcribed sequence (ITS) using the method described by White, *et al*. (1990) with some modifications. To prepare the samples for colony-PCR, a small yeast colony was removed and spread on the walls of a PCR tube, then a thermal shock was applied and immediately the tubes were placed on ice to inhibit the action of the enzymes that degrade DNA. The primers used were the following: ITS1 (5´- TCCGTAGGTGAACCTGCGG-3´), as forward and ITS4 (5´- TCCTCCGCTTATTGATATGC-3´) as reverse. In addition, a negative control (without any microorganisms) and positive control (*Candida albicans*) were added. PCR amplification was performed in a thermocycler and then confirmed with a 1.5 % agarose gel in 1x Tris-acetate-EDTA (TAE) buffer (45 min, 100 V and 400 mA). After confirming the presence of bands, the purification of the PCR products was performed using the NZYGelpure kit. Subsequently, the samples were quantified in a NanoDrop ™ ND-1000 spectrophotometer, using 2 µL of DNA sample. For sequencing, 5 µL (20-80 ng / µL) of PCR product and 5 µL ITS1 and ITS4 (5 µM each) primers were added to 1.5 mL microtubes. The analysis of results, it was made using MEGABLAST with NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Table 3. List of the 21 yeast strains used in this work. "TB" code attributed to each yeast which is part of TRANSBIO, "DB" code attributed to each yeast which is part of the department of biology and "T" code attributed to each yeast which is part of the collection of *Torulaspora delbrueckii* available at CBMA.

Yeast ID	Yeast species
TB87	*Pichia fermentans*
TB100	*Pichia kudriavzevii*
TB103	*Candida tropicalis*
TB115	*Cyberlindnera jadinii*
TB132	*Apiotrichum brassicae*
TB148	*Wickeranomyces anomalus*
TB210	*Hanseniaspora uvarum*
TB215	*Geotrichum candidum*
TB512	*Rhodotorula mucilagin*
DB34	*Saccharomyces cerevisiae*
DB387	*Schizosaccharomyces pombe*
DB600	*Debaromyces hansenii*
T11	*Torulaspora delbrueckii*
T12	*Torulaspora delbrueckii*
T13	*Torulaspora delbrueckii*
T14	*Torulaspora delbrueckii*
T15	*Torulaspora delbrueckii*
T21	*Torulaspora delbrueckii*
T22	*Torulaspora delbrueckii*
T25	*Torulaspora delbrueckii*
T26	*Torulaspora delbrueckii*

2. Culture media

The culture media used throughout this work are listed and detailed in Table 4 and Table 5. The maintenance of the yeast cells was made in a solid YPD medium at 30 °C. Almost all the experiments started with pre-grown cultures prepared by transferring a portion of cell biomass of each strain in to 40 mL of YPD medium (Table 4) in a 100 mL Erlenmeyer flask, and incubated at 30 °C, 180 rpm, for 24 h. After incubation, the seed culture was inoculated in different media, considering the purposes:

1) Screening from different yeast species for SA production;

2) Optimization of experimental conditions for SA production;

3) SA production by yeast under stress conditions;

4) Growth of yeast in a medium with SA as sole carbon source.

For the first experiment, two media were tested, M1 and M2 (Table 5). These two media are very rich regarding the carbon source since M1 contains 20 % w/v of glucose and M2 contains 20 % w/v of sucrose. For media preparation, some solutions were added after autoclave through filtration, to prevent volatilization. The pH in the M1 medium was adjusted to 3.3 or 3.4, with the addition of an adequate amount of sodium hydroxide (1 - 12 M), and in M2 pH was not adjusted. After the process of sterilization was finished (autoclave), it was necessary to filter the solutions with 0.22 μm filters in asepsis conditions.

In order to optimize the experimental conditions (experiment 2), five media were tested: YPD, YPG, M3, and M4. The media M3 and M4 were prepared exactly like M2, but the carbon source was changed from sucrose to glucose and M4 instead of having 20 % w/v has 2 % w/v glucose. Two YPG medium was tested with different nitrogen sources (peptone and tryptone).

Afterwards, yeasts were subject to stress conditions (experiment 3). The medium used was M3 with two different percentages of ethanol, 4 % and 8 %. Ethanol was added to the medium, after autoclave, also with 0.22 μm filters in asepsis conditions.

For the growth of yeasts in solid and aqueous medium containing SA as a sole carbon source (experiment 4), the medium used was M6. For this medium, two solutions were prepared independently. SA was dissolved in deionized water and the pH was adjusted to 5.0 and sterilized by autoclave. When the solid medium was prepared, agar was also added. The other solution was prepared with 0.67 % of YNB that was also dissolved in deionized water. Finally, YNB was added to the previously autoclaved SA solution with 0.22 μm filters in asepsis conditions.

Table 4. Composition of culture media used throughout experiments.

	Concentration (g/L)		
Medium composition[a]	YPD	YPG	YPG
Glucose	20	-	-
Glycerol	-	20	20
Bacto-peptone	10	10	-
Tryptone	-	-	10
Yeast-Extract	10	10	10

a) When solid media were prepared 20g/L of Agar was added to the medium

Table 5. Composition of complex culture media used throughout experiments.

Medium nr	Medium	Composition	pH
M1	SM	Glucose 200 g/L + HMa 6 g/L + HTa 6g/L + KH_2PO_4 0.750 g/L + K_2SO_4 0.500 g/L + $MgSO_4.7H_2O$ 0.250 g/L + $CaCl_2.2H_2O$ 0.155 g/L + NaCl 0.200 g/L + NH_4Cl 0.460 g/L + MSA 13,09 mL/L + MST 1 mL/L + MSV 10 mL/L + MSF 1 mL /L	3.3-3.4
M2	sSM 20% S	Sucrose 200 g/L + HTa 2 g/L + HMa 3 g/L + HCi 0.5 g/L + KH_2PO_4 1.14 g/L + $CaCl_2.2H_2O$ 0.44 g/L + $MgSO_4.7H_2O$ 1.23 g/L + Yeast Extract 5 g/L + Trace elements 10 mL/L	NA
M3	sSM 20% G	Glucose 200 g/L + HTa 2 g/L + HMa 3 g/L + HCi 0.5 g/L + KH_2PO_4 1.14 g/L + $CaCl_2.2H_2O$ 0.44 g/L + $MgSO_4.7H_2O$ 1.23 g/L + Yeast Extract 5 g/L + Trace elements 10 mL/L	NA
M4	sSM 2% G	Glucose 20 g/L + HTa 2 g/L + HMa 3 g/L + HCi 0.5 g/L + KH_2PO_4 1.14 g/L + $CaCl_2.2H_2O$ 0.44 g/L + $MgSO_4.7H_2O$ 1.23 g/L + Yeast Extract 5 g/L + Trace elements 10 mL/L	NA
M6	YNB+suc	YNB 50 mL/L + Succinic acid 10 g/L	5.0

NA: Not adjusted;
(Appendix1)- MSA: Mother solution of Amino acids; MST: Trace elements mother solution; MSV: Vitamin mother solution; MSF: Anaerobic factors mother solution; Trace elements

3. Yeast growth conditions

3.1. Metabolite quantifications

3.1.1. Screening from different yeast species for SA production

For the screening with different yeast species, a total of 13 yeast strains were randomly selected. As mention above two media were tested, M1 and M2. For the inoculum, approximately 10 mL of the yeast pre-grown cultures were transferred to falcon tubes and centrifuged (3000 rpm, 2 min). The supernatant was discarded, and cells were washed twice with 10 mL of sterile deionized H_20. This culture was diluted in 24 mL fresh medium in a 100 mL Erlenmeyer flask to obtain an OD of 0.1 and incubation was performed at 30 °C, at 180 rpm for 8 days. The cultures growth and evolution were monitored by optical density at 640nm (OD) during the 8 days and samples were collected after 120 h and 192 h.

3.1.2. Optimization of experimental conditions for SA production

T. delbrueckii strains were used in order to optimize some experimental conditions, with the objective to select the best conditions for microbial SA production. The inoculum was performed using the same protocol described above (3.1.1), however, different media and agitations rates were tested. The strains T11 and T14 were initially tested on three different media: YPD, M4, and M3 at 30 °C at 180 rpm for 8 days. Then, a total of 7 yeasts were analyzed in two media: YPG and M3 and at two agitation rates: 75 rpm and 180 rpm at 30 °C for 8 days. For all these experiments, samples were also collected after 120 h and 192 h.

3.1.3. Production of SA by yeast under optimized conditions and under stress conditions

The strain T12 was chosen to test the optimized conditions and stress conditions. To test the optimized experimental conditions, yeast was grown in M3 at 180 rpm for 8 days. Every 24 h, 1.4 mL of samples were collected. In order to test the effect of stressful conditions in the amount of SA produced, an experimental design was elaborated, as illustrated in Figure 5. The inoculum (I1) was the same as the previous section (3.1.1), the medium used was M3 but with a percentage of ethanol, 4 % and 8 % as mention above and the agitation rate was 180 rpm. An Erlenmeyer with only M3 was also included, used

as control. For HPLC, samples were collected every 24 h until 96 h. After 96 h (I2), the cells grown in medium with ethanol were used as pre-culture and a new inoculum with only M3 medium was made, collecting samples every 24 h for 7 days.

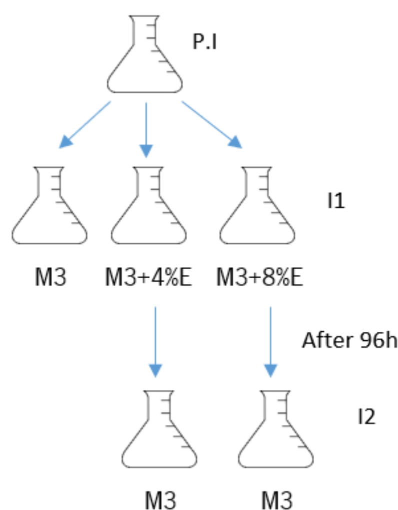

Figure 5. Illustration of T12 stress experiment.

3.1.4. Chromatography analysis of SA production

As mentioned before, samples were collected in every section above. The samples were transferred into microtubes and centrifuged at 13200 rpm, 4 ºC for 2 min in order to separate the cells from the metabolite contents. The supernatant was collected, and the samples were stored and frozen at -20 °C until the moment of HPLC analyzes. Before HPLC analysis, culture supernatant samples were prepared in our laboratory using a revised version of the protein precipitation book. This method removes protein contaminants in the samples through the addition of 10 % Trichloroacetic acid (99.5% Panreac), leaving for 24 h at 4 °C. After 24 hours, the samples were centrifuged at 13200 rpm, 4 ºC for 15 min. Finally, the supernatant was filtered directly to an HPLC vial, through a 0.22 μm filter.

Finally, in order to quantify the concentrations of SA and other metabolites, such as acetic acid, citric acid, tartaric acid, malic acid and ethanol, a Rezex 8 μm ROA-organic acid H+ (8 %) high performance liquid chromatography column (Phenomenex) with an Elite LaChrom (VWR Hitachi) chromatography system was used. The column is preheated to 60 °C and refractive index measurement with an Elite LaChrom L-2490 RI detector (VWR Hitachi) temperature was 40 °C. Isocratic elution was performed using an aqueous solution of H_2SO_4 (2.5 mM) as a solvent with a flow rate of 0.150 mL/min (Collins, *et al.*, 2014).

3.1.5. Data analysis

The experiments were conducted in triplicate and results are presented as a mean and a standard deviation (SD) of these three replicates. The optical density (OD) was measured in the Thermo Sprectronic spectrophotometer (Genesys 20). Statistical analysis was performed using a data analysis pack in excel. The significance of the differences between the yeasts, media, agitation rates and times was assessed using a two-way analysis of variance (ANOVA test) followed by the Tukey's test through GraphPad prism 6 software (GraphPad Software, Inc., La Jolla, CA). Asterisks indicate differences considered statistically significant: * means $p < 0.05$, ** means $p < 0.01$, and *** means $p < 0.001$, when compared to the respective controls.

3.2. Growth of yeasts in a medium that contains SA as a sole carbon source

For yeast growth in medium containing SA as carbon source, cells were grown in YNB supplemented with 1 % succinic acid in solid (30 °C and 18 °C) and aqueous media (30 °C, 180 rpm). For aqueous medium, cells were prepared as the previous section (3.1.1), for 8 days and cultures growth and evolution were monitored by optical density at 640nm (OD). For solid medium cells were washed twice with PBS 1X, then for the first drop a diluted to an OD of 0.2 was performed, followed a set of three 1:10 dilutions. After dilutions, drops of 3 µL of each suspension were transferred in plates with SA and in YPDA, as control. Finally, plates were incubated for 144 h, watching every day if there were a growth of colonies. PBS solution was prepared by diluting 10 times the stock PBS solution composed by 80 g/L sodium chloride (NaCl), 2 g/L potassium chloride (KCl), 14.4 g/L disodium hydrogen phosphate (Na_2HPO_4) and 2.4 g/L potassium dihydrogen phosphate (KH_2PO_4), with an adjusted pH of 7.4.

4. The concentration of SA in metabolic engineered strains

In order to evaluate the concentration of SA in EUROSCARF collection, Enzymatic kits (Megazyme internationality, Ireland) were used. A total of eight yeast strains of EUROSCARF collection were selected, all with a different gene deleted, and one *Saccharomyces cerevisiae* (DB34) strain without any mutated gene.

The procedure for obtaining the samples was the same used for the samples for HPLC (3.1). The medium used in this part of the study was M1 at 180 rpm. A total of 38 samples were analyzed, 9 yeasts, samples collected in two time points (120 h and 192 h) with 2 biological replicates, two controls, and one enzymatic standard. For enzymatic kit, the first step was the preparation of six reagents solutions/suspensions (Table 6). Bottle 1, 4 and 5 were already provided as they should be used, whereas Bottle 2 and 3 need to be dissolved in 2.4 mL of distilled water. Bottle 6 is weighed to approximately 200 mg into 1 L of distilled water. The sample preparation was the second step. All samples need to have a range between 0.8 and 40 µg, if the sample does not have these values, it is necessary to make a dilution. The last step was to prepare the microplate.

Table 6. Composition of the 6 bottles presented in the enzymatic kit.

Bottle	Solutions
Bottle 1	Buffer (8 mL, pH 8.4) plus sodium azide (0.02 % w/v) as a preservative
Bottle 2	NADH plus stabilizer
Bottle 3	ATP plus PEP and CoA
Bottle 4	Pyruvate kinase plus L-lactate dehydrogenase suspension, 55 µL
Bottle 5	Succinyl-CoA synthetase suspension (55 µL)
Bottle 6	Succinic acid (≈2 g)

In order to prepare 96-well microplate flat bottom, firstly it was added a control sample in duplicate, prepared with 210 µL of distilled water and 20 µL of the solutions 1,2,3 and 2 µL of solution 4. Then, a standard sample was included, arranged with 200 µL of distilled water, and 10 µL of the standard solution (bottle 6) and the rest of the solutions used in control samples. Finally, the sample solution was transferred to the microplate, where the procedure is the same as the standard sample but instead of adding the standard solution, the solution of the samples of EUROSCARF was added. In every

well, 274 µL were added. After 3 minutes of agitation, the absorbance was read in a microplate reader (SpectraMax Plux, Molecular devices) at the wavelength of 340 nm and at a temperature of approximately 25 °C (A_1). After measuring the absorbances of the solutions, 2 µL of the bottle 5 was added in all the wells and after 6 minutes, the microplate was read again (A_2). Lastly, equation 1 was applied in order to obtain the concentration of the SA present in each sample.

