Exploitation of *Torulaspora delbrueckii* for winemaking focusing on biochemical and phenotypic traits

Fábio Rafael Moutinho Pereira

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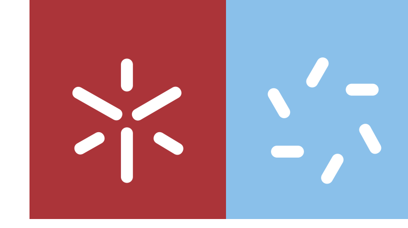

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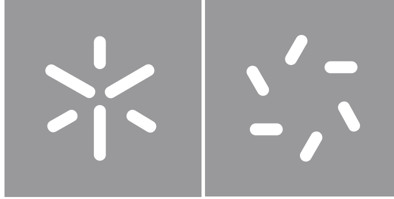

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Exploitation of *Torulaspora delbrueckii* for winemaking focusing on biochemical and phenotypic traits

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Doutor Ricardo Franco-Duarte
Professora Doutora Maria João Marques Ferreira Sousa Moreira

março de 2022

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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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Exploração de *Torulaspora delbrueckii* para a vinificação focando-se nos seus traços bioquímicos e fenotípicos

RESUMO

Um dos processos biotecnológicos mais antigos do mundo é a produção de vinho a partir de uvas, sendo a fermentação alcoólica o processo metabólico principal. Nesse processo, as leveduras desempenham um papel na conversão do açúcar da uva em álcool e CO_2, e na produção de metabolitos secundários. Dada a sua pureza de fermentação, acompanhada por uma baixa produção de subprodutos negativos para a qualidade do vinho, tanto em inoculação mista como sequencial com *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* representa uma excelente alternativa para reduzir a acidez volátil do vinho. Para além da sua utilização no processo de produção do vinho, diversos estudos têm mostrado o uso de *T. delbrueckii* na produção de pão, cerveja e chocolate. No âmbito do presente trabalho, procedeu-se à caracterização do perfil fermentativo de uma coleção de estirpes de *T. delbrueckii,* selecionadas de diferentes origens geográficas e tecnológicas, de forma a obter um melhor conhecimento da diversidade fenotípica da espécie. Para esse propósito, foi avaliado o caráter fermentativo de todas as estirpes da coleção, através de fermentações individuais em meio sintético que mimetiza o mosto de uva. Para avaliar o perfil metabólico das estirpes, foi utilizada a cromatografia líquida de alta eficiência (HPLC) para determinar o teor em glucose, etanol, acidez volátil, glicerol e ácidos orgânicos. A caracterização desta coleção permitirá uma exploração mais consistente da levedura, facilitando a sua aplicação em diferentes áreas de interesse, assim como compreender o desenvolvimento do processo fermentativo nesta espécie. A análise dos resultados mostrou uma produção variável de etanol, glicerol e ácido cítrico, apresentando estes compostos a maior contribuição para a variabilidade entre estirpes. Contudo, contrariamente ao descrito na literatura, foi detetada uma produção elevada de ácido acético (até 4.64 g/L) para algumas estirpes de *T. delbrueckii*. Com os nossos resultados, surge também o interesse de compreender melhor o consumo ou a produção de ácido málico, e a forma como este afeta a qualidade do vinho.

Palavras-chave: Perfil fermentativo, Leveduras não *Saccharomyces*, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, Vinho.

Exploitation of *Torulaspora delbrueckii* for winemaking focusing on biochemical and phenotypic traits

ABSTRACT

One of the oldest biotechnological processes in the world is the production of wine from grapes, with alcoholic fermentation being the main metabolic process occurring. In this process, yeasts play a key role in the conversion of grape sugar into alcohol and CO_2, and in the production of secondary metabolites. Given its high purity of fermentation, accompanied by a low production of negative sub-products for the quality of the wine, either in mixed or sequential culture with *Saccharomyces cerevisiae*, *Torulaspora delbrueckii* presents an excellent alternative for reducing the volatile acidity of wine. In addition to its use in the wine production process, several studies have shown the potential of *T. delbrueckii* for the production of bread, beer and chocolate. Regarding the development of the present work, it was intended to obtain a better characterization of the fermentative profile produced by a set of *T. delbrueckii* strains, selected from different geographical and technological origins to allow the evaluation of the phenotypic behavior of this yeast. For this purpose, individual fermentations in synthetic medium simulating grape must were performed. In order to evaluate the metabolic profile of the strains, high performance liquid chromatography (HPLC) was used to determine glucose, ethanol, volatile acidity, glycerol and organic acids concentrations. The results from this work will allow an easier exploration of *T. delbrueckii*, facilitating its application in different areas of interest, as well as understanding the development of the fermentative process in this yeast. HPLC analysis reflected variable results, with ethanol production, glycerol and citric acid concentrations, contributing the most to the variability between strains. However, contrary to what is found in the literature, a high acetic acid production (up to 4.64 g/L) was detected for some *T. delbrueckii* strains. With our results also arises the interest to better understand the consumption or the production of malic acid, and how it affects wine quality.

Keywords: Fermentative profile, non-*Saccharomyces* yeasts, *Saccharomyces cerevisiae*, *Torulaspora delbrueckii*, Wine

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ABBREVIATIONS, ACRONYMS AND SYMBOLS

Acetyl-CoA – Acetyl coenzyme A

ATP – Adenosine triphosphate

CBMA – Centro de Biologia Molecular e Ambiental

cDNA – Complementary Deoxyribonucleic acid

DNA – Deoxyribonucleic acid

FCT – Fundação para a Ciência e a Tecnologia

GC – Gas Chromatography

GC-O – Gas Chromatography-Olfactometry

g/L – Gram per liter

h – Hours

HPLC – High-Performance Liquid Chromatography

INIAV – Instituto Nacional de Investigação Agrária e Veterinária

INRA – Institut national de la recherche agronomique

LP- Lag Phase

min – Minutes

mL- Milliliter

mL/min - Milliliter per minute

MS – Synthetic must medium

MSA – Mother Solution of Amino acids

MSF – Mother Solution of Anaerobic Factors

MST – Mother Solution of Trace elements

MSV – Mother Solution of Vitamins

NADH - Nicotinamide adenine dinucleotide hydride

NAD^+ - Nicotinamide adenine dinucleotide

PCA – Principal Component Analysis

PCR- Polymerase Chain Reaction

REA-PFGE – Restriction Endonuclease Analysis Pulse-Field Gel Electrophoresis

RNA – Ribonucleic acid

RNAseq – RNA sequencing

rpm – revolutions per minute

SNP – Single nucleotide polymorphism

TCA - Tricarboxylic acid cycle

UNL – Universidade Nova de Lisboa

YPD – Yeast extract-peptone-dextrose

µm - Micrometer

v/v – Volume per volume

CHAPTER 1

INTRODUCTION

1.1. Alcoholic fermentation

The current wine production process aims to achieve greater control over the cost, yield and quality of the whole process. Therefore, it has been necessary to consider two distinct phases: the grape production phase, which is the viticulture phase, and the winemaking phase, where the grapes are transformed into wine (Grainger *&* Tattersall, 2007). The way the grape juice is obtained varies according to the characteristics of the grapes and the type of desired wine. In the case of red and rosé wines, prolonged macerations take place so that the compounds present in the grapes skin can be extracted for the juice. The grapes may be pressed, and the juice clarified. Sometimes, it is necessary to adjust the acidity of the must, which can be reached by the addition of tartaric acid or calcium carbonate (Jackson, 2008).