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L\ standard \qquad \text{Equation 1}$$

Chapter 4: RESULTS

1. Screening of different yeast species for SA production

In order to analyze the production of succinic acid (SA) by different yeast species and identify promising yeasts with capacity for an improved production of succinate, a set of 13 distinct yeast isolates were randomly selected from the CBMA yeast collection and cultivated in two different synthetic media: M1 (glucose as carbon source) and M2 (sucrose as carbon source). After 120 h and 192 h, samples were collected, to measure the concentration of SA and other metabolites by HPLC. The goal of this experience was to identify which yeast species produce higher concentrations of SA. The used set of yeasts included *Pichia fermentans* (TB87), *Pichia kudriavzevii* (TB100), *Candida tropicalis* (TB103), *Cyberlindnera jadinii* (TB115), *Apiotrichum brassicae* (TB132), *Wickeranomyces anomalus* (TB148), *Hanseniaspora uvarum* (TB210), *Geotrichum candidum* (TB215), *Rhodotorula mucilagin* (TB512) and *Torulaspora delbrueckii* (T11, T12, T13, T14). From previous projects *T. delbrueckii* had already been identified as a promising yeast for succinate production, therefore more isolates of this species (four strains) were included to confirm this information and to evaluate if this production was higher than when using other yeast species. Initially, two culture media were tested, as mentioned before. The first one - M1- was chosen based on the fact that when Klerk and collaborators (2010) studied the production of SA in yeasts, they claimed that a synthetic grape must, was the most completed medium to produce organic acids. However, this medium is quite expensive and for this reason, in previous works by our group, this medium was optimized in order to decrease its prize, leading in this way to M2 medium. In particular, the amino acids and vitamins in the M1 medium were replaced by yeast extract in M2.

1.1. Evaluating of SA production

Results of SA (g/L) production in 13 yeast isolates that were grown in the two media after 120 h and 192 h are shown in Figure 6. Regarding the production of SA in the medium M1, values ranged between 0.012 g/L and 2.0 g/L. When using M2 medium, values ranged between 0.1 g/L and 1.4 g/L. Considering the two media, yeasts T11 and T13 had the highest values (≈2.0 g/L) and yeasts T14, TB87 and TB100 the lowest values (≈0.2 g/L). Two-way ANOVA analysis showed statistical differences ($p > 0.05$) between T11 yeast with all the other yeasts, excluding T13. Considering the differences between the two media, although not statistically significant, a large heterogeneity between the two media was obtained in terms of SA production. For 62% of the yeasts tested (8 out of 13), the SA production was

superior in M2 medium than in M1 medium and although the growth had a similar profile in both media (Appendix2-Figure A6.2), M2 medium was used in the experiments that followed. Considering results in different time points, it is possible to observe variability in the SA production at 120 h and 192 h, but these values are not statistically different. Regarding standard deviation (SD), for most of the yeasts tested this value was low, confirming the consistency of replicates. Considering that the aim was choosing the yeast species capable of producing SA in relevant amounts and since the two yeasts with the highest SA production were isolates of *T. delbrueckii* (T11 and T13), only this species was used in the following experiments. However, results showed also that, even though *T. delbrueckii* is a promising yeast for SA production, this production seems to be strain dependent, since the obtained concentrations were very different between the tested strains.

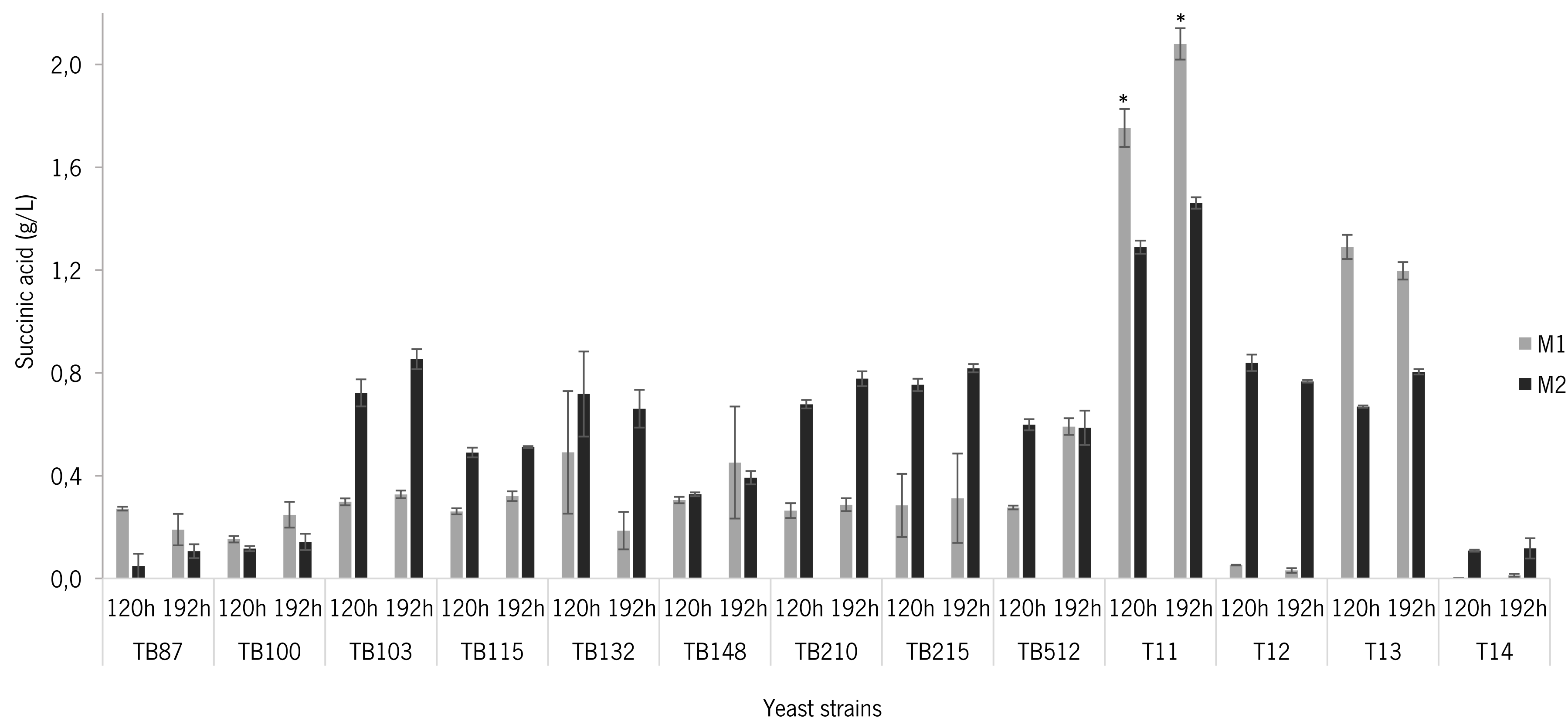

Figure 6. Concentration of succinic acid obtained by HPLC analysis using 13 distinct yeast isolates, in two media (M1- glucose as carbon source, and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h). The experiments were performed in triplicate.

1.2. Evaluation of other compounds of interest

In addition to the SA production, other metabolites were evaluated, namely citric acid, acetic acid, tartaric acid, malic acid and ethanol (Appendix2). Formic acid and glycerol were also quantified, however, the HPLC column couldn`t effectively separate the two compounds, eluting together as one peak, therefore the data is not shown in any of the following experiences. Regarding the production of citric acid (Appendix2-Figure A1.2), present in the constitution of both media, it was possible to observe that in M1 medium almost all yeasts consumed 50 % of this acid, apart from TB148 which produces negligible amounts, while in M2 half of the yeasts consumed citric acid, the other half showed production of this acid. In M1, values range from 2.4 g/L to 6.3 g/L while in M1 the highest value was 0.8 g/L. Tartaric acid (Appendix2-Figure A2.2) was barely produced in both media; in M1 the highest value produced was 0.1 g/L. Regarding M2 medium, it is important to note that it has about 2 g/L of tartaric acid in its constitution, therefore there is only consumption but not production. All the yeasts produced acetic acid (Appendix2-Figure A3.2) in both media but there was a higher production in M1, with the highest value being 6.7 g/L as produced by strain TB87. Regarding malic acid (Appendix2- Figure A4.2), also present in the constitution of both media, some yeasts consumed all the acid and others consumed only a small percentage, aside from TB215 which in M2 produced a small amount. Relevant concentrations of ethanol (Appendix2-Figure A5.2) were registered, especially when considering yeast T11 growing in medium M1, which achieved 56.9 g/L of ethanol.

2. Optimization of experimental conditions for SA production

2.1. Optimization of the medium

After choosing *T. delbrueckii* isolates as the most able to produce SA, we further tested some other media to determine the most favorable experimental conditions for SA production. To perform that, two *T. delbrueckii* strains were randomly selected for this study: T11 and T14. Experimental conditions were optimized, considering two different time points (120 h and 192 h) and four culture media (YPD, M2, M3, and M4, having different carbon sources and in different percentages). Media M3, M2, and M4 have the same constitution, but in M2 medium the carbon source is sucrose, being glucose the carbon source in media M3 and M4. The main difference between M3 and M4 was that, the first one had 20 % (w/v) of glucose and the second one 2 % (w/v) of glucose. The carbon source was switched from sucrose (the cheaper) to glucose (most expensive) because although the two sugars use the same transporter to enter the cell, they have more affinity to carry glucose than fructose so the glucose is utilized at a faster rate (Verstrepen, *et al.*, 2004). Moreover, sucrose is a complex molecule (glucose + fructose) whereas glucose is simpler. At the same time, we intended to compare these media to understand how the carbon source influences the production of the metabolites.

Regarding the SA production (Figure 7), when using M3 medium values ranged from 0.8 g/L to 1.3 g/L. Comparing this result with the results from the YPD and M4 it is remarkable to see that there is a discrepancy. In YPD, the production of SA ranged between 0.2 g/L to 0.3 g/L. In M4 the values were even lower, 0.1 g/L to 0.2 g/L. In the M2 medium, when the carbon source is sucrose, values ranged between 0.1 g/L to 1.4 g/L. Even though the highest value has been achieved in this medium, the lowest value was also obtained, depending on the yeast in question. It is important to refer again that M2, M3, and M4 media have the same constituents, the only difference is in the carbon source, so we can conclude that carbon source is very relevant, once results are so distinct. Observing the results, it is doable to affirm that of these four media, M3 is the best one to produce SA due to the higher values obtained when using this medium. Statistical analysis (one-way ANOVA) showed no statistical differences ($p < 0.05$) between the different media, times and yeasts and once again SD was very low, showing consistence between replicates.

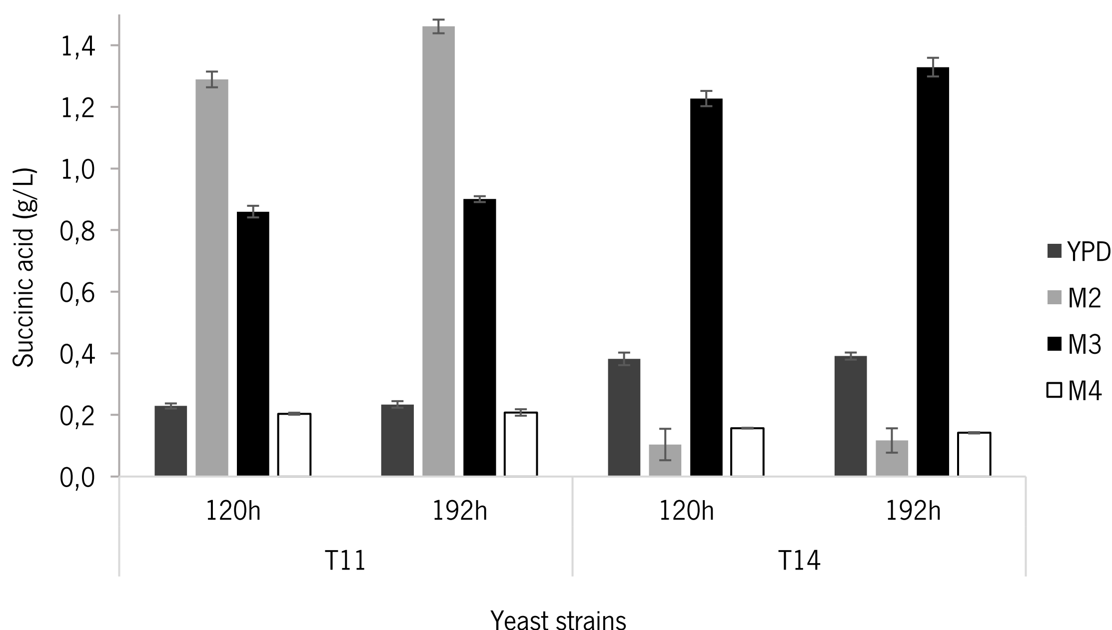

Figure 7. Concentration of succinic acid obtained by HPLC analysis using two *T. delbrueckii* strains, in the four tested media (YPD, M3, and M4- glucose as carbon source and M2- sucrose as carbon source) in two time-points of growth (120h and 192h). The experiments were performed in triplicate.

Regarding other organic acids (Appendix3-Figure A1.3), in YPD, there was almost no production of the tested acids. In all the other media, containing malic, citric and tartaric acid in its composition, it was possible to verify that there is consumption but no production of any tested acids. Regarding the production of acetic acid, the highest values were obtained in the M2 medium, whose carbon source is sucrose. Observing the results from ethanol production (Appendix3-Figure A2.3), the yeasts T11 and T14 only produced ethanol in M3 and M2 media, which contains 20 % (w/v) of the carbon source in their composition. Ethanol production varied between 0 g/L (YPD and M4) to 55 g/L (M3).

The growth of these two yeasts was also tested in these four media. Cell density was measured every 2 h, during the day, at 640 nm. As can be seen in the Appendix3-Figure A3.3, no differences in the growth curve of the two yeasts in the four media were visible, although the SA production was clearly different between them. The stationary phase has been reached after 20 h, being the OD values close to 10.

2.2. Optimization of the agitation rates

To perform this optimization, two agitation rates were tested: 180 rpm and 75 rpm. Cells were collected after 120 h and 192 h of incubation at 30 °C. After the choice of M3 as the best medium to produce SA in the previous experiment, this medium was chosen to optimize other experimental conditions. However, since some recent works (Li, *et al.*, 2018) focused in the SA production using glycerol as carbon source, obtaining for example high amounts of SA by *Yarrowia lipolytica*, we decided to include this medium to compare our results using a medium already suitable for SA production. As shown before, the SA production is strain dependent, which can be explained by the fact that different strains have differences in their metabolism (Benito, 2018), so we included a set of seven *T. delbrueckii* strains randomly selected, in order to identify the best conditions to produce SA. One extra factor that we have tested was the nitrogen source, since several studies showed some differences when using peptone (Yuzbashev, *et al.*, 2010) or tryptone (Yang, Wang, Li, & Lin, 2017) as nitrogen sources.

Results of SA production (Figure 8) showed than T26 was the strain that produced more succinate in M3 at 75 rpm (1.5 g/L), followed by T13 with 1.1 g/L. Comparing the two media, we can state that to produce SA, yeasts prefer glucose as carbon source (maximum value reached was 1.5 g/L) than glycerol (maximum value achieved was 0.4 g/L). Regarding the agitation rates, no clear patterns can be observed when comparing the results obtained at 75 rpm and 180 rpm. This fact seems to be somehow irrelevant since some strains prefer lower agitation rates, while other prefer higher ones. When performing statistical analysis (ANOVA) there was no differences between the values of these two agitation rates, just as SD does not showed visible differences between the two replicates. Since no statistical differences were found to choose one or the other, we continued the experiments using 180 rpm, the higher agitation rate, since we think it would lead to a better oxygenation of the medium and would prevent cells to deposit in the bottom of the flask.

Regarding the use of different nitrogen sources (Appendix4-Figure A6.4), no differences in terms of SA concentration were observed when changing from peptone to tryptone. When analyzing the growth curves (data not show), it was possible to conclude that the only condition causing change in the yeast growth was the use of YPG as growth medium, since in this case yeasts take more time to reach the stationary phase, especially when using 75 rpm as agitation rate.