During wine production, alcoholic fermentation is the main metabolic process occurring. It involves the anaerobic transformation of the fermentable sugars present in the must, particularly glucose and fructose, into ethanol and carbon dioxide (CO_2) (Aranda *et al.,* 2011). However, this is a much more complex process. While sugar is being consumed, many other biochemical reactions may take place, giving rise to several other compounds that will contribute to the organoleptic properties of the wine (Grainger *&* Tattersall, 2007). These substances can be of different types, such as organic acids, higher alcohols, aldehydes, volatile fatty acids, ethyl esters or acetates. The volatile compounds detain a high importance in this context since, without their production, the wine would have very little organoleptic interest (Aranda *et al.*, 2011; Genisheva *et al.*, 2014).

Yeasts are responsible for metabolizing the sugars and nutrients present in the must to obtain energy and thus increase its population. The process has 4 different phases: lag phase, exponential phase, stationary phase, and declining phase (Moreno-Arribas *&* Polo, 2009). The Lag Phase (LP) duration, T50 parameter (time necessary to ferment half the sugar content), and ethanol production are three important technological parameters that must be considered for industrial selection of yeasts, particularly the LP duration (Renault *et al.,* 2009).

During the first hours of the alcoholic fermentation process, yeasts undergo a period of adaptation to the new environment, which is known as the lag phase. Once well-adapted, the cells enter the exponential phase and begin to multiply until reaching their maximum rate of multiplication. This phase can be influenced by temperature, amount of oxygen and the concentration of available nutrients. Toxic secondary metabolites can also influence the stationary phase, which occurs when conditions start to become unfavourable, not only because of a lack of nutrients but also because of accumulation of toxic

metabolites. These cause a decrease in the rate of cell division of yeasts, which will decline until it equals the cell dead rate, leading to a constant population number. Then, the higher concentration of ethanol and other toxic substances and the lower availability of nutrients cause a decrease in the population, which enters the declining phase. The population of available yeasts should be sufficient to allow the total consumption of remaining sugars, thus avoiding slow or stuck alcoholic fermentations (Moreno-Arribas & Polo, 2009). It can thus be said that the quality of a wine depends on several factors, including viticultural practices, winemaking techniques and the types of yeasts used (Ciani *et al.*, 2010).

1.2. *Saccharomyces cerevisiae* and its role in alcoholic fermentation

One of the oldest biotechnological processes in the world is the production of wine from grapes. This process of converting sugars present in the grapes into ethanol is usually conducted by *Saccharomyces* yeasts. *Saccharomyces cerevisiae* is the most notorious yeast, since it plays a fundamental role not only in food production-related areas, such as bread, beer, and wine, but also in studies regarding the fields of genetics, molecular biology and biochemistry (Maturano *et al.*, 2012; García *et al.,* 2016). When it comes to wine production, the main yeasts involved in vinification within the *Saccharomyces* genus are *S. cerevisiae* and *S. uvarum* (Ribéreau-Gayon *et al.*, 2006).

In the beginning of the fermentation process, there are several yeast species present. However, *S. cerevisiae* rapidly becomes predominant, as it is more tolerant to alcohol (Andorrà *et al.*, 2012; Beltran *et al.*, 2002; Capece *et al.*, 2013; Ciani *et* Comitini, 2015). Accordingly, it is often the only one found in a final phase of the fermentation process, as the highly osmotic environment, the high acidity of the grape must, and the accumulation of toxic metabolites restrict the growth of most microorganisms. In fact, the dominance of *S. cerevisiae* in relation to other non-*Saccharomyces* yeasts is due to several factors, not only related to competition for nutrients, but also to the production of toxic compounds. The main factor that conditions the growth of non-*Saccharomyces* species during fermentation is the production of ethanol by *S. cerevisiae* (Fleet, 2003).

Ethanol is not the only metabolite to exert selective pressure on populations. In fact, the production of medium-chain fatty acids, as well as high amounts of acetic acid, may additionally lead to inhibition of the growth of some species. Another important factor is the rapid development of anaerobic conditions, which limit the growth of yeasts that, compared to *S. cerevisiae*, are poorly tolerant to reduced oxygen availability. Besides these factors inherent to the fermentation process, the addition of sulfur dioxide (SO_2) to musts has a strong selective effect on the development of microorganisms, thus favoring the

predominance of *S. cerevisiae*, which is more resistant than most species (Andorra et al. , 2011; Ciani *et* Comitini, 2015). The diversity of microorganisms present at the beginning of the alcoholic fermentation is also influenced by the contact of the grapes during harvest, as well as their transport and handling in the winery (Aranda *et al.*, 2011; González-Royo *et al.*, 2015).

In this way, at the beginning of the winemaking process, several species can be present in the must and participate in the fermentation, influencing, in a positive or negative way, the composition and final quality of the wine. In reality, indigenous yeasts have the capacity to produce sensory profiles that are difficult to replicate (Capozzi *et al.*, 2015). The characterization of *Saccharomyces* species has allowed the selection of strains that are less susceptible to give rise to undesirable flavors, ultimately allowing the different attributes of a wine to be emphasized. Due to this genetic and metabolic variability, there are strains of *S. cerevisiae* available on the market suitable for the most different situations, which gives producers the ability to modulate their wines according to the desired characteristics.

1.3. Non-*Saccharomyces* yeasts

In healthy grapes, the presence of *S. cerevisiae* strains is rarely detected and, when present, they only appear in small quantities. However, yeasts of other genera are often found in grapes such as *Brettanomyces, Candida, Dekkera, Pichia, Saccharomycodes, Schizosaccharomyces, Torulaspora* or *Zygosaccharomyces* (Capozzi *et al.*, 2015; De Koker, 2015; Fleet, 2008). The species whose presence has been detected are numerous (Table 1). Their particular contribution to wine quality can take several forms. For instance, *Candida stellata* and *Candida pulcherrima* produce glycerol and esters, respectively. In contrast*, Kloeckera apiculata* and other yeasts lead to the production of acetic acid, which reduces wine quality (Mateo *et* Maicas, 2016).

Table 1- Main non-*Saccharomyces* yeasts found in winemaking (Mateo & Maicas, 2016)

Aureobasidium pullulans	*Hansenula* sp
Brettanomyces sp	*Issatchenkia terricola*
B. anomalus	*Kluyveromyces thermotolerans*
Candida guilliermondii	*Lanchancea thermotolerans*
C. molsichiana	*Metschnikowia pulcherrima/C. pulcherrima*
C. stellata	*Pichia angusta*
C. utilis	*P. anomala*
C. zemplinina	*P. capsulata*
Debaryomyces castellii	*P. guilliermondii*
D. hansenii	*P. kluyvery*
D. polymorphus	*P. membranifaciens*
D. pseudopolymorphus	*Saccharomycodes ludwigii*
D. vanriji	*Schizosaccharomyces pombe*
Hanseniaspora sp. (Kloeckera)	*Sporidiobolus pararoseus*
H. guilliermondii	*Torulaspora delbrueckii*
H. osmophila	*Trichosporon asahii*
H. vineae	*Wickerhamomyces anomalus*
H. uvarum	*Zygosaccharomyces bailii*

Non-*Saccharomyces* yeasts, in the past, were essentially considered spoilage microorganisms but today they are viewed as beneficial. In recent years, the utilization of non-*Saccharomyces* yeasts has been considered for industrial wine production as several species have shown high oenological potential. Nonetheless, a problem arises with their use, as these non-*Saccharomyces* yeasts are only active in the first part of the fermentation, in which the ethanol concentration is still not very high, bringing some consequences to the quality of the wine (Romano *et al.*, 2003). They contribute to a better sensory profile of the wine, especially when grown in controlled mixed fermentations together with *S. cerevisiae*.

The presence of non-*Saccharomyces* wild yeasts in fermentation has been traditionally associated with high levels of acetic acid and other off-flavors. another relatively important compound is acetoin, which despite being odorless, can act as an intermediary in the metabolic pathways of aroma compounds, playing an important role in the formation of wine flavours. Previous studies have also shown that non-*Saccharomyces* yeasts affect the concentration of mannoproteins in wine (Domizio *et al.*, 2014). These have many beneficial oenological properties, such as improved taste, decreased astringency, added aromatic complexity and increased sweetness (Carvalho *et al.*, 2006; Chalier *et al.*, 2007; Guadalupe *et al.*, 2007). In addition, they can adsorb some toxic compounds present in wines, such as ochratoxin A (Caridi, 2007), while in sparkling wines, they can improve their sparkling properties (Núñez *et al.*, 2006). Some studies have reported the influence of non-*Saccharomyces* yeast species on wine quality, evaluating

the biotechnological interest of their enzymatic activities (e.g., esterases, β-glucosidase, and proteases), which are assumed to enhance fruit aromas in wine (Renault *et al.*, 2009; Sanoppa *et al.,* 2020; Tondini *et al.*, 2020; Tufariello *et al.,* 2021). For *Debaryomyces hansenii,* it was possible to prove that the enzyme D-glucosidase, whose activity is not inhibited by glucose or ethanol during the fermentation of Muscatel grape juice, results in an increase in the concentration of monoterpenols in wine (Domizio *et al.*, 2014).

The use of non-*Saccharomyces* yeasts as initiators of fermentation in the wine production process, besides presenting beneficial characteristics, also presents negative aspects, such as the production of acetaldehyde or acetoin in high concentrations (Ciani *et al.*, 2006; Viana *et al.*, 2008). Another parameter to be considered is the fact that most of the species from wine-producing environments have a limited fermentation potential evidenced by low power and fermentation rates, and low resistance to SO_2 (Nadai *et al.*, 2016). It is believed that non-*Saccharomyces* yeasts, besides contributing to the diversity of fermentation, also have a great influence on the organoleptic properties of wine in proportion to its relative abundance during fermentation. Due to the potential use of non-*Saccharomyces* yeasts in wine fermentation, several studies have been carried out and most wine distribution companies already use non-*Saccharomyces* strains in wineries (Jolly *et al.*, 2014; Lleixà *et al.*, 2016).

1.4. Torulaspora delbrueckii

Torulaspora delbrueckii is particularly useful in fermentations of grape musts with high sugar concentration since this yeast does not increase the production of acetic acid in response to the hyperosmotic environment like *S. cerevisae*. On the contrary, it maintains the ability to have a high purity fermentation with low production of volatile acidity. However, it has to be used together with *S. cerevisiae* because, although it can survive at high concentrations of ethanol due to its slow fermentation, it cannot reach the alcohol content required in wine fermentation (Bely *et al.*, 2008, Ciani *et al.*, 2006). This has led to the use of mixed fermentations of *T. delbrueckii* and *S. cerevisiae* in order to improve the quality of the aroma and reduce the concentration of acetic acid in wines (Taillandier *et al.*, 2014).

Several studies have described that, besides acetic acid, *T. delbrueckii* is responsible for low production of acetaldehyde, acetoin and ethyl acetate, though it also leads to high levels of beneficial compounds with great taste properties (Liu *et al.*, 2020; Martinez *et al.*, 1990; Padilla *et al.*, 2016). The amount of volatile acidity plays an important role in the wine's aroma since excessive concentrations of these by-products greatly impair the fermentation process. Furthermore, its fermentation capacity allows it to be implemented at the early stage of the fermentation process, an aspect that becomes quite

advantageous compared to other non-*Saccharomyces* species that are specifically oxidizing (Quirós *et al.*, 2014).

The phenotypic characteristics of *T. delbrueckii* should be illustrated by an analysis of fermentation traits and aromatic profiles in accordance with oenological practice. Previous studies on the aromatic potential of this species have shown that *T. delbrueckii* has a low capacity to produce esters, and also allowed Hernández-Orte and colleagues (2008) to suggest that this species significantly modulates the levels of various aromatic compounds, such as volatile phenols, vanillin, and lactones through hydrolysis of their precursors (Hernández-Orte *et al.*, 2008). Michel and colleagues (2016) studied different strains of *T. delbrueckii* from different habitats and found that one of these strains could produce a fruity, floral aroma (Michel *et al.*, 2016; Tufariello *et al.*, 2021). Thus, it is important to discriminate *T. delbrueckii* at the strain level due to the advantages associated with the different strains that may present peculiar properties to the wine's attributes or even lead to the introduction of new wines on the market.

There are currently six accepted *Torulaspora* species: *T. delbrueckii, Torulaspora globosa*, *Torulaspora franciscae*, *Torulaspora microellipsoides*, *Torulaspora maleeae,* and *Torulaspora pretoriensis* (Van Breda *et al.*, 2013), but only *T. delbrueckii* has been exploit for wine fermentation.

Yeasts are not only used in winemaking processes, but in other interesting fields like in baking and brewing areas, whose production processes include the same basic ingredients: cereals, yeast, and water (Canonico *et al.*, 2016; Fernandes *et al.*, 2021). Both fermentation processes use *S. cerevisiae* as a biocatalyst throughout evolution. Bread production requires three main ingredients: flour, water and yeast. In this process, the main role of yeast is the rapid fermentation of the sugars available in the dough flour. As a result of efficient fermentation, yeast produces CO_2 and ethanol. The amount of CO_2 that is retained in the gluten matrix of the dough is responsible for causing the fermentation. Consequently, ethanol contributes to the development of flavor, along with other volatile compounds and other flavor precursors that are formed during the fermentation process. The use of *T. delbrueckii* yeasts in bread production aims to increase tolerance to different stresses, such as osmotic stress or even thawing (Randez *et al.*, 1999; Pacheco *et al.*, 2012).

The modification of the volatile profile of beers to obtain a more complex aroma profile can easily be achieved through the use of *T. delbrueckii*. Some strains in this process have the peculiarity of producing, in a natural way, a lower ethanol content than *S. cerevisiae*, the main yeast species used in this sector, keeping in it all the improved properties. In this case, the use of *T. delbrueckii* has a positive effect on the aroma and taste of the alcoholic beverage obtained at the end (De La Cruz *et al.*, 2019). In

this context, Michel *et al.* (2016) found two strains suitable for producing low-alcohol and good flavored beer, despite its inability to ferment maltose and maltotriose (Michel *et al.*, 2016).