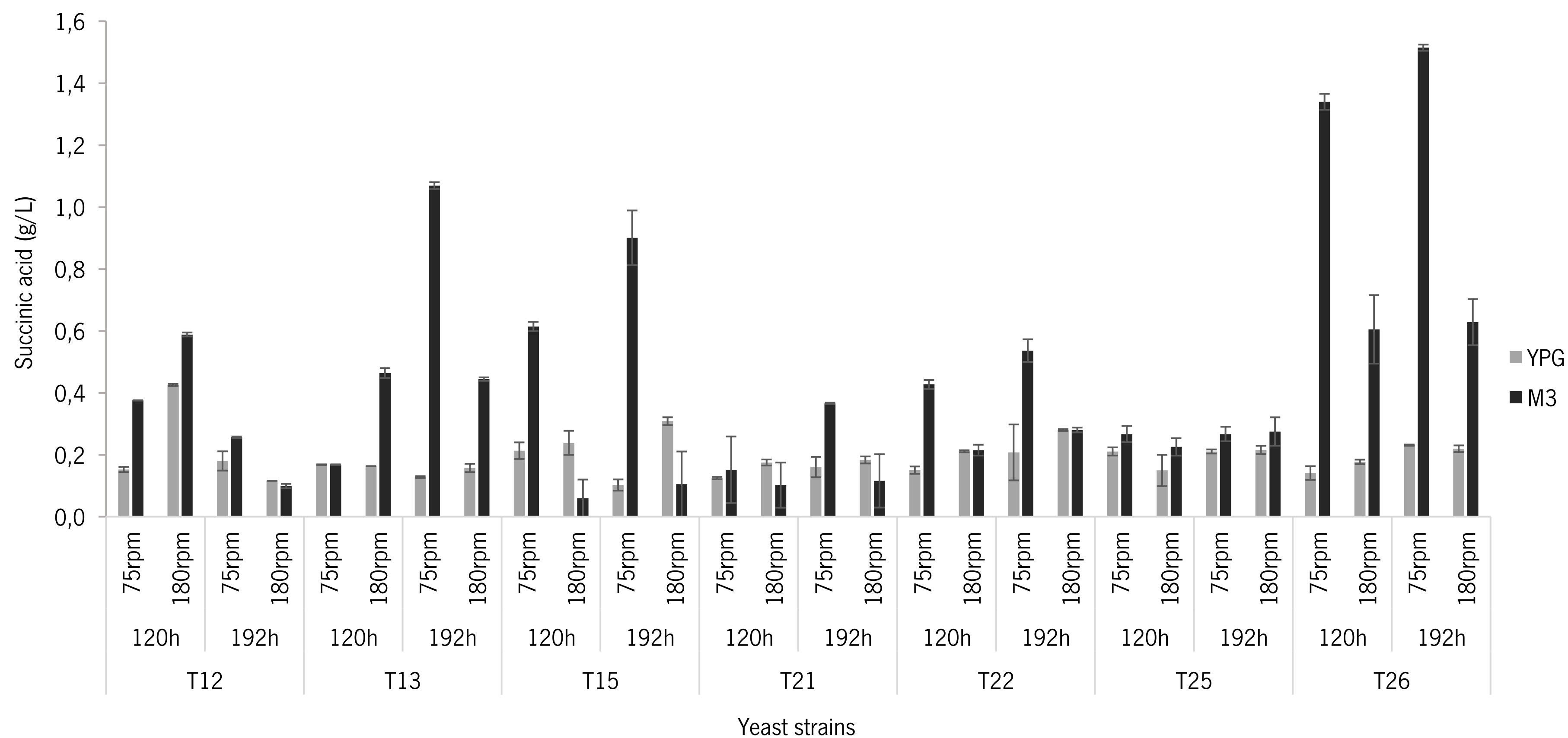

Figure 8. Concentration of succinic acid obtained by HPLC analysis using seven *T. delbrueckii* strains in two media (YPG- glycerol as carbon source and M3- glucose as carbon source) in two time-points of growth (120 h and 192 h) at two agitation rates: 75 rpm and 180 rpm. The experiments were performed in triplicate.

Regarding other metabolites produced (Appendix4), it is important to be aware that the YPG medium does not feature in its composition any of the tested acids, while M3 medium has tartaric, malic and citric acids in is composition. Therefore, in the YPG medium, there was almost no production of organic acids, in fact, the yeast with more production was T26, which had a production average of only 0.1 g/L. In M3 medium even though it has already an initial value of 0.5 g/L of citric acid (Appendix4-Figure A1.4), some yeasts produced this acid, while the same did not happen with tartaric acid (Appendix4- Figure A2.4), that was only slightly consumed, but not produced. Acetic acid (Appendix4-Figure A3.4) was the acid most produced by yeasts, up to a yield of 3 g/L. This may have happened due to the absence of this acid in the medium. Malic acid (Appendix4-Figure A4.4) available in the medium (3 g/L) was consumed in its totality by all the yeasts, except T26 which at 75 rpm still presented final values of malic acid of 1.9 g/L and 2.2 g/L. The highest amount of produced ethanol was detected in M3 by isolate T12, reaching 43 g/L (Appendix4-Figure A5.4).

With the results obtained, the best combination of experimental conditions was chosen in order to obtain the highest concentration of SA possible in the subsequent experiments. Furthermore, we also chose T12 strain as the most promising isolate for further studies using the optimized conditions.

3. Production of SA by the selected yeast

3.1. Selected yeast under optimized conditions

The optimized experimental conditions previously chosen were applied to reassess the SA production using only one *T. delbrueckii* strain (T12), selected having in account our previous results. Samples were collected every 24 h until 192 h and analyzed by HPLC.

When analyzing the results of SA production presented in Figure 9, it is possible to see that the lowest value obtained was at 24 h (0.04 g/L). The highest accumulation was achieved at 192 h with a maximum concentration of 0.5 g/L. The concentration of SA at 120 h, 144 h, and 168 h remained almost constant. The statistical analysis (One-way ANOVA) showed no significant differences ($p < 0.05$) between the SA production at different time-points, and the values of SD were once again lower, proving the consistency and reproducibility of the replicates.

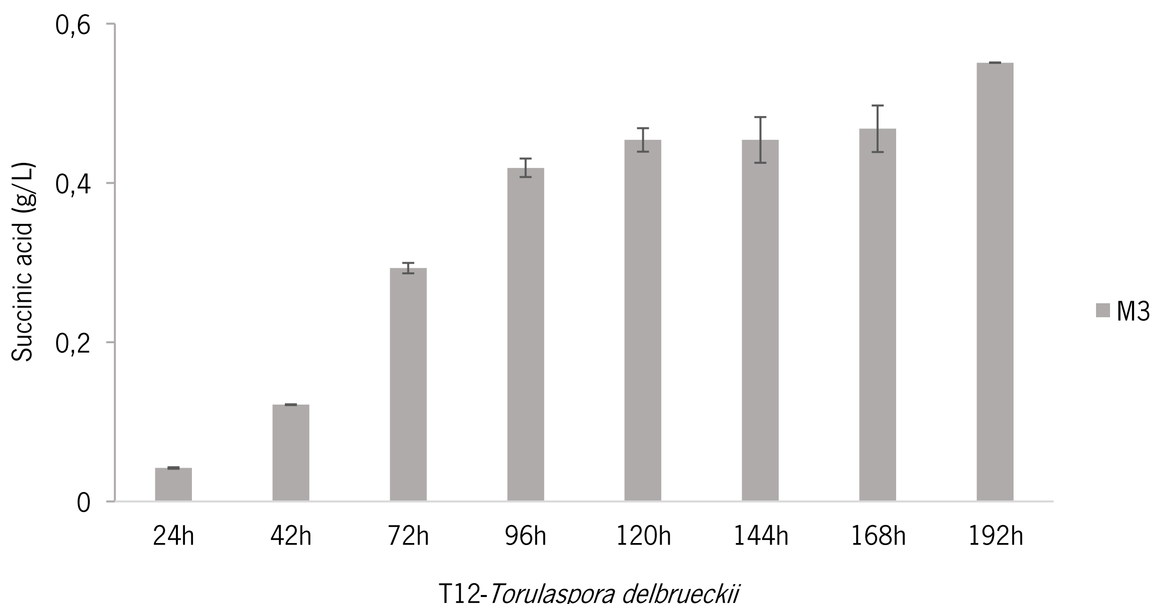

Figure 9. Concentration of succinic acid obtained by HPLC analysis using *T. delbrueckii* (T12) strain in M3 medium, in 8 time-points of growth (24,48,72,96,120,192 h). The experiments were performed in triplicate.

Concentration of other organic acids is presented in Appendix5, where we can see that there is no production of malic acid, being this acid also completely consumed after 24h. Regarding the production of citric acid, there was a small production, except at 72h, tartaric acid was only slightly consumed and acetic acid, which is the only acid that was not present in the composition of the M3 medium, was the most produced acid, with a maximum value reached after 72 h (1.6 g/L), but with a decrease in the subsequent time-points as the hours pass by. Regarding ethanol production (Appendix5-Figure A2.5), the maximum was achieved at 96 h by yeast T12 (34 g/L).

The OD results (Appendix5-Figure A3.5) from T12 yeast in optimal conditions gradually increased to approximately 8.7. The cell populations of *T. delbrueckii* yeast reached the stationary phase after 20 h of growth.

3.2. Effects of ethanol concentration in SA production

With the objective of trying to increase SA yields, *T. delbrueckii* strain (T12) was subjected to conditions of stress, in particular by the presence of high ethanol concentrations, in order to direct its metabolism towards the production of this acid. The goal of this experiment was to prevent the yeast from follow the alcoholic fermentation pathway and, instead, follow the pathway that leads to the SA production. In order to achieve that, ethanol was added to the medium, at concentrations of 4 % and 8 % (v/v), using M3 medium, and collecting supernatants every 24 h, until 96 h. A control Erlenmeyer with only M3 medium was also included.

Results, presented in Figure 10, showed that when the yeast was exposed to 4 % and 8 % of ethanol, failed to produce SA. However, in the control medium (M3) the same was not observed, having an average production of 0.4 g/L. These results suggested that the presence of ethanol leads to a repression of SA production. Although these differences between M3 medium (with ethanol) and M3 medium (without ethanol) are visible, the statistical analysis ANOVA does not consider these differences significant.

The microbial cell growth in these media is present in Figure 11. It is possible to see that the presence of 8 % ethanol in the medium suppressed the growth of the yeast but when yeast is subjected to 4 % ethanol, cells grow almost as well as in the control medium, reaching in both media the stationary phase after 24 h.

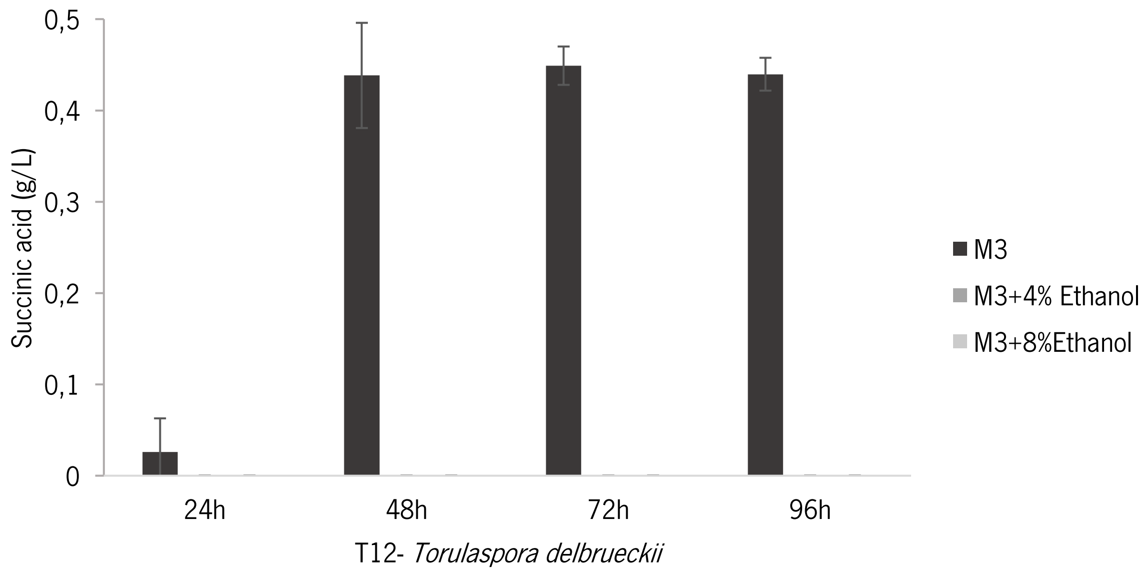

Figure 10. Concentration of succinic acid obtained by HPLC analysis using *T. delbrueckii* (T12) strain, in three media (M3- the control medium and M3+4 %- medium with 4 % of ethanol and M3+8 %- medium with 8 % of ethanol), in four time-points of growth (24 h,48 h,72 h,96 h). The experiments were performed in triplicate.

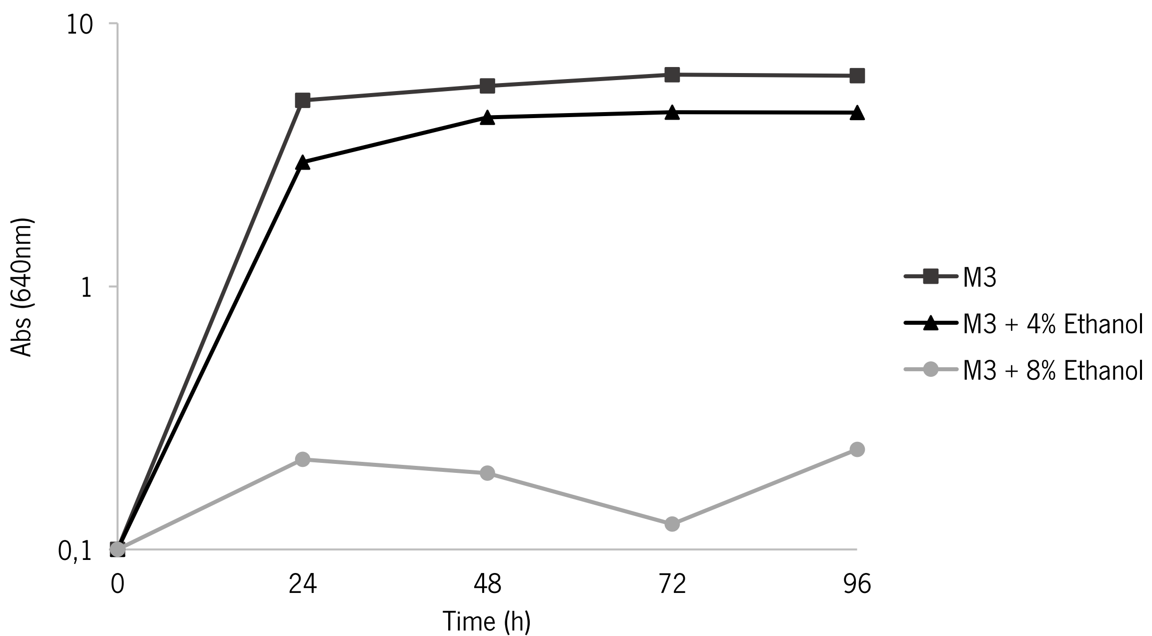

Figure 11. Growth curve of the *T. delbrueckii* (T12) strain, in the control medium (M3) and in medium with 4 % and 8 % of ethanol. The growth was monitored in the course of 96 h. The experiments were performed in triplicate.

An additional analysis was performed for the same selected yeast, restoring growth in M3 medium (without ethanol as stressor) after growth during 96 h in the presence of ethanol, in order to see if the production of SA increased after yeasts were subjected to a stressful condition. A control was also included using cells that grew during 96 h in YPD and also inoculating them into the M3 medium.

With the results from Figure 12, it was possible to conclude that although the presence of ethanol in the medium prevents SA production, there is an increase in the concentration of succinate when cells initially grow in ethanol. This conclusion was drawn due to the comparison of control results with those obtained in M3(4 %) and M3(8 %). At all time-points, apart from 24 h, there is a higher yield of SA in M3(4 %) and M3(8 %) that in M3 (control). The best value was achieved at 168 h by M3(4 %) with 0.9 g/L of SA, while at the same time in M3 medium, yeast produced ≈0.4 g/L of the same metabolite, indicating, in this way, a two-times higher production in this medium.