1.5. Mixed fermentations

Over the years, in order to improve the specific characteristics of the different styles of wine that is produced every day in the industry, and increase the complexity of the wines produced, the use of mixed cultures has been introduced, instead of the use of pure cultures (Capece *et al.*, 2013). In fact, the effects of pure and mixed cultures on the wine aroma have often been evaluated under various conditions (e.g., laboratory-scale tests carried out on synthetic media or sterilized grape must) very different from those normally found in the winery (Azzolini *et al.*, 2015). This controlled fermentation using mixed cultures is usually composed of yeasts, which, in combination, have good fermentative properties and allow high production of aromatic compounds and others of interest (Barrajón *et al.*, 2011). The yeast population found on the grape surface, and on winery equipment, usually consists of a mixture of *Saccharomyces* and different species of non-*Saccharomyces* yeasts. In fact, it is believed that, by inoculating non-*Saccharomyces* yeasts with strains of *S. cerevisiae,* the negative contribution of the former may not be expressed due to the metabolic activity of the strains of *S. cerevisiae* or could even be modified by this one (Ciani *et al.*, 2010, 2016; Claus, 2019).

Several studies have described the performance of alcoholic fermentations of both natural and synthetic grape must, with mixed starter cultures composed of a non-*Saccharomyces* yeast and a strain of *S. cerevisiae*, of two yeasts of the genus *Saccharomyces*, or of two different strains of *S. cerevisiae* (Andorrà *et al.*, 2012; Comitini *et al.*, 2011; Howell *et al.*, 2006; Loira *et al.*, 2014; Rodríguez *et al.*, 2010; Saberi *et al.*, 2012). Each yeast species presents a specific metabolic activity, but within each species, there may be some differences. For instance, within *S. cerevisiae*, different strains have different effects on the taste of wine (Molina *et al.*, 2009; Romano *et al.*, 2003). Of note, it has been proved that wines with mixed cultures have a different composition from wines produced by inoculating the must with pure strains. In fact, when wines produced by two different pure cultures are mixed in an attempt to reproduce a wine produced by these two yeasts, the result is not the same (Capece *et al.*, 2013; Ciani *et al.*, 2010).

Mixed fermentations with *T. delbrueckii* and *S. cerevisiae* culture constitute the best combination of yeasts for improving the analytical profile of sweet wine, particularly in terms of volatile acidity. A mixed culture of *T. delbrueckii* and *S. cerevisiae*, when in a proportion of 20 to 1, can produce 53% less volatile

acidity and 60% less acetaldehyde (Belly *et al.*, 2008). It has also been shown that a co-culture of *Candida zemplinina* and *S. cerevisiae* produces a lower concentration of aromatic compounds than a single culture of *C. zemplinina*, thereby suggesting a negative interaction between these two strains. When it comes to chocolate production, the combination of *S. cerevisiae* and *T. delbrueckii* has had a positive influence in the product, with the production of chocolate in terms of its analytical profile. The characteristic taste of chocolate is determined by the genetic potential of the cocoa variety, as well as by the way that the chocolate fermentation, drying, and processing phases are carried out (Afoakwa *et al.*, 2008; Crafack *et al.*, 2014; Visintin *et al.*, 2017). In addition, as in wine production, fermentation is considered to be a very important factor influencing the quality of cocoa and the taste of chocolate.

It is thus possible to state that the interactions between different strains, despite having positive effects, can also present negative effects on the final product. As a result, it is necessary to evaluate and test these effects in order to be able to select yeasts with synergistic effects on the sensory profile of the wine (Cheraiti *et al.*, 2005; Sadoudi *et al.,* 2012). Taking this into consideration, it is possible to say that combined fermentations are very useful to improve the wine fermentations, in which it is intended to unite the aromatic complexity of the spontaneous fermentations and the security of the oriented industrial fermentations. (Ciani *et al.,* 2010; Belda *et al.*, 2015).

1.6. The metabolism behind alcoholic fermentation

For the production of wine, it is necessary to always consider the phase of production of the grapes (viticulture) and the phase involving the transformation of grapes into wine (winemaking phase) (Grainger *et* Tattersall, 2007). Today, wine production depends on the ability of producers to make the most appropriate choices from the large set of options available for performing each of the steps in the process (Jackson, 2008). The main metabolic process that occurs during wine production, is alcoholic fermentation. This catabolic pathway involves the anaerobic transformation of fermentable sugars present in the must, mainly glucose and fructose, into products such as ethanol and carbon dioxide (Aranda *et al.* , 2011; Ugliano *et* Henschke, 2009). This process occurs in the cytoplasm of cells and can be expressed by the reaction depicted in Figure 1 (Genisheva *et al.*, 2014; Zamora, 2009).

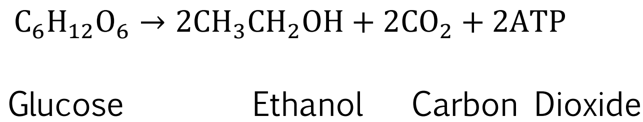

Figure 1 - Simplified reaction of alcoholic fermentation.

The conversion of the must sugars into ethanol first involves glycolysis. For most organisms, including yeast, this metabolic pathway represents the main initial process of carbohydrate catabolism. This process consists of a sequence of reactions that result in the transformation of hexoses (glucose and fructose) into pyruvate, with energy release in the form of ATP, as we can see in figure 2 (Aranda *et al.*, 2011; Jackson, 2008; Ribéreau- Gayon *et al.*, 2006; Zamora, 2009). The transport of hexoses across the membrane into cells can occur by facilitated diffusion. In fact, since the sugar concentration inside the cells is lower than the concentration in the must, this transport system does not require energy (Ribéreau-Gayon *et al.*, 2006; Zamora, 2009).

The first reaction of glycolysis consists of the phosphorylation of glucose and fructose by the action of enzymes called hexokinases, forming glucose-6-phosphate and fructose-6-phosphate, respectively, as represent in figure 2.

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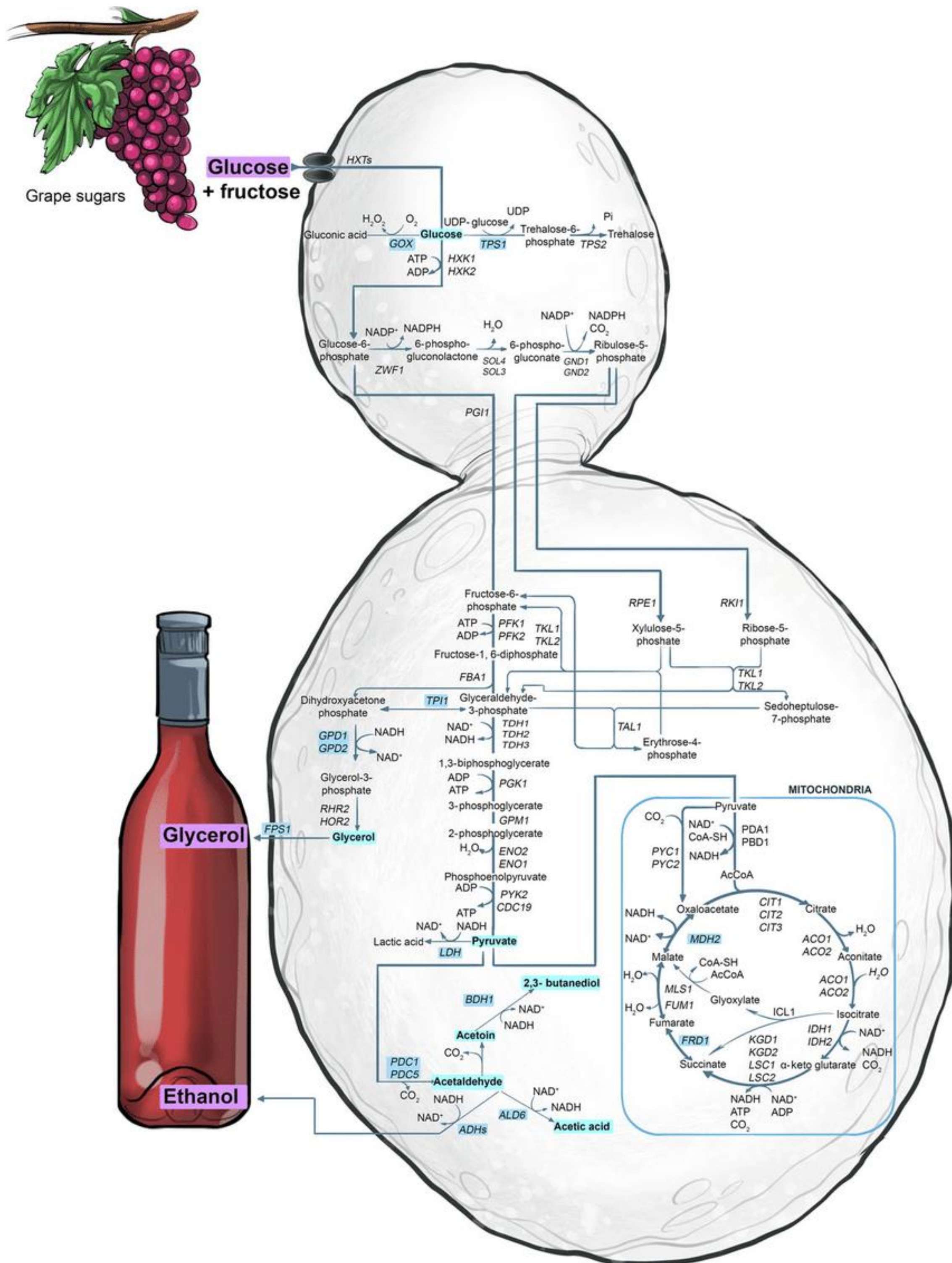

Figure 2- Representation of carbon metabolism in wine yeast, schematizing the processes of glycolysis, pentose phosphate pathway, and tricarboxylic acid cycle (TCA) (Goold et al., 2017, Microbial Biotechnology)

This reaction requires the consumption of ATP, but allows the preservation of the hexose concentration gradient, favoring the transport of sugars into the cell. Besides being involved in energy production for yeast, this metabolic pathway is also related to the production of metabolites involved in the biosynthesis of important molecules for the cells (Aranda *et al.*, 2011). The pyruvate produced by glycolysis can be used in various metabolic pathways, however, in order to restore its redox potential and ensure the continuation of glycolysis, cells must reoxidize NADH to NAD^+. Yeasts such as *S. cerevisiae* tend to direct pyruvate to ethanol production, allowing them to regenerate NAD^+ through two additional reactions that constitute alcoholic fermentation (Aranda *et al.*, 2011; Ribéreau-Gayon *et al.*, 2006; Zamora, 2009)

Regarding the transport of the end products of alcoholic fermentation, ethanol and carbon dioxide, this occurs by simple diffusion to the outside of the cell (Zamora, 2009). However, alcoholic fermentation is a much more complex process. At the same time as these general reactions occur, numerous other biochemical and chemical reactions can also take place, which in turn result in the production of various compounds responsible for contributing to the organoleptic properties of the wine. These compounds, produced in small quantities, are acetates, aldehydes, ethyl esters, higher alcohols, organic acids and volatile fatty acids. These are of high interest since without their production, the wines produced would have very little organoleptic interest (Aranda *et al.*, 2011; Genisheva *et al.*, 2014; Zamora, 2009).

1.7. Biochemical and genetic characterization of wine yeasts and species selection

As referred above, during the alcoholic fermentation process, yeast cells are subjected to various stress conditions, which leads them to develop molecular mechanisms to increase resistance to these adverse conditions. Studies have shown that this process of adaptation to environments with specific oenological conditions is reflected in the transcriptome, proteome, and metabolome (Mendes *et al.*, 2017; Rossouw *et al.*, 2010). In fact, the different properties that yeasts display during wine production determine their fermentative ability and allow the establishment of criteria for the selection of future wine yeasts (Carrasco *et al.*, 2001; Ivorra *et al.*, 1999). It can thus be said that monitoring the population dynamics of different yeast strains during an alcoholic fermentation process, can provide valuable information for the optimization of the process.

There are different selection criteria for yeasts. Some are favorable, such as tolerance to ethanol, good yield in the transformation of sugars into ethanol, and the ability to survive in high sugar concentrations, but others may be unfavorable, as the production of hydrogen sulphide, volatile acidity, and even foam

production (Belda, *et al.*, 2015; Ciani *et* Picciotti, 1995; Loira *et al.*, 2014). Starting from a large number of different yeasts, a selection process can be applied by competition in order to select only one type of yeast as the initiator of a fermentation process. As in a spontaneous fermentation process, this method is based on yeast competition for the nutrients available in the medium, so that the yeasts with the best fermentation capacity can be chosen (Torija *et al.*, 2001). This method may prove to be sufficient, but there is usually a need to use other methods, such as the temperature at which the fermentation process takes place, as well as its correct conclusion and the organoleptic analysis of the wines produced.

With this, the need has arisen to differentiate genera and species that are taxonomically very close, but which in turn have quite different properties as regards their fermentative and organoleptic characteristics. Yeast identification involves methods such as isolation of yeast from the environment, determination of morphological and physiological properties, and even comparison with standard microorganisms (Matienzo *et al.*, 2002). With the information obtained, it is possible to select native yeasts with high fermentation potential, as well as better adaptation to different needs. It was, necessary to start using different molecular methods in order to compare the genetic profile of yeasts. Examples of these methods are digital satellite microprinting, which consists of PCR amplification of microsatellite loci using primers that flank repeated fluorescence-labeled sequences, and REA-PFGE (Restriction Endonuclease Analysis Pulse-Field Gel Electrophoresis), which is carried out by cutting rate endonucleases. In addition to this type of approach, functional genomics approaches are also able to provide a comprehensive view of yeast physiology.

Approaches such as microarray or RNA sequencing (RNAseq) are ideal tools for the analysis of gene expression (Rossignol *et al.*, 2003; Spellman *et al.*, 1998). Although microarray is an efficient technique to measure the expression levels of several genes simultaneously, it has some limitations (Li *et al.*, 2010; Royce *et al.*, 2007). In order to overcome the difficulties that this process may present, an alternative is the RNAseq technique (Holt *et al.,* 2008; Wang *et al.*, 2009). With the aim of determining the sequence of a large number of cDNA fragments, RNAseq uses high-performance sequencing. In fact, studies performed with microarrays or RNAseq on the transcriptome level have contributed to a better understanding of the networks involved in the regulation of the whole genome, as well as the mechanisms involved in the expression of genes in yeasts, when subjected to various stress conditions (Taymaz-Nikerel *et al.*, 2016).