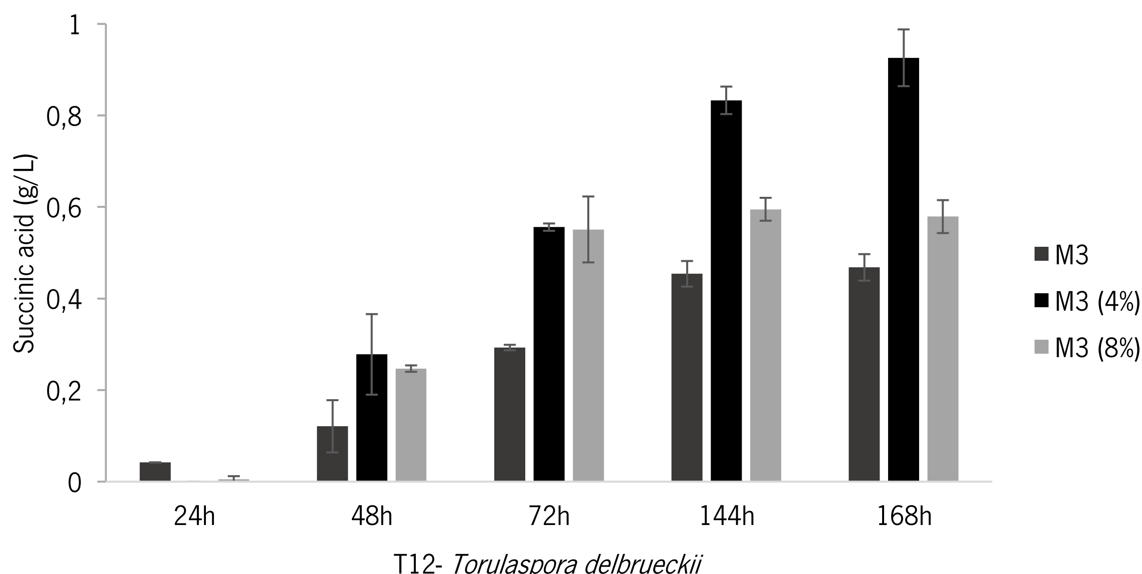

Figure 12. Concentration of succinic acid obtained by HPLC analysis using *T. delbrueckii* (T12) yeast strain, in M3 medium but with three different pre-inoculum: cells grown of YPD (M3), as control; cells grown of medium with 4 % of ethanol- M3(4 %); cells of medium with 8% of ethanol- M3(8 %). The experiments were performed in triplicate.

4. Growth of yeasts in medium that contains SA as sole carbon source

4.1. Solid medium

After analysis of SA production, it was important to evaluate the growth of *T. delbrueckii* in medium containing SA as sole carbon and energy source, to understand if this yeast could grow well in the presence of this acid, which could be an indication of some transport system that could carry the acid to the inside of the cell. Once the aim of this work consists in the study of *T. delbrueckii* isolates, four strains of this species were tested (T11, T12, T13, T14). In addition, three other species were included as positive (*Debaryomyces hansenii*-DB600 and *Schizosaccharomyces pombe*-DB387), or as negative (*Saccharomyces cerevisiae*-DB34) controls. To perform this test, yeasts were cultivated in Petri dishes with YNB supplemented with 1 % of SA (w/v), at pH 5.0, during 144 h. Yeasts were also cultivated in YPDA, as control, to ensure that the yeast cells were viable. We also tested two temperatures, 18 °C and 30 °C. As all previous experiences in this work were performed at 30 °C, this condition was still used, although according to Soares-Silva, *et al.* (2015) at 18 °C the growth is slower and therefore it is possible to observe the evolution in more detail.

The results of yeast growth after 144 h are shown in Figure 13. When glucose was the carbon source, all the yeasts were able to grow, as expected. However, only T12 (*T. delbrueckii*) and DB600 (*D. hansenii*) were able to efficiently use SA as sole carbon and energy source. Once *D. hansenii* is the positive control, the growth in medium with SA as sole carbon source was already expected however, the considerable growth of *T. delbrueckii* could indicate that the SA is being transported through the plasma membrane, considering that this yeast could have the presence of a transportation system. This result can be explained by the existence of a new transporter able to uptake the SA and cross the cell membranes or for the existence of a known transporter or even a transporter from other acid. If this transportation system exists, it must be strain dependent, since only one of the four *T. delbrueckii* strains was able to grow in a similar way of *D. hansenii* (positive control). *S. pombe* was the only yeast where no yeast colonies were detected on the petri dishes, when SA was the sole carbon and energy source. It was expected *S. cerevisiae* (DB34) not to grow when SA was the carbon source, nevertheless, at 30 °C it was possible to observe slightly growth, detected by some cellular colonies, similar as the ones obtained for *T. delbrueckii* (T11, T13, T14). Regarding the temperature, there was a higher growth at 30 °C than at 18 °C.

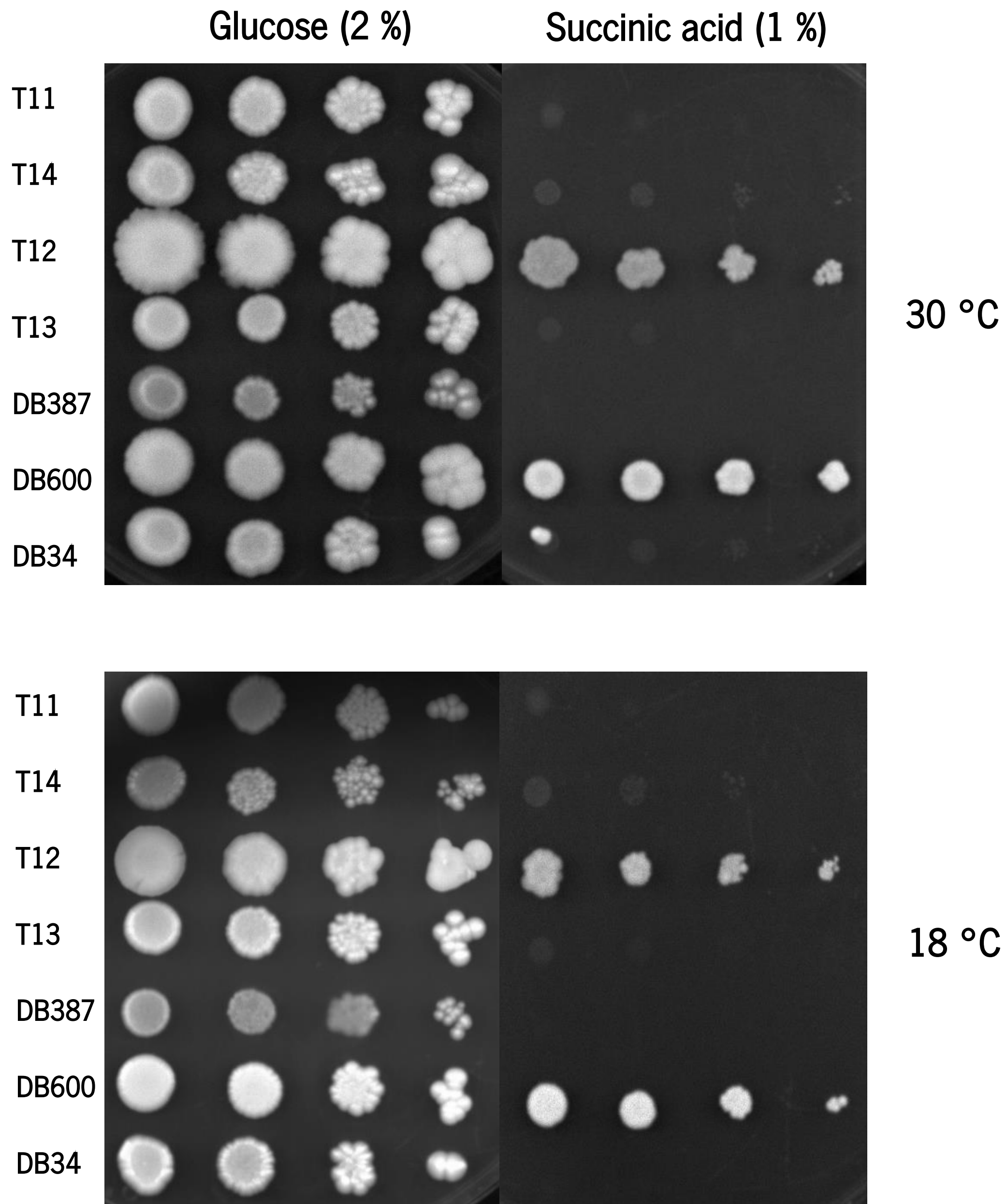

Figure 13. Drop tests at 30 °C and 18 °C, of four *Torulaspora delbrueckii* strains (T11, T12, T13, T14) and three other species: *Schizosaccharomyces pombe* (DB387) and *Debaryomyces hansenii* as positive control (DB600) *and* *Saccharomyces cerevisiae* as negative control (DB34). Incubated in the following solid media: YPD (glucose as carbon source) and YNB 1 % succinic acid (succinic acid as carbon source, pH 5.0). Cells were diluted, 3 μL of drops of each dilution were placed in the plates. The picture was taken after 144 h incubation.

4.2. Aqueous medium

In the meantime, we have also tested these seven yeasts in aqueous medium containing SA as sole carbon source (Figure 14). Cell density was measured every 2 h, during the day, until 180 h. Although in solid medium only two yeasts have grown, in aqueous medium all the yeasts have grown except T11. Nevertheless, only DB600 and T12 entered the stationary phase after 24 h, the other yeasts took much longer than 24 h. However, DB600 (positive control) had an OD of 3.4 and T12 of 2.3, but in solid media, T12 had higher growth than DB600.

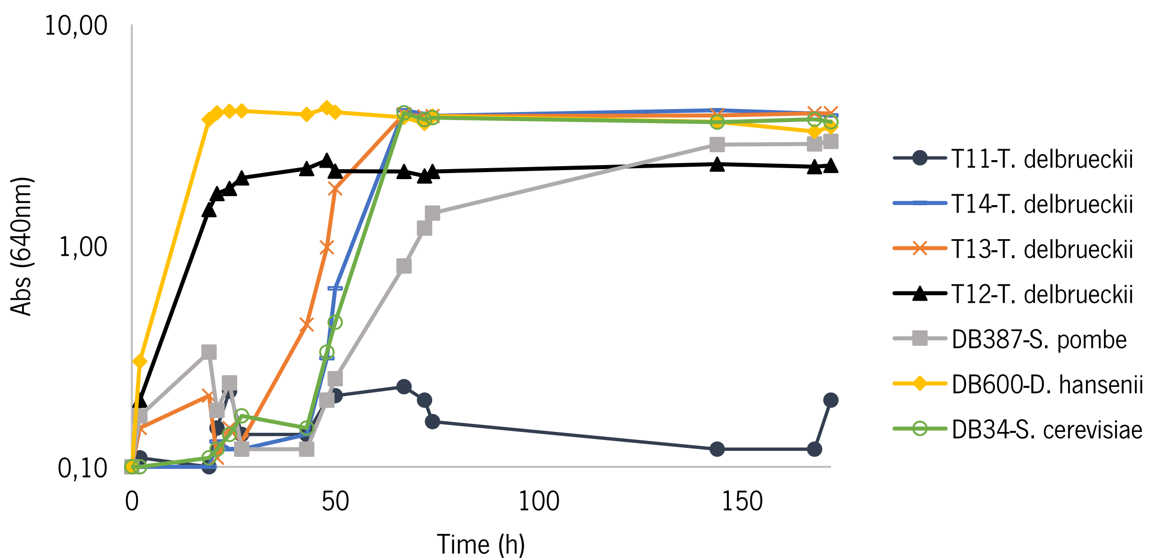

Figure 14. Cell growth of 7 yeast strains in YNB supplemented with 1 % of succinic acid as carbon source, with a pH of 5.0. The yeasts were: four *Torulaspora delbrueckii* strains (T11, T12, T13, T14), *Schizosaccharomyces pombe* (DB387), *Debaryomyces hansenii* as positive control (DB600) and *Saccharomyces cerevisiae* as negative control. Cell density was measured until 180 h.

5. SA production by genetically modified yeasts

In order to understand whether the deletion of some genes contributes to an increase of SA production, a set of eight EUROSCARF collection yeasts and one *S. cerevisiae* strain without any deleted genes, as control were tested. The concentration of SA in these yeasts were quantified by using enzymatic kit. The mutated genes are represented in Table 7. To perform this experiment, yeasts were cultivated in M1 medium and samples were collected at 120 h and 192 h. After all the samples were collected, the absorbance was read by a microplate reader in a 96-well microplate. Through this value the concentration of SA was calculated.

Table 7. List of the eight mutated genes and the corresponding genes

Code	Gene
E1	HAP4
E3	ADH1
E5	ADH3
E9	SDH1
E11	FUM1
E13	MDH2
E16	SER3
E18	ICL1

Based on the results from Figure 15, no statistical differences were detected by the statistical analysis ANOVA, contrarily to what was expected, nevertheless it is possible to observe some variability. The strain E16, which presents the gene *SER3* deleted, at 192 h had the higher value of SA production (0.6 g/L) which compared to the control (0.3 g/L) was twice the production. While E5, which has the *ADH3* gene mutated, has the lowest value of SA production (0.1 g/L).

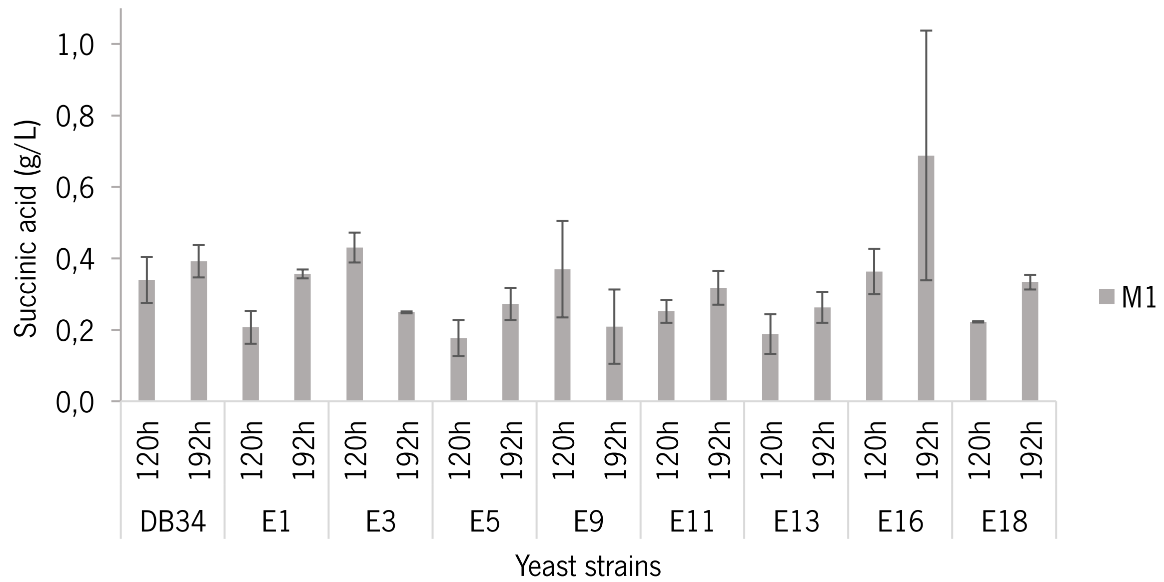

Figure 15. Concentration of succinic acid obtained by enzymatic kit in nine *S. cerevisiae* strains; eight strains with mutated genes and one *S. cerevisiae* under normal conditions, as control. The medium used was M1 and the samples were collected at two time-points of growth (120 h and 192 h).