1.8. Aromatic compounds and their identification

Depending on the production capacity of the yeasts, it is possible to detect the occurrence of various types of aromas, from neutral, floral, fruity and spirituous as well as cheesy flavours (Cordente *et al.*, 2012; Goldner *et al.*, 2009). Generally, three different functional groups of compounds constitute grape must, the nutrients, the flavor-precursors, and the non-flavor-active-precursor compounds. In addition to the superior alcohols, the acetate esters and the ethyl esters are the main aromatic compounds to contribute to a floral and fruity flavour (Zalacain, 2007). This classification is based on their mode of formation. As far as acetate esters are concerned, they are the result of the reaction of AcetylCoA with the higher alcohols that are formed from the degradation of amino acids or carbohydrates. Ethyl esters are, in turn, produced enzymatically by yeast during fermentation and by the autolysis of Acetyl-CoA that is formed during the synthesis or degradation of fatty acids. In the former, the acyl group is derived from acetate and the alcohol group is derived from ethanol or a complex alcohol resulting from amino acid metabolism, in the case of ethyl esters, the alcohol group is the same as the former, but the acyl group is derived from medium-chain fatty acids (Cordente *et al.*, 2012). Regarding the first group mentioned, the most important contributors to the active flavor present in fermented beverages are: ethyl acetate, which contributes to a solvent aroma; isoamyl acetate, to a fruity aroma with banana or pear flavor; and phenylethyl acetate, which provides a rose and honey aroma, a more floral aroma (Lilly *et al.,* 2006; Nedović *et al.,* 2015). As far as ethyl esters are concerned, they are associated with a sour apple aroma (Verstrepen *et al.*, 2003).

However, as mentioned before, there are other compounds that may affect wine aroma. Ethanol can indeed affect the chemical and sensory properties of wine, such as the perception of viscosity, sweetness, acidity, aroma, flavor intensity, as well as textural properties (Gawel *et al.*, 2007). It also influences the sensory perception of aroma, when it is present at high concentrations, demonstrating a herbaceous rather than fruity odor (Goldner *et al.,* 2009). During S. cerevisiae fermentation the main polyol produced is glycerol, but its impact on wine aroma is not yet fully understood. However, studies have shown that this compound has some effects on the perceived viscosity of wine (Gawel *et al.*, 2007). Another aromatic compound directly related to alcoholic fermentation is acetaldehyde, constituting more than 90% of the total aldehyde content of wine (Salton *et al.,* 2000). This compound in high concentrations results in a herbaceous aroma, which is not desirable for wine (De Azevêdo *et al.,* 1968). Wine also has a large number of organic acids in its constitution and their metabolism plays an important role for wine, as it helps in maintaining the redox balance, as well as in the production of precursors for biosynthetic pathways. Malic acid is the most important non-volatile organic acid, contributing 90% to the acidity of

the must. In fact, acetic, malic, citric and succinic acids can affect the aroma and taste of wine in a positive or negative way, depending on their concentration and the type of wine desired (Swiegers *et al.,* 2005). Succinic acid is produced during anaerobic fermentation via the reducing branch of the tricarboxylic acid (TCA) cycle and is the main organic acid resulting from yeast metabolism. Other compounds that derive directly from pyruvate through the limited functioning of the TCA cycle are acetic and citric acids, which also have an effect on the pH value as well as on the organoleptic characteristics of the wine (Carolina, 2006).

The ability of yeasts to adapt to different oenological environments is reflected not only in their transcriptome, proteome and metabolome (Rossouw *et al.,* 2010), but also in their genome. However, the latter is difficult to track, due to the presence of heterozygosity, single nucleotide polymorphisms (SNPs) and a large variation in the number of genetic copies among the different wine strains. In order to study the metabolome, the analysis of a wide variety of chemical compounds present in low concentrations is usually used. In this way, to analyze the metabolic profile, several analytical platforms are used, such as gas chromatography (GC) or liquid chromatography coupled with mass spectrometry, being GC-MS the most accepted approach to study the metabolome because of its sensitivity, robustness, easy use and wide linear range (Kleijn *et al.*, 2007; Villas-Bôas *et al.*, 2005). In addition to these techniques, it is also possible to use other methods, such as capillary electrophoresis coupled with Mass spectometry, nuclear magnetic resonance spectroscopy and infrared and Raman spectroscopy (García *et al.*, 2008; Bjerrum *et al.*, 2010; Tanaka *et al.*, 2007; Ellis et Goodacre, 2006). Gas-liquid balances are used, as they make it possible to calculate the amount accumulated in the liquid and the amount lost in the gas, values that when added allow to determine the biological production and physical evaporation of volatile compounds such as propanol, isobutanol, isoamyl alcohol, isoamyl acetate, ethyl hexanoate and ethyl octanoate. In fact, GC allows the identification and comparison of compounds that constitute the aroma of different alcoholic beverages, such as wine (Kotseridis *et al.*, 2000; Pectka *et al.*, 2006).

The composition and content of flavour compounds determine the quality of alcoholic products, namely wine (Benito, 2018). As far as the chemical composition of the odors is concerned, it depends on the quality and type of raw materials that are used, as well as the conditions behind the alcoholic fermentation process. The odor of an alcoholic beverage is the effect of many chemical compounds with properties, such as different polarity or volatility that occur at different concentrations. Thus, the need to study the odor of alcoholic beverages also arises, for which gas chromatography-olfactometry (GC-O) studies are available. GC-O studies aim to determine the relationship between the composition and

content of volatile compounds and the organoleptic properties of products such as beer, wines and cognac spirits (Campo *et al.*, 2005; Culleré *et al.*, 2004; Gürbüz *et al.*, 2006; Lablanquie *et al.*, 2002; Soares da Costa *et al.*, 2004; Wanikawa *et al.*, 2002).

1.9. TODOMICS: Contextualization

Due to the growing interest in using *T. delbrueckii* in grape must fermentation, it is very important to increase the knowledge about this yeast and the use of new sequencing technologies and omics approaches will certainly be very beneficial to this end. Within the TODOMICS project (POCI-01-0145-FEDER-030488, *Omics approaches towards the industrial exploitation of Torulaspora delbrueckii: Elucidation of the molecular basis underlying complex cellular traits*), the genomes of about 50 strains were sequenced, considering strains from different technological and geographic origins. Besides the genomic information and the phenotypic characterization, the dynamic regulation of gene expression is also very important. By analyzing the genome and transcriptomic data of the different strains, it will be possible to determine the genetic basis of phenotypes and to design modifications in order to improve certain specific properties and thus achieve a genetic improved strain, either *T. delbrueckii* or *S. cerevisiae*. After obtaining the functional genetic information and selecting the genes to be modified, the strategies may include the regulation of genes directly involved in the production of aromas and secretion pathways. With the results obtained in this project, an easier exploitation of this species will be possible, facilitating its application in the different areas of interest.

CHAPTER 2

AIMS

This thesis project presents as main objective the characterization of a collection of 41 *T. delbrueckii* strains regarding its wine fermentation potential, taking into account the production and the consumption of sugar and the production of ethanol and organic acids. For this, we:

1. Used a set of *T. delbrueckii* strains from different geographical locations and technological groups, previously constituted in our laboratory.
2. Carried out small scale fermentations with the *T. delbrueckii* strains in liquid media using a synthetic grape must medium.
3. Analyzed sugar consumption and ethanol, glycerol and organic acids produced during the fermentation process using high-performance liquid chromatography (HPLC).
4. Analyzed data using orange software and assessed the biotechnological potential of the *T. delbrueckii* strains.

CHAPTER 3

MATERIALS AND METHODS

3.1. The *Torulaspora delbrueckii* collection

A *T. delbrueckii* set of 41 strains from different geographic locations and technological applications was compiled, gathering some strains available at Centro de Biologia Molecular e Ambiental (CBMA) and others kindly provided by Carole Camarasa (Institut national de la recherche agronomique (INRA) – Montpellier, France), Filomena Duarte (Instituto Nacional de Investigação Agrária e Veterinária (INIAV) – Dois Portos, Portugal), Javier Ruiz (Facultad de Biología, Universidad Complutense de Madrid, Spain) and José Paulo Sampaio (Faculdade de Ciências e Tecnologia (FCT); Universidade Nova de Lisboa (UNL), Portugal). The strains were categorized into different groups according to their biotechnological status or origin: wine (11 isolates), water (3 isolates), bread (4 isolates), arboreal and soil (11 isolates), food (6 isolates), other beverages (2 isolates), clinical (1 isolate) and 3 with an unknown origin (Table 2). Each strain was preserved at –80 °C in cryotubes containing 1 mL of glycerol (30%, v/v), to maintain membranes integrity.

Table 2 - Collection of *Torulaspora delbrueckii* strains used in this study. Each yeast has been attributed a letter "T" for being part of the collection of *Torulaspora delbrueckii* available at CBMA.

Internal Code	Strain Code	Substrate of isolation	Category of substrate	Geography
T01	ISA 1229	Wine fermentation, José Maria da Fonseca	Wine	Portugal
T02	PYCC 3209	Potato starch factory	Unknown	Netherlands
T03	PYCC 2916	Rhagi	Other beverages	Indonesia
T04	PYCC 2477	Unknown	Unknown	Unknown
T05	ISA 1549	Aestuary water from Guadiana river	Water	Portugal
T08	PYCC 5323	Homemade corn and rye bread dough	Bread	Portugal
T09	PYCC 5321	Homemade corn and rye bread dough	Bread	Portugal
T11	V187/ TB193	Green beans	Food	Portugal
T13	V393/ TB509	Artichoke	Food	Portugal
T14	V405/TB522	Strawberry	Food	Portugal
T15	MTF 1142	Grape berries	Wine	France
T19	MTF 3799	Fermenting grape juice (Sauvignon)	Wine	France
T22	MTF 3985	Bakery	Bread	France
T23	MTF 3987	Bakery	Bread	France
T26	MTF 4301	Green oak bark	Arboreal / Soil	France
T27	MTF 4303	Pedunculated oak bark	Arboreal / Soil	France
T28	MTF 4307	Pedunculated oak bark	Arboreal / Soil	France
T30	Zymaflore® Alpha	Wine	Wine	Unknown
T34	EVN 1129	Grape must of portuguese wine Castelão	Wine	Portugal
T35	EVN 1141	Grape must of portuguese wine Castelão	Wine	Portugal
T36	EVN 1155	Grape must of portuguese wine Castelão	Wine	Portugal
T38	NS-G-9	Grape must of Prieto Picu	Wine	Spain
T39	NS-G-62	Grape must of Prieto Picu	Wine	Spain
T40	NS-G-72	Grape must of Prieto Picu	Wine	Spain
T41	NS-PDC-169	Grape must of Prieto Picu	Wine	Spain
T42	PYCC 2478	Souring milk	Food	Japan
T43	PYCC 2713	Unknown	Unknown	Unknown
T44	PYCC 2844	Skin lesion on 3-month-old girl	Clinical	Brazil
T45	PYCC 2913	Sorghum brandy (kaoliang-chui)	Other beverages	Manchuria, China
T46	PYCC 2999	Coastal sea water (Florida, near Miami)	Water	USA
T47	PYCC 4739	Coastal sea water (shore near Lisbon)	Water	Portugal
T49	PYCC 6792	Cheese	Food	Azores, Portugal
T50	PYCC 6819	Soil	Arboreal / Soil	Portugal
T51	PYCC 7193	Fallen leaf from olive tree	Arboreal / Soil	Portugal
T56	PYCC 8309	Olives washing water	Food	Portugal
T57	PYCC 8413	Bark of *Quercus rubra*	Arboreal / Soil	Canada
T58	PYCC 8414	Bark of *Quercus acutissima*	Arboreal / Soil	Japan
T59	PYCC 8415	Bark of *Quercus velutina*	Arboreal / Soil	Canada
T60	PYCC 8416	Soil	Arboreal / Soil	Portugal
T63	PYCC 8419	Soil	Arboreal / Soil	France
T64	PYCC 8420	Soil underneath *Quercus petraea*	Arboreal / Soil	Romania

3.2. Media and Cultures

Cultures were pre-inoculated in yeast extract-peptone-dextrose (YPD) medium (2% glucose, 1% bacto-peptone and 0.5% yeast extract) and incubated at 30 °C, 200 rpm, for approximately 24 h, in order to allow strains to grow after thawing. This ensured that the inoculum would start with the same amount of biomass for all strains. In order to perform the individual fermentations, a synthetic grape must medium (MS) was used to simulate the natural grape must. The composition of the MS medium employed is described in table 3.

Table 3- Synthetic must medium (MS) composition.

MS Medium	
Compound	**Concentration**
Glucose	200 g/L
Malic acid	6 g/L
Citric acid	6 g/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

Appendix 1) – MSA – Mother Solution of Amino acids; MSF – Mother Solution of Anaerobic Factors; MST – Mother Solution of Trace elements; MSV – Mother Solution of Vitamins.

Regarding the preparation of the medium, all the individual compounds described in the table were added, after which the pH was adjusted to 3.3 – 3.4 and the medium was sterilized. Finally, before carrying out each individual fermentation, the MS solution was supplemented with amino acids, trace elements, vitamins, and anaerobic factors (MSA, MSF, MST, MSV, respectively). The addition of these supplements was performed by filtration (0.22 µm) in order to avoid thermal denaturation of the prementioned compounds.

3.3. Individual fermentations and HPLC quantifications

Each strain from the *T. delbrueckii* collection was subjected to individual fermentations, which were conducted in triplicate. These were performed at 18 °C in 100 mL Erlenmeyer flasks, using 50 mL of MS Medium, in order to have a ratio of 1:2 of liquid to void volume (Franco-Duarte *et al.,* 2016). Upon 120 h and 192 h of fermentation, samples were taken, deproteinized with perchloric acid ($HClO_4$) to obtain a final concentration of 2% (v/v) and left to rest on ice for 30 min. The samples were then centrifuged at 4°C for 10 min at 12000 G, and a volume of 1.5 mL of supernatant was transferred in triplicate to new eppendorfs and stored at -20 °C.