Chapter 5: DISCUSSION

Currently, there is a higher concern about the environmental pollution, that in part can be met by the reduction of fossil fuel use. Succinic acid (SA) is currently produced by petrochemical processes but in the last years, the use of yeasts to produce this acid has become a great challenge for researchers. Although the majority of the studies were developed using *S. cerevisiae*, there is already an interest in non-*Saccharomyces* yeasts, such as the species *Torulaspora delbrueckii* (Li, Gao, Yang, & Lin, 2017). The first part of this thesis consists in a screening comprising a group of 13 distinct non- *Saccharomyces* yeasts in two different synthetic media, M1 (glucose as carbon source) and M2 (sucrose as carbon source), in order to identify yeasts with capacity for an improved production of succinate. In previous works, *T. delbrueckii* was considered as a promising yeast for SA production, therefore more isolates of this species (4 strains) were included. The two yeasts with the higher SA production were *T. delbrueckii* strains T11 and T14, reaching values of 1.2 g/L and 2.0 g/L, being categorized as the most promising SA producer yeasts in the entire selection. However, this production must be strain dependent, since the obtained concentrations were very different among the tested strains of *T. delbrueckii*. Although the production was not the highest ever achieved, it must be taken into attention that these values were reached with natural isolates. When comparing our results with results from studies with strains with deleted genes that obtained values of 0.9 g/L and 0.23 g/L of SA, our strains show a great potential (Otero, *et al.*, 2013). Additionally, some articles also refer to *T. delbrueckii* as a good SA producer, mentioning that this species has the ability to produce more succinate than *Saccharomyces cerevisiae* (Ciani & Maccarelli, 1997; Contreras, *et al.*, 2015; Puertas, Jiménez, Cantos-Villar, Cantoral, & Rodríguez, 2016; Liu, Laaksonen, Kortesniemi, Kalpio, & Yang, 2018). In fact, Franco-Duarte *et al.* (2017) obtained in natural isolates of *S. cerevisiae* a maximum yield of 1.13 g/L, when we compare this result with the production of SA in natural isolates of *T. delbrueckii* we can see that our two best producers had higher values (1.2 g/L and 2.0 g/L). Some studies also claim that this yeast produces large amounts of SA due to its reduced production of undesirable by-products mainly acetic acid and ethanol (Puertas, Jiménez, Cantos-Villar, Cantoral, & Rodríguez, 2016; Belda, *et al.*, 2017; Liu, Laaksonen, Kortesniemi, Kalpio, & Yang, 2018). In the literature, it is stated that to obtain an efficient SA production it is necessary to eliminate the ethanol production. In other words, it is necessary to prevent yeast from following the path of alcoholic fermentation, redirecting the carbon flux into the TCA cycle, leading to the succinate production (Drewke, Thielen, & Ciriacy, 1990). However, comparing the results from the SA production with the ethanol production, in the 13 yeast isolates studied, it is possible to observe an interesting correlation between these two metabolites, but in some cases, we found the opposite to what is reported in the literature. For example, for the best SA producer strain (*T. delbrueckii*), the amounts of ethanol

produced were very high, about 56 g/L. Nevertheless, acetic acid production was lower in the best SA producer (*T. delbrueckii*), which is in agreement with the literature. This antagonism in the production pathways of these two acids is very important as it is very difficult to obtain when using bacteria (Franco-Duarte, *et al.*, 2017). Since *T. delbrueckii* showed to be promising as a good producer of SA, we used only isolates of *T. delbrueckii* in the following experiments.

Further on, once the goal is to improve SA production for industry purposes, we need to find a way to increase the production rates through natural processes. A very important factor for increasing SA concentrations are the growing conditions, such as media, glucose and nitrogen sources, agitation rates, pH, incubation time, etc. Therefore, the optimization of experimental conditions was required. To do so, we tested different experimental conditions, in order to optimize the ones that led to the obtainment of higher concentrations of SA. In the first experiment, the previously mentioned two media were tested, M1 (glucose as carbon source, pH 3.3-3.4) and M2 (sucrose as carbon source). We concluded that M2 brings more advantages for the SA production, since 62 % of the yeasts (8 out of 13) produced more SA in this medium. Two *T. delbrueckii* strains were selected (T11 and T14) and tested in three more media, YPD, M3 and M4. The last two media have the same composition as M2 but with glucose as carbon source, M3 (20 % w/v glucose) and M4 (2 % w/v glucose). The carbon source has been changed from fructose to glucose because there are already studies on optimization of yeast growth conditions that conclude that glucose is the energy source preferred by yeasts (Angulo-Montoya, *et al.*, 2019). The influence that the carbon source has on cell growth and SA production was studied, concluding that with glucose as sole carbon source, yeasts produce higher succinate values. Another conclusion was that the higher the glucose percentage, the higher the SA production since in M4 (2 % w/v glucose as carbon source) the maximum value of SA produced was 0.2 g/L while in M3 (20 % w/v glucose as carbon source) was 1.3 g/L. Nevertheless, Ong *et al.* (2019) studied SA production in various glucose concentrations in *Y. lipolytica* and came to the conclusion that the highest SA yield achieved was with 4 % glucose. Although this study was performed in *Y. lipolytica* and not in *T. delbrueckii*, this topic needs to be deeply explored in the future, testing intermediate glucose concentrations, also because Angulo-Montoya *et al.* (2019) state that media with high glucose concentrations can lead to an unexpected loss of plasmids or even cause a change in chromossomes. Recently, some studies also started to use xylose as carbon source for the SA production, concluding that a co-utilization of glucose and xylose leads to an increase of SA production (Ong, *et al.*, 2019). For this reason, in the future it is also important to test more carbon sources, namely xylose. Notwithstanding, more media need to be tested at laboratory scale to ensure which is the most adequate one. Comparing the results from SA production with the production of other

metabolites, once again results showed a correlation, sometimes contradictory to what is described in the literature, between the production of SA and ethanol. Yeast with more SA produced had more ethanol production. With the other organic acids, it was possible to confirm the influence that acetic acid production had in succinate production, since T14 yeast in M2 medium only produced 0.2 g/L and yet produced the highest values of acetic acid (1.9 g/L), although the same strain but in M3 medium produced 1.3 g/L of SA and 0.9 g/L of acetic acid.

The last step of this optimization was to test different agitation rates and also test more *T. delbrueckii* strains in order to confirm that SA production is strain dependent and consequently understand the differences in the metabolism of this yeast species. For this, seven isolates were tested using two agitation rates (75 rpm, 180 rpm) and in two media: the medium previously elected (M3) and other medium having glycerol as carbon source (YPG). The last medium was appended because studies confirm that glycerol presents several advantages, since is an attractive carbon source for biological conversions, is considered a good osmotic regulator of the cell, maintain an intracellular redox balance and most important lead to the production of high SA yields while avoiding the formation of by-products such as acetic acid (Chayabutra, Wu, & Ju, 2001; Albers, Larsson, Lidén, Niklasson, & Gustafsson, 1996). Moreover, Gao *et al*. (2016) also tested distinct media, including YPG, using a *Y. lipolytica* strain, and concluded that YPG was the optimal medium for SA production and cell growth, achieving a production of 5.5 g/L of SA. Observing our results of organic acids and ethanol production in the YPG medium it was possible to see a scarce production of these metabolites. Therefore, a higher SA production would be expected but surprisingly this was not the case, the highest value achieved was 0.4 g/L, whereas yeasts in M3 medium produced about 3.8 times more. When comparing the value obtained in YPG medium (0.4 g/L) with results obtained by Gao *et al*. (2016), obtaining 5.5 g/L, our result was very low, however one should take into attention that these concentration values also depend on the yeast species, that was not the same and the strain used by Gao and co-workers was not in natural conditions, was genetically modified with the deletion in the gene encoding *SDH5*. Once again, the yeast with the highest SA production also obtained higher ethanol yield. In conclusion, comparing all the media tested, it was very clear that the best medium for SA production is the M3, having achieved a production of 1.5 g/L. Regarding the agitation rates, the results were not very conclusive; for some yeasts, it was observed more SA production at 75 rpm than at 180 rpm, in others, the opposite occurred. For this reason, it was not possible to draw conclusions and further work will be necessary to test more agitation rates and, eventually, identify the best one. However, in our following experiments we used 180 rpm, the higher

agitation rate, since we think would lead to a better oxygenation of the medium and would prevent cells to deposit in the bottom of the flask.

A parallel experiment was made with only two of the six yeasts previously tested (T21 and T26), comparing their growth and SA production in two YPG media but with different nitrogen source. Initially, we tested YPG with peptone as nitrogen source, nevertheless, some studies claim that yeasts prefer tryptone as carbon source because of the large amount of tryptophan that this nitrogen source presents, which is an essential amino acid for microorganisms to produce pyruvic acid, indole and ammonia (Angulo-Montoya, *et al.*, 2019). However, our results showed that no differences were found in SA production and in yeast growth using either of the nitrogen sources mentioned above.

Following the optimization of the experimental conditions, only strain T12 (*T. delbrueckii*) was chosen for further experiments. This yeast was chosen due to intermediate SA productions and information of this strain from previous works (personal communications). This strain produced values among 0.04 g/L to 0.5 g/L. It is important to note that 0.04 g/L were produced at 24 h, which is usually the time-point in which the yeast begins to grow and only from this point further, it does start to produce metabolites. In this experiment, the relation between the SA production and the production of acetic acid and ethanol is already in agreement with what is described in literature: more SA production, lower production of acetic acid and ethanol. However, we can conclude that an improvement in cultural conditions such as the use of glucose as carbon source, controlling temperature and rotation does not increase the SA production.

After all the optimization of experimental conditions were complete, the SA titer and yield still could not meet the need of industrial application. On account of that, we decide to study and understand the resistance of yeasts to stress and the complexity of their reactions. There is a lot of stresses that yeasts can be subjected to, such as heating stress, osmotic perturbations, air drying processes, nutrients availability and ethanol concentrations (Câmara, Maréchal, Tourdot-Maréchal, & Husson, 2019). According to the literature, one of the steps to obtain an efficient SA production is the elimination of alcoholic fermentation and consequently end ethanol production (Drewke, Thielen, & Ciriacy, 1990). In the present study, the tested yeast produced very high amounts of ethanol (60 g/L). However, a study by Belda, *et al.* (2017) claim that one of the reasons why *T. delbrueckii* is considered a promisor yeast to produce SA, is due to its reduced production of ethanol. Kamzolova, Yusupova, Vinokurova, & Morgunov. (2009) state that media with ethanol for the microbial production of SA have some advantages over other possible carbon sources because ethanol facilates the isolation and purification of SA due to the low contect of by-products. Considering this, we added ethanol to the culture medium to understand if T12

yeast (*T. delbrueckii*) when exposed to stress conditions follow the metabolic pathway of the TCA cycle. None SA production was obtained in media with ethanol, concluding that the presence of ethanol on the medium makes no difference to increase the SA production, contradicting what was stated in the literature. Even though Kamzolova, Yusupova, Vinokurova, & Morgunov. (2009) have confirmed the opposite, however, the medium that these authors used had ethanol as a sole carbon source, while ours also had glucose in its composition. An additional experiment was accomplished, using cells adapted to ethanol after 96 h, transferring them to a medium without ethanol (M3). In this case, SA production was obtained. Although the SA yield was low (0.9 g/L), it is possible to observe an increase when comparing these results with the results from SA production when yeast is initially grown in a medium without ethanol (YPD), having twice its production. Nevertheless, more SA production was obtained when the pre-culture contained 4 % of ethanol (0.9 g/L) than in 8 % of ethanol (0.5 g/L).

Another aim of this work was to understand if *T. delbrueckii* is able to grow in the presence of SA, which could be an indication of some transportation system that could carry the acid to the inside of the cell. Four *T. delbrueckii* strains were tested along with three more yeasts of distinct species: 1) *Debaryomyces hansenii* (DB600) as positive control, because Soares-Silva *et al.* (2015) reviewed the existence of two succinate transporters in this species; 2) *Schizosaccharomyces pombe* also as positive control because it is already known that *S. pombe* has transporters for SA and since exist a study by Ito and co-workers that proposed an improvement in the SA yield by expressing the gene encoding the *S. pombe* malic acid (*MAE*) transporter, achieved in the end and increase in SA concentration, concluding that the *MAE* carrier is also capable of transporting SA; 3) *Saccharomyces cerevisiae*, as negative control, since so far, no carrier for SA was found in this yeast (Raab, Gebhardt, Bolotina, Weuster-botz, & Lang, 2010). The growth was tested in solid and aqueous media and yeasts were also cultivated in YPD, a medium containing glucose as sole carbon and energy source as a control, to be sure there are no problems with cell viability. The results observed showed that only two yeast strains were able to use SA as sole carbon source, DB600 (*D. hansenii*) and T12 (*T. delbrueckii*). The first one was expected once there are already studies on their SA transporters, but *T. delbrueckii* was a surprising result due to the inexistence of information about this topic in the literature, making this, one of the first studies showing the capacity of *T. delbrueckii* yeasts to grow in a medium containing SA as sole carbon and energy source. This fact could indicate the presence of a transport system, not described until now for this species. However, if this transport system exists, its presence must be strain dependent, since only one of the four *T. delbrueckii* strains was able to display growth similar to the control. According to the literature, the transporters are involved not only in the export of the SA but also in the decrease of cell toxicity and the

increase of the productivity of this acid (Soares-Silva, *et al.*, 2015). However, the *T. delbrueckii* strain (T12) which is able to grow in SA was not the yeast that produced the most SA, in fact under natural conditions only produced 0.5 g/L. Apparently, the presence of transporters doesn`t lead to an increased SA production, showing that there is no direct relationship between the transport inside the cell and outside of the cell. These results can be explained in three ways. First, the existence of a new transporter able to uptake the SA and cross the cell membranes. Second, the existence of a known transporter, for example, *JEN2*, as there are already studies that confirm that this carrier has the ability to uptake SA (Vieira, *et al.*, 2010). Third, even a carrier of another acid which in this case is also transporting the SA. However, in the study by Ito and co-workers beyond the introduction of the *S. pombe* gene, they also deleted other genes and what may have contributed to the increase of SA production was the deletion of the genes and not the intrusion of *MAE* gene (Ito, Hirasawa, & Shimizu, 2014). Another surprising result was related to the fact that this yeast strain (T12- *T. delbrueckii*) in the control medium (YPD) also had higher growth than the other yeasts so this strain may have some alteration in its metabolism. Therefore, further experiments are already underway to sequence the complete genome of these isolates in order to better understand what differentiates this strain from the others.

Comparing growth in solid and aqueous media it is possible to observe major differences. While in solid media only two yeasts have grown, in liquid media all yeasts eventually started to grow, with an exception of T11. It is important to mention that the two yeast strains that showed growth in solid medium (*D. hansenii* and *T. delbrueckii*) were the first two to achieve the stationary phase in the aqueous medium, while the others only reached this phase after approximately 50 h. However, it should also be noted that cells grown in the SA medium only reached OD of ≈3, whereas, in YPD, yeast growth reached OD of 10. *S. cerevisiae* yeast was the negative control because studies with this isolate until now have never found a transporter for SA, so the growth of this yeast in SA aqueous medium must be explained. One possible explanation for this difference is the production of other metabolites after some time, using these metabolites as a carbon source and not the SA present in the medium and that is why these yeasts only started to grow after 48 h. Nonetheless, there is little information about the growth of yeasts in aqueous medium with SA as sole carbon source in the literature.

The last step was to evaluate the SA production in yeasts from the EUROSCARF (European *S. cerevisiae* archive for functional analysis) collection. As the name implies this collection consists of *S. cerevisiae* strains with a deleted gene, thus we can see the influence that the deleted gene has in the SA production. In this part of the work, as it was a smaller number of samples, we use enzymatic kit instead of HPLC because according to the theorical information enzymatic kit was more accurate but up to this

point we had a lot of samples to analyze and so it would be impractical to use the enzymatic kit. We also test if there are some differences between the concentration of SA obtained by enzymatic kit with that obtained by HPLC (date not shown) but the results were inconclusive. In the future a new exploration will be needed. A set of 8 important genes was selected (*HAP4*, *ADH1*, *ADH3*, *SDH1*, *FUM1*, *MDH2*, *SER3*, and *ICL1*) and a comparison of succinate production was done comparing the strains with the mutated genes with an *S. cerevisiae* strain in natural conditions. These 8 genes were chosen due to previous works that sequenced the whole genome from different strains and identified these genes as important and relevant in the pathway of SA production (Franco-Duarte, *et al.*, 2017; Pais, *et al.*, 2016). *HAP4* is a transcription factor and a regulator of the respiratory gene expression. This gene is involved in the change from fermentative to oxidative status and its higher activity will decrease the ethanol production redirecting flow to the oxidative part of the TCA cycle (Franco-Duarte, *et al.*, 2017). The alcohol dehydrogenase (*ADH1* and *ADH3*), as the name implies is the key enzyme of alcoholic fermentation. Deleting these genes leads to a decrease in ethanol production, preventing yeast to follow the alcoholic fermentation path (Drewke, Thielen, & Ciriacy, 1990). Gene *SDH1* is involved in the oxidation of succinate to fumarate, and deleting this gene prevents the accumulation of fumarate. The elimination of gene *FUM1*, involved in the interconversion of fumarate to malate, does not allow the production of malate. Then, in the TCA cycle, malate is transformed in oxaloacetate, gene *MDH2* codifying enzyme responsible for this process, deletion of this gene prevents the accumulation of oxaloacetate (Franco-Duarte, *et al.*, 2017). Gene *SER3* (3-phosphoglycerate dehydrogenase) when suffering a deletion interrupts the production of serine derived by glycolysis. Deleting the gene *ICL1*, that converts isocitrate into glyoxylate, does not allow the yeast to follow the glyoxylate shunt, redirecting the flow to the oxidative pathway of the TCA cycle (Rezaei, Aslankoohi, Verstrepen, & Courtin, 2015). Results showed no major differences between control (*S. cerevisiae* wild type) and the eight tested strains with deleted genes. However, all strains with deleted genes should have higher SA values than control because these genes were selected due to previous information confirming that the deletion of these genes, leads to an increase of SA yield. One possible reason behind this outcome is the fact that just deleting a gene is not enough to increase SA production since most of the studies focused on the metabolic engineering strategies, consist of the elimination of several genes. As for example, in this study with only a deletion of *SDH1* there was a SA yield of 0.3 g/L wich was equal to the control yield, in strain E16 (deletion of *SER3* gene) the maximum yield obtained was 0.6 g/L and with the *ICL1* gene mutation the production was similar to the control. When comparing these results with the results obtained by Otero, *et al.* (2013) that with the deletion of the genes (*SDH*, *SER3*/*SER33*, overexpression of native *ICL1*) only obtain 0.9 g/L of SA, our results were not that different.

In the study by Yan, *et al.* (2014), 3 genes were delected (*FUM1*, *PDC*, *GPD1*), leading to a final product of 8.09 g/L of succinate. The strain E11, of this study, presented a deletion of the gene *FUM1*, in the end, had a SA production of ≈0.3 g/L. The difference between these two results is very high, however, this difference may be due to the influence of the other two genes. Although the metabolism in which these genes are involved are very complex to study, in the future, it would be interesting to manipulate new genes and delete more than one gene in the same strain, in order to understand if the elimination of more than one gene leads to a increase in SA production.

Chapter 6: REFERENCES

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Chapter 7: APPENDIX

Appendix 1- Composition of the mother solutions (amino acids, trace elements, vitamins and anaerobic factors) presented in M1 medium and the trace elements present in M2 medium.

Table A1.1. Composition of the amino acids mother solution.

Amino Acids	Amount in grams (1 L)
L-Tyrosine	1.4
DL-Tryptophan B	13.7
L-Isoleucine	2.5
L-Aspartic acid	3.4
L-Glutaminic acid	9.2
L-Arginine	28.6
L-Leucine	3.7
DL-Threonine	5.8
Glycine	1.4
L-Glutaminic	38.6
DL-Alanine	11.1
L-Valine B	3.4
DL-Methionine	2.4
DL-Phenylalanine	2.9
L-Serine B	6.0
L-Histidine B	2.5
L-Lysine B	1.3
L-Cysteine	1.0
L-Proline B	46.8
Total	138.9

Table A2.1. Composition of the trace elements mother solution.

Elements	Amount in grams (1 L)
$MnSo_4.H_2O$	4
$ZnSO_4.7H_2O$	4
$CuSO_4.5H_2O$	1
KI	1
$CoCl_2.6H_2O$	0.4
H_3BO_3	1
$(NH_4)_6Mo_7O_{24}$	1

Table A3.1. Composition of the vitamin mother solution.

Elements	Amount in grams (1 L)
Myo-Inositol	2.0
Pantothenic acid	0.15
Thiamine, hydrochloride	0.025
Nicotinic acid	0.2
Pyridoxine	0.025
Biotin	0.0003

Table A4.1. Composition of the anaerobic factors mother solution.

Elements	Amount in grams (1 L)
Ergosterol	1.5
Oleic acid	0.5
Pure ethanol + Tween 80	50mL + 50mL

Table A5.1. Composition of trace elements solution.

Components	Concentration (g/L)
$MnSO_4.H_2O$	0.1708
$ZnSO_4.7H_2O$	0.2848
$FeSO_4.7H_2O$	0.0658
$CuSO_4.5H_2O$	0.0279
$Co(NO_3)_2.H_2O$	0.0367
$Na_2MoO_4.2H_2O$	0.0250

Appendix 2- HPLC analysis results regarding the concentration of metabolites (citric acid, tartaric acid, acetic acid, and malic acid, ethanol) and growth curve of a total of 13 yeast isolates.

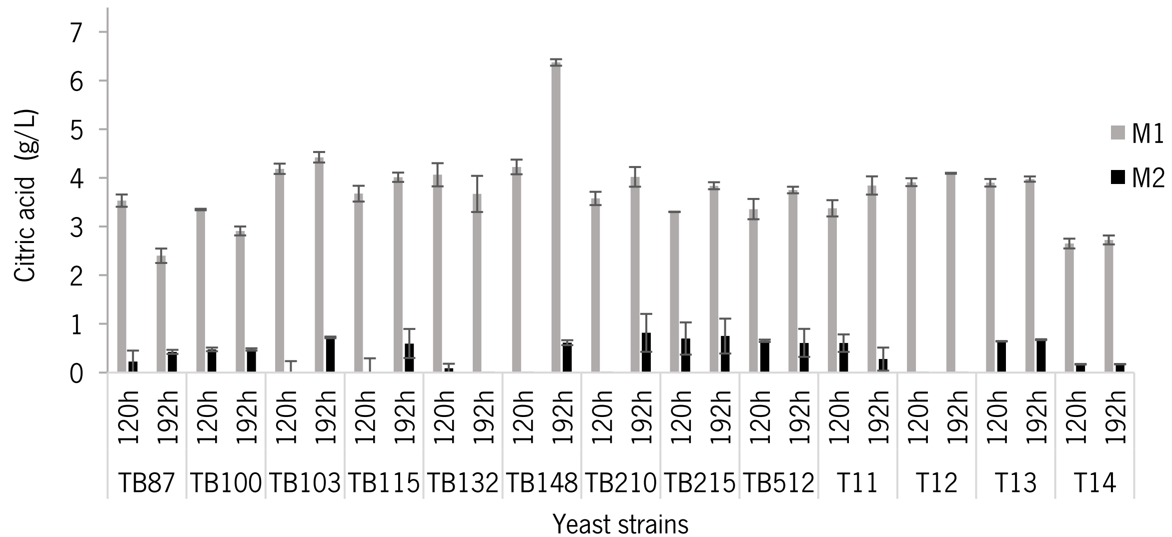

Figure A1.2. Concentration of citric acid obtained by HPLC analysis using 13 distinct yeast isolates, in two media (M1- glucose as carbon source, and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h). The experiments were performed in triplicate.

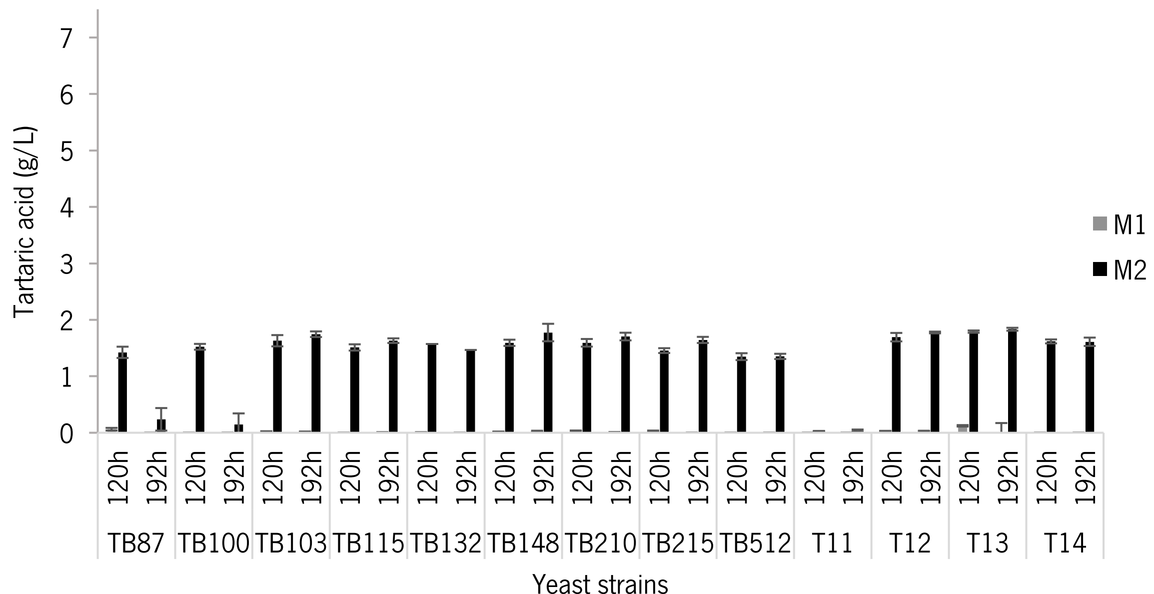

Figure A2.2. Concentration of tartaric acid obtained by HPLC analysis using 13 distinct yeast isolates, in two media (M1- glucose as carbon source, and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h). The experiments were performed in triplicate.

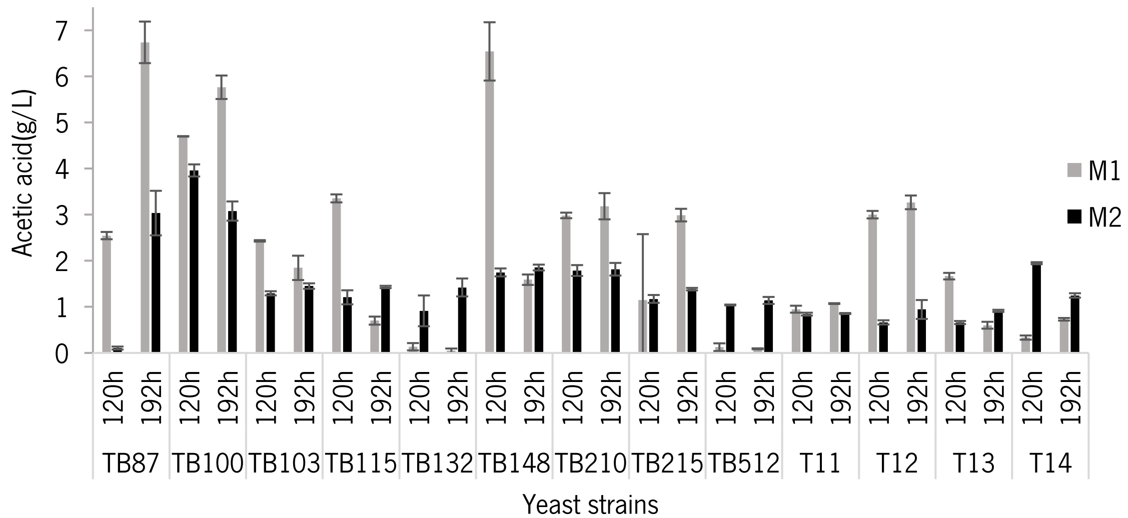

Figure A3.2. Concentration of acetic acid obtained by HPLC analysis using 13 distinct yeast isolates, in two media (M1- glucose as carbon source, and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h). The experiments were performed in triplicate

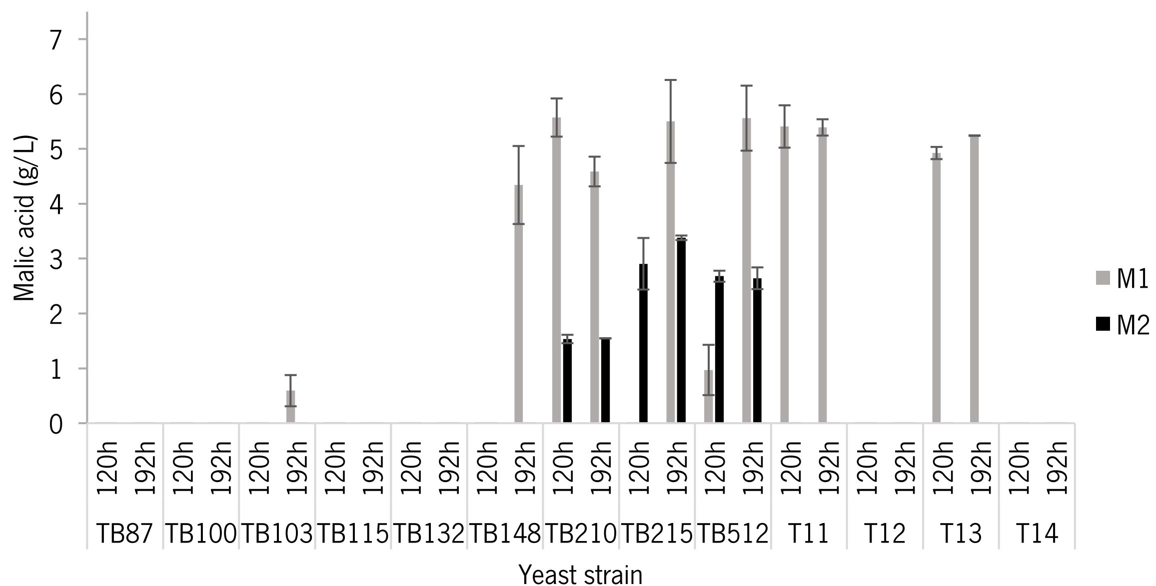

Figure A4.2. Concentration of malic acid obtained by HPLC analysis using 13 distinct yeast isolates, in two media (M1- glucose as carbon source, and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h). The experiments were performed in triplicate

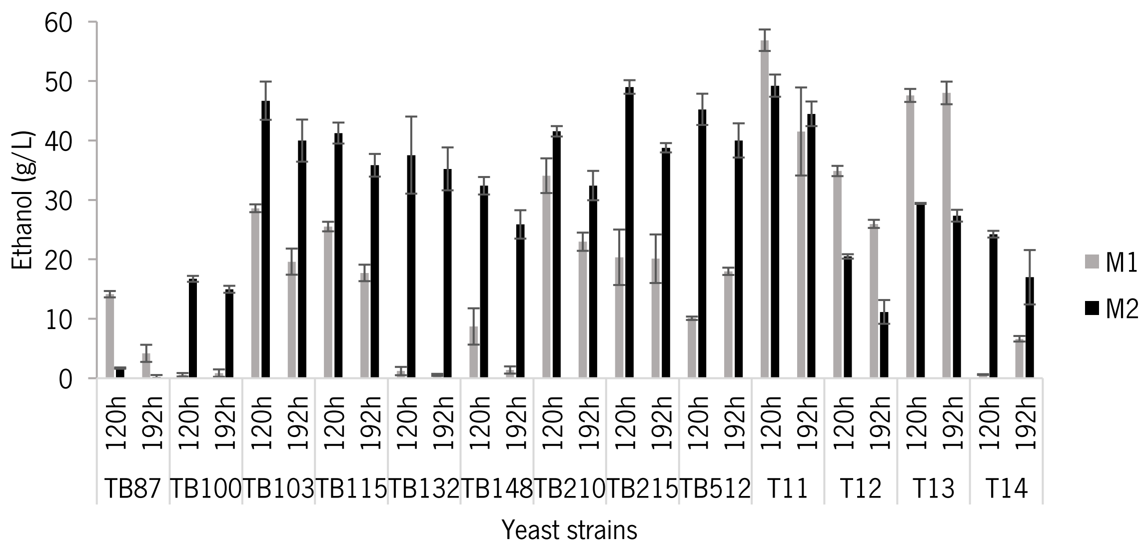

Figure A5.2. Concentration of ethanol obtained by HPLC analysis using 13 distinct yeast isolates, in two media (M1- glucose as carbon source, and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h). The experiments were performed in triplicate.

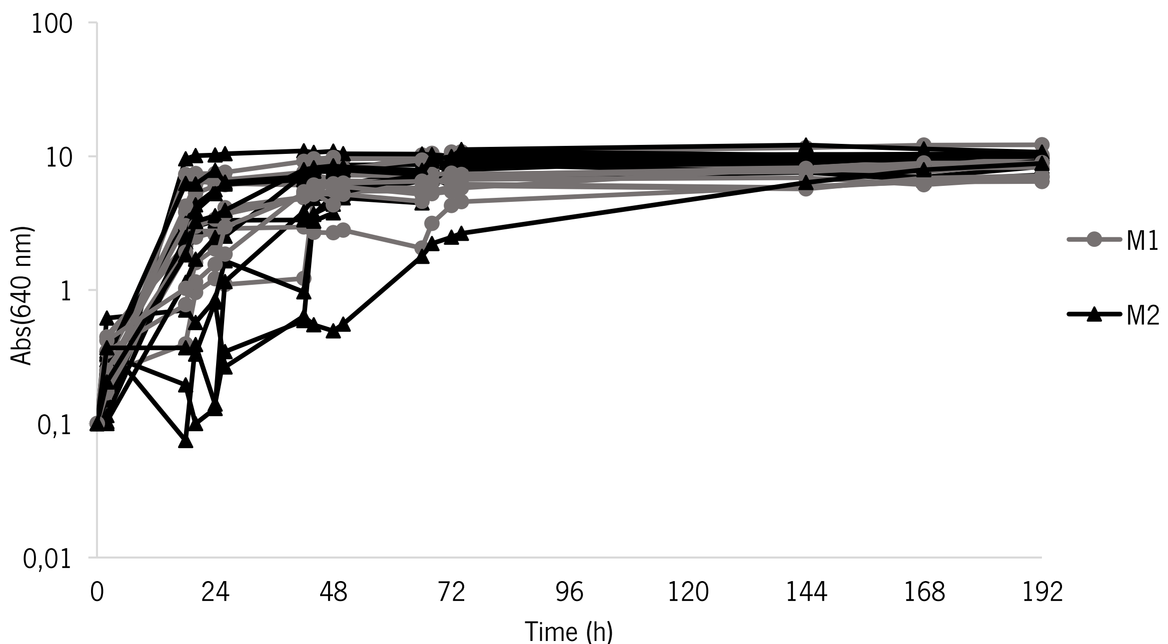

Figure A6.2. Growth curve of the 13 distinct yeast isolates, in two media (M1- glucose as carbon source and M2- sucrose as carbon source). The growth was monitored in the course of 192 h. The experiments were performed in triplicate.

Appendix 3- HPLC analysis results regarding the concentration of metabolites (citric acid, tartaric acid, acetic acid, and malic acid, ethanol) and growth curve of a total of two *Torulaspora delbrueckii* strains (T11 and T14).

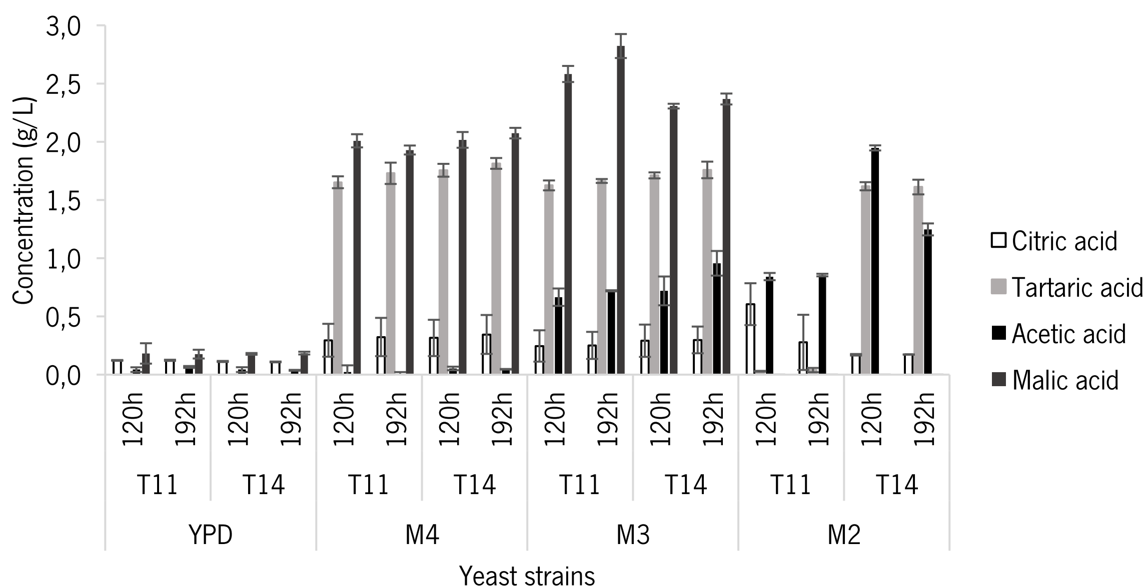

Figure A1.3. Concentration of organic acids (citric acids, tartaric acid, acetic acid, and malic acid) obtained by HPLC analysis using two *T. delbrueckii* strains, in four media (YPD, M3, and M4- glucose as carbon source and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h) The experiments were performed in triplicate.

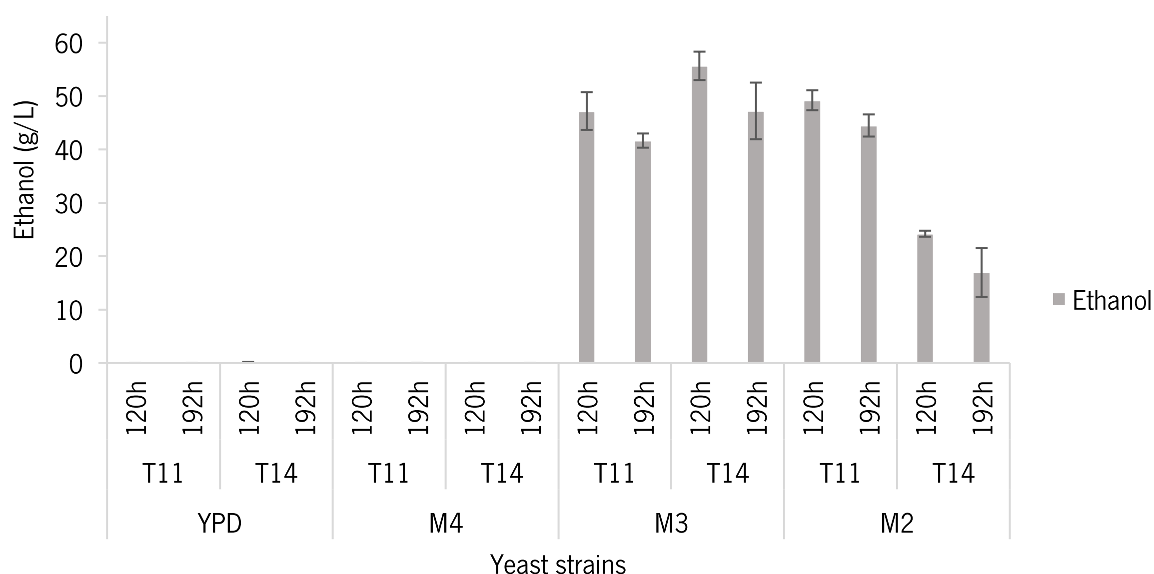

Figure A2.3. Concentration of ethanol obtained by HPLC analysis using two *T. delbrueckii* strains, in four media (YPD, M3, and M4- glucose as carbon source and M2- sucrose as carbon source) in two time-points of growth (120 h and 192 h) The experiments were performed in triplicate.

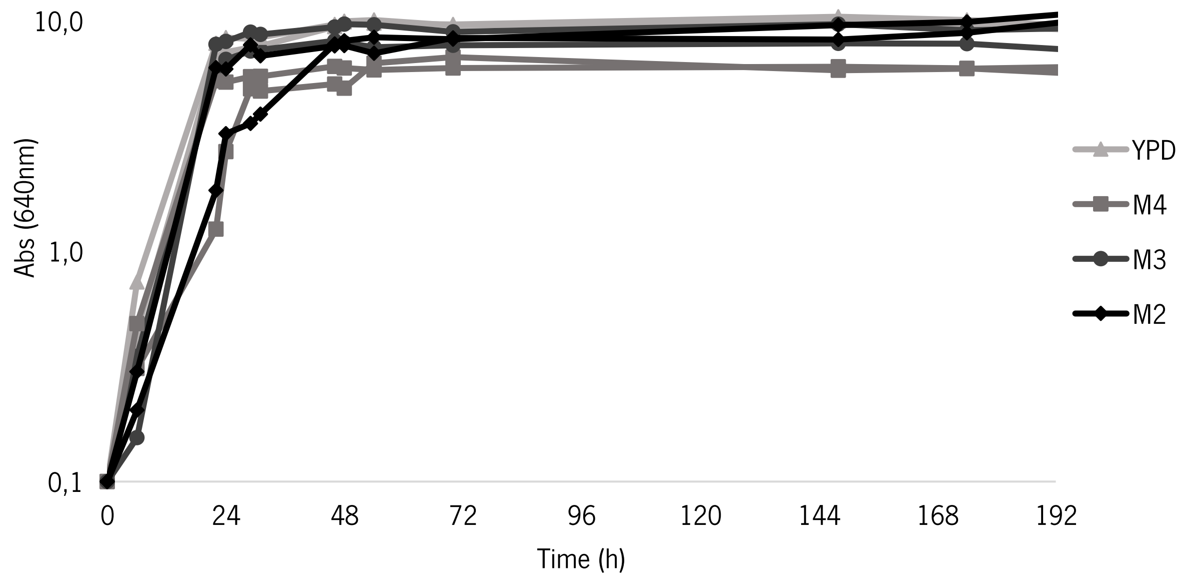

Figure A3.3. Growth curve of two *T. delbrueckii* strains (T11 and T12), in four media (YPD, M4, and M3- glucose as carbon source and M2- sucrose as carbon source). The growth was monitored in the course of 192 h. The experiments were performed in triplicate.

Appendix 4- HPLC analysis results regarding the concentration of metabolites (citric acid, tartaric acid, acetic acid, and malic acid, ethanol) of a total of seven *T. delbrueckii* strains.

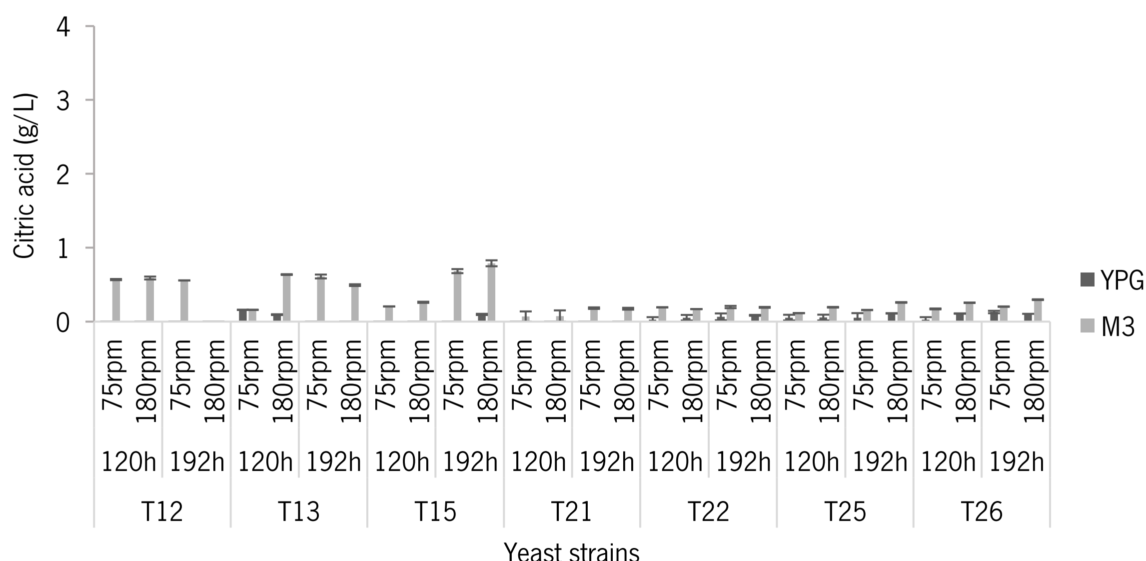

Figure A1.4. Concentration of citric acid obtained by HPLC analysis using seven *T. delbrueckii* strains in two media (YPG- glycerol as carbon source and M3- glucose as carbon source) in two time-points of growth (120 h and 192 h) at two agitation rates: 75 rpm and 180 rpm. The experiments were performed in triplicate.

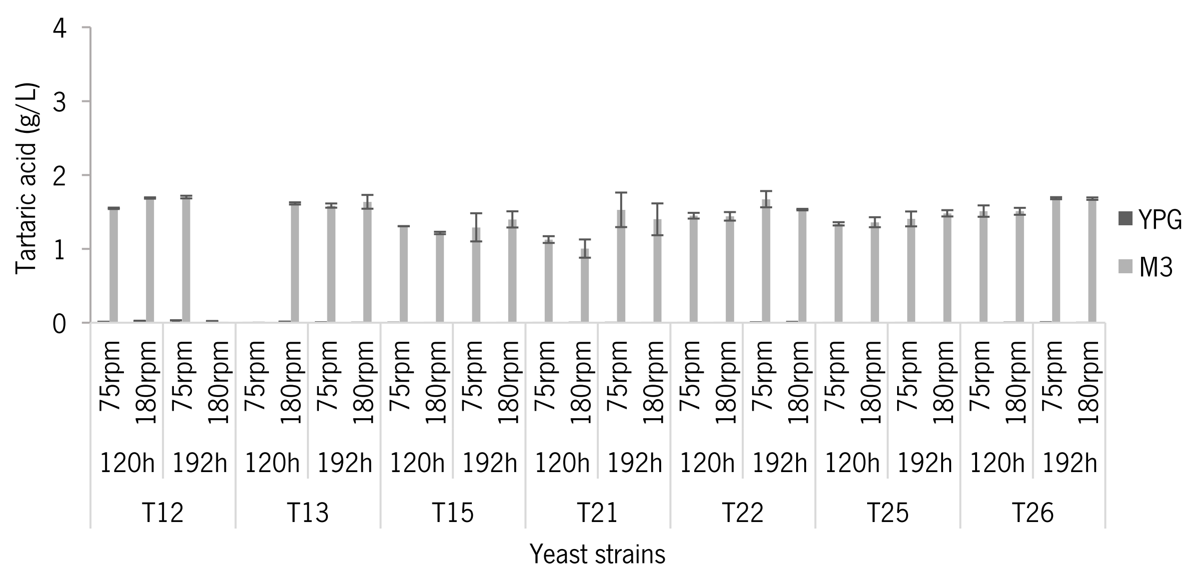

Figure A2.4. Concentration of tartaric acid obtained by HPLC analysis using seven *T. delbrueckii* strains in two media (YPG- glycerol as carbon source and M3- glucose as carbon source) in two time-points of growth (120 h and 192 h) at two agitation rates: 75 rpm and 180 rpm. The experiments were performed in triplicate.

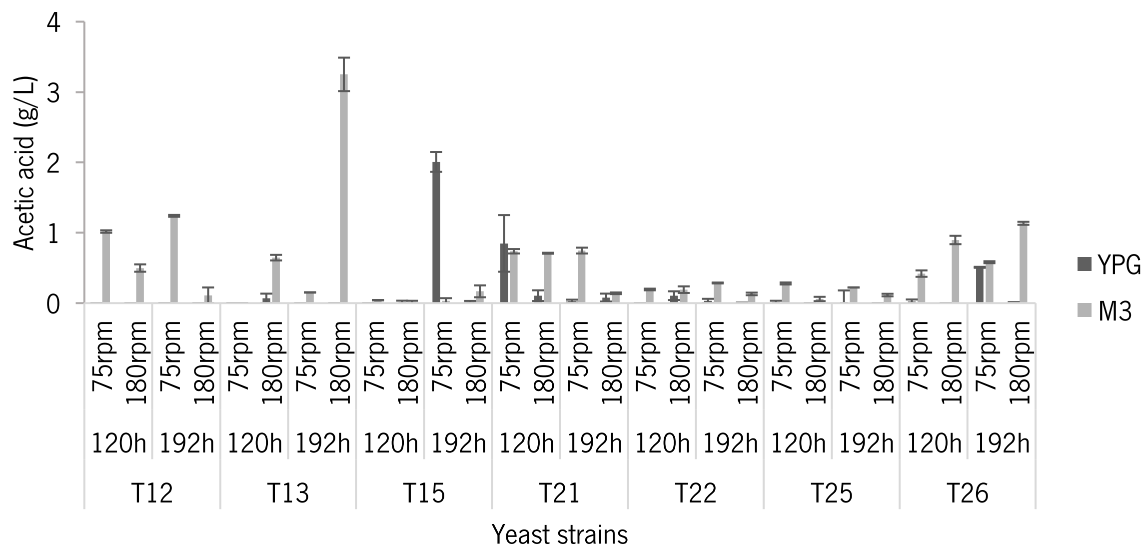

Figure A3.4. Concentration of acetic acid obtained by HPLC analysis using seven *T. delbrueckii* strains in two media (YPG- glycerol as carbon source and M3- glucose as carbon source) in two time-points of growth (120 h and 192 h) at two agitation rates: 75 rpm and 180 rpm. The experiments were performed in triplicate.

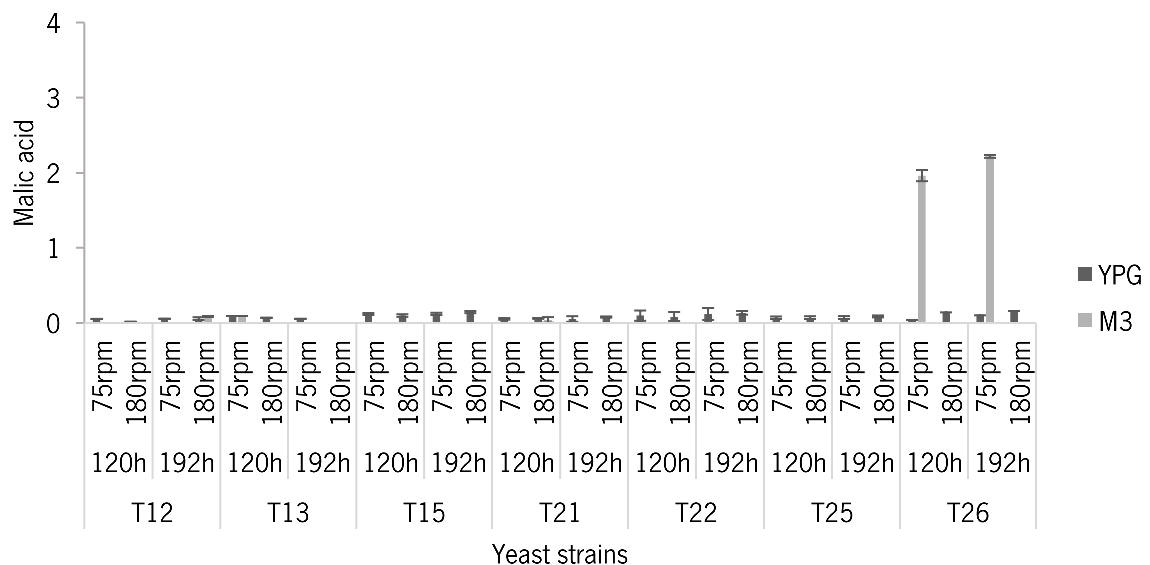

Figure A4.4. Concentration of malic acid obtained by HPLC analysis using seven *T. delbrueckii* strains in two media (YPG- glycerol as carbon source and M3- glucose as carbon source) in two time-points of growth (120 h and 192 h) at two agitation rates: 75 rpm and 180 rpm. The experiments were performed in triplicate.

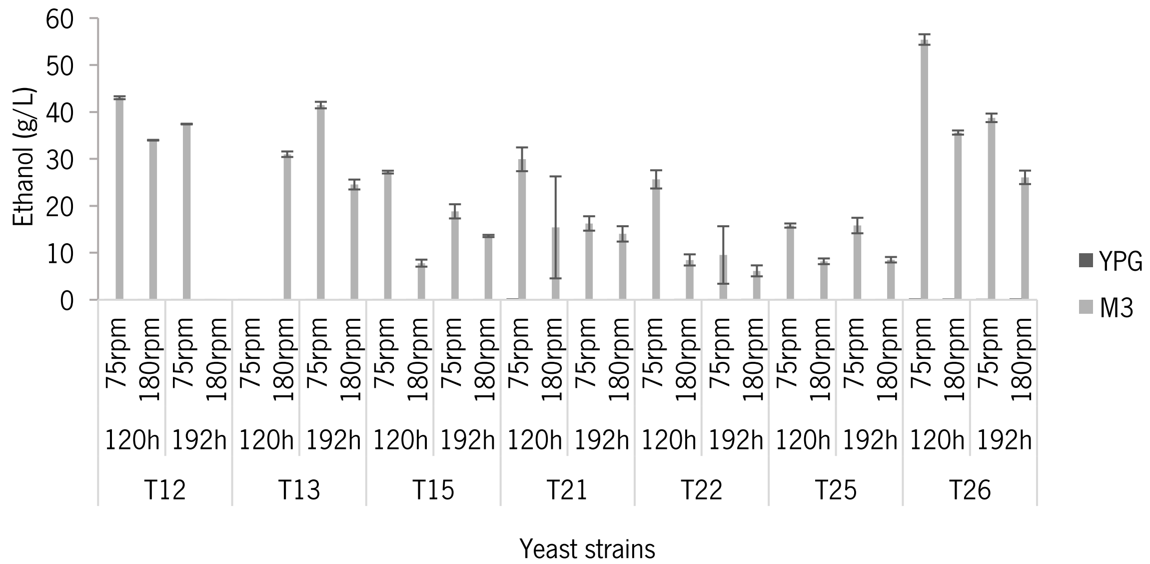

Figure A5.4. Concentration of ethanol obtained by HPLC analysis using seven *T. delbrueckii* strains in two media (YPG- glycerol as carbon source and M3- glucose as carbon source) in two time-points of growth (120 h and 192 h) at two agitation rates: 75 rpm and 180 rpm. The experiments were performed in triplicate.

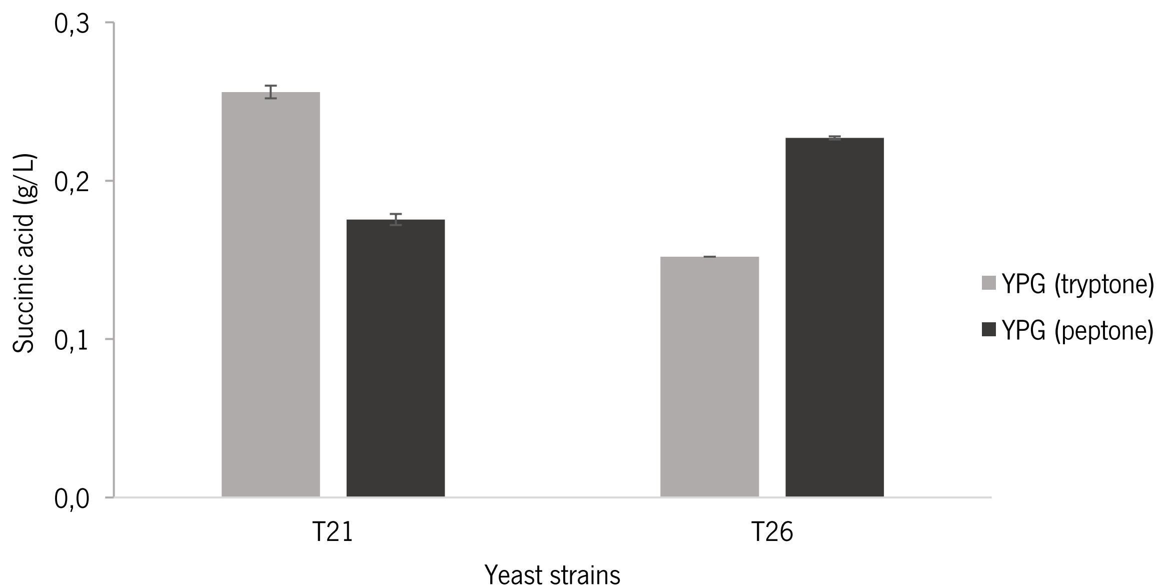

Figure A6.4. Concentration of succinic acid obtained by HPLC analysis using two *T. delbrueckii* strains, in YPG medium with different nitrogen sources (tryptone and peptone), in one time-point of growth (192 h) The experiments were performed in triplicate.

Appendix 5- HPLC analysis results regarding the concentration of metabolites (citric acid, tartaric acid, acetic acid, and malic acid, ethanol) and growth curve in one *Torulaspora delbrueckii* strain (T12).

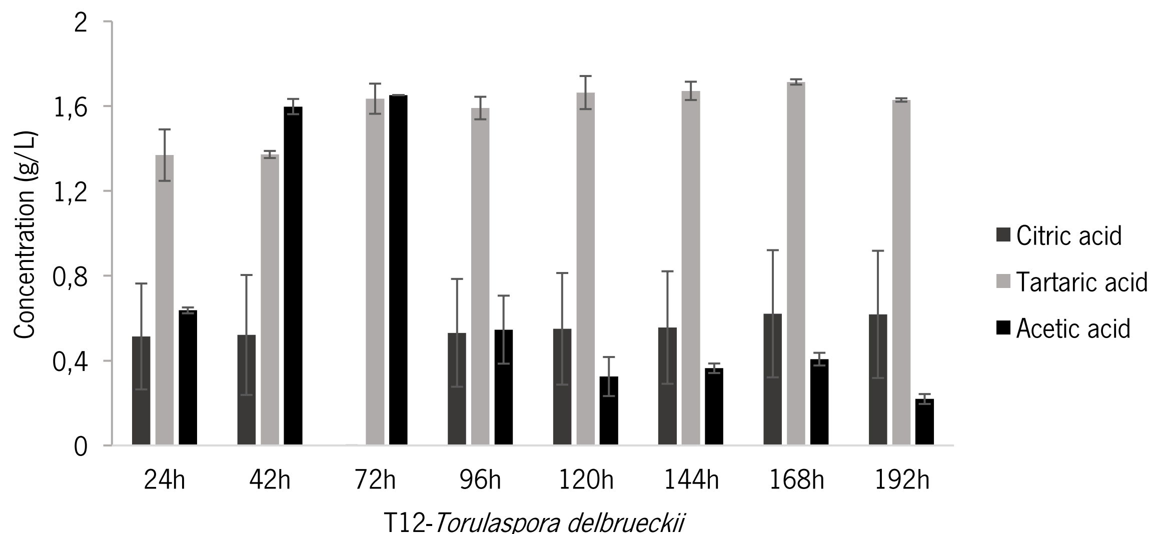

Figure A1.5. Concentration of organic acids (Citric acid, Tartaric acid, Acetic acid, and Malic acid) obtained by HPLC analysis using *T. delbrueckii* (T12) yeast strain in M3 medium, in 8 time-points of growth (24,48,72,96,120,192 h). The experiments were performed in triplicate.

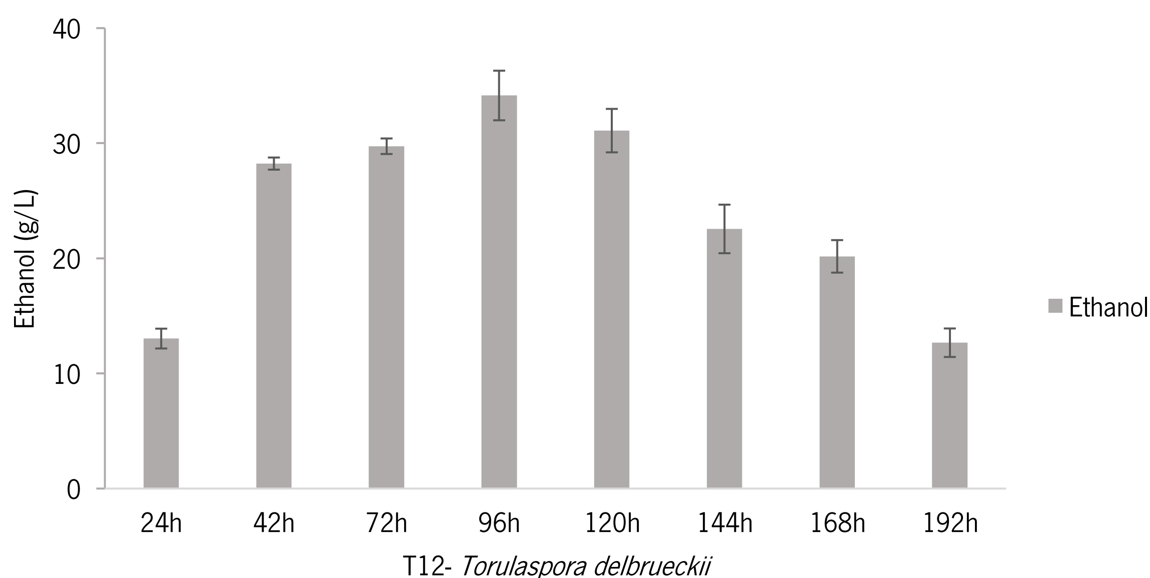

Figure A2.5. Concentration of ethanol obtained by HPLC analysis using T. delbrueckii (T12) yeast strain in M3 medium, in 8 time-points of growth (24,48,72,96,120,192 h). The experiments were performed in triplicate.

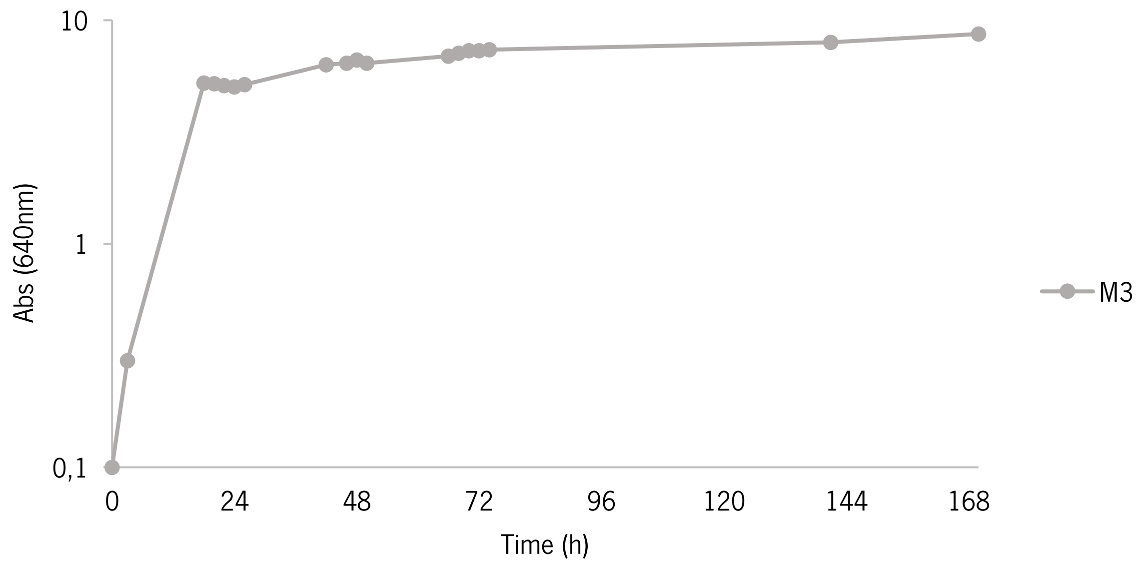

Figure A3.5. Growth curve of *T. delbrueckii* strain (T12), in M3 medium at 180 rpm. The growth was monitored in the course of 168 h. The experiments were performed in triplicate.