The metabolic profile of *T. delbrueckii* isolates was assessed by HPLC methodology. After deproteinization, the collected supernatants were filtered using a 0.22 μm pore size filter and analyzed using a HyperRez XP H+ 9 μm Carbohydrate column suitable to quantify specific organic acids, such as acetic acid, citric acid, formic acid, malic acid and succinic acid, alongside with glucose, glycerol and ethanol. The analysis of the samples was conducted using a solution with sulfuric acid (H_2SO_4) as the mobile phase, at a constant flow of 0.5 mL/min, at a temperature of 40 °C and for 30 min. Lastly, in order to be able to measure the concentration of the compounds, we performed the internal standard method using arabinose (20 g/L) as standard, and the Chromeleon 7.2.9 software was employed for data collection.

3.4. Data Analysis

The variability between *T. delbrueckii* strains was evaluated through a principal component analysis (PCA) performed using the Orange data mining suite software (version 3.25.0; Demsar, Zupan, & Leban, 2005). This assessment was applied to the entire group of strains under study, taking into account all the compounds analyzed.

CHAPTER 4

RESULTS

4.1. Analysis of individual fermentation profiles of *T. delbrueckii* strains

The strains under study were subjected to small-scale fermentations on MS medium. All strains were pre-inoculated with YPD medium, and all fermentations started with the same initial optical density. After, supernatant samples were collected at 120 h and 192 h of fermentation and analyzed by HPLC. To quantify the different compounds, the HPLC analysis was based on the method of internal standard, using arabinose at a concentration of 20 g/L as a standard. The aim was to visualize differences between the several strains considering the concentration of specific chemical compounds, such as organic acids (citric, malic, succinic, acetic, and formic acid), as well as glycerol, glucose, and ethanol and with it, analyze the fermentation process and verify how the fermentation occurs and develops over time. The data obtained was organized and represented in figures 3 and 4. To facilitate the analysis of our results, it was necessary to separate the data obtained into two groups. The results for glucose and ethanol were organized separately from the other compounds analyzed, since these two compounds presented concentrations in a range significantly higher than the other compounds. After that, the three highest and the three lowest concentrations for each compound were organized in the table 4, where the data of each timepoint analyzed are represented. In order to better analyze the behavior of each strain, the individual graphs are represented in appendix 2.

4.1.1. Glucose

Starting with glucose, this compound is present in the medium at a high concentration (200 g/L) since it is very important for the fermentation process. By analyzing the results shown in figure 3B and table 4, it is possible to verify that, at 120 h, the glucose concentration varied between 4.02 ± 2.93 g/L and 101.19 ± 1.27 g/L. The data represented in table 4 enables to obtain information about the three lowest concentrations, as well as the highest concentrations that were detected for each compound. Regarding the strains in which the lowest glucose concentrations were detected, strain T50, which belongs to the arboreal/soil category, showed a concentration of 4.02 ± 2.93 g/L (Figure 3B). Next, the two lowest concentrations were 4.29 ± 0.23 g/L and 5.90 ± 0.69 g/L, corresponding to strains T26 and T15, respectively. As far as their categories are concerned, strain T26 belongs to the same category as the previous one, arboreal/soil, while strain T15 is part of the wine category. The strains with the highest glucose concentrations were T11, T04, and T59, belonging to the food, unknown, and arboreal/soil categories, respectively. The type strain, T04, showed a glucose concentration of 85.72 ± 2.51 g/L after 120 h of fermentation, whereas strain T11 showed the highest concentration, with a concentration of 101.19 g ± 1.27 g/L. The strain T59 showed a glucose concentration of 66.62 ± 1.80 g/L, which shows

that, within the arboreal/soil category, there is a great variability, since there are strains that degrade glucose much better than others.

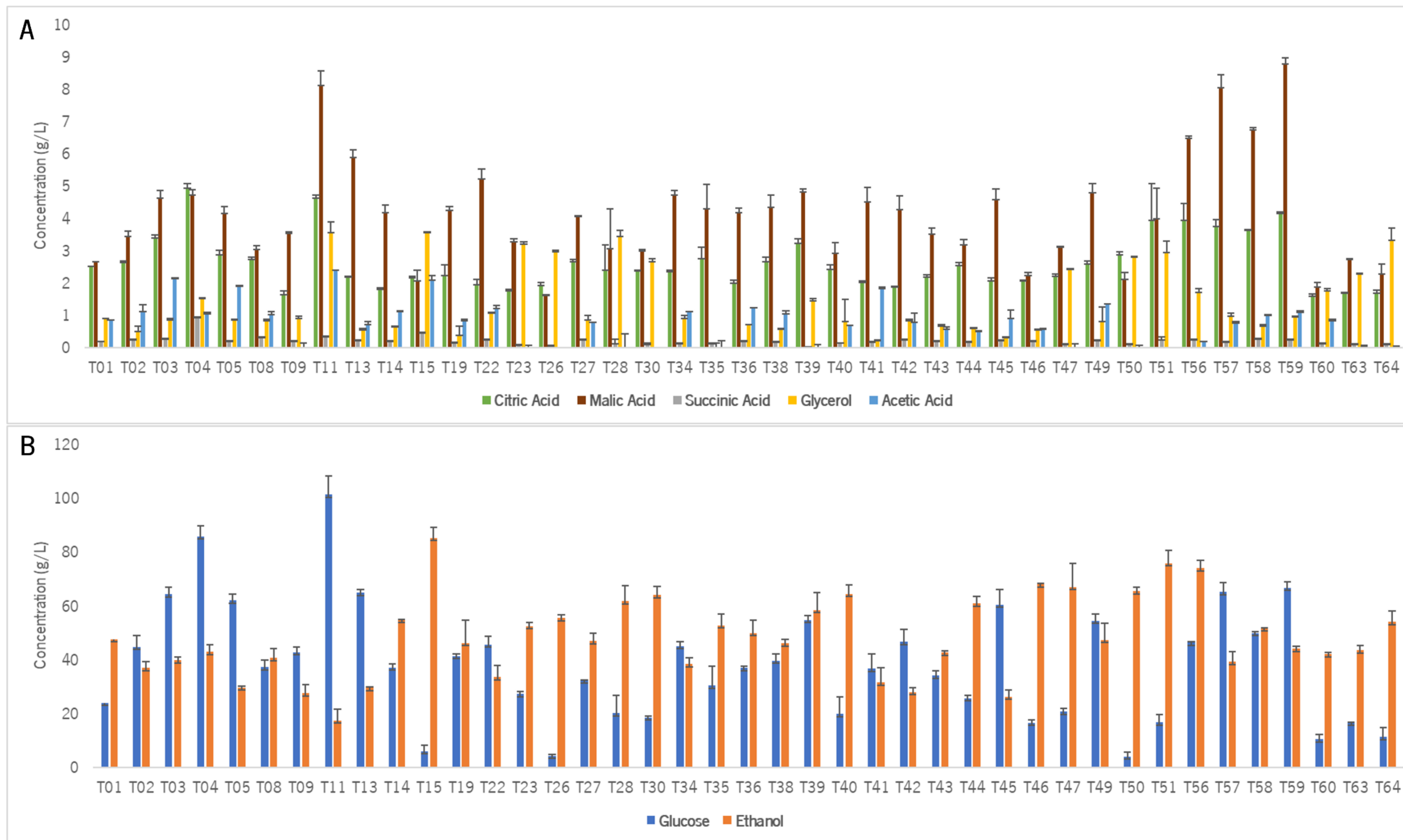

Figure 3 - HPLC analysis results of the *T. delbrueckii* set after 120 h of incubation. **A:** concentration (g/L) of citric, malic, succinic, glycerol and acetic acids. **B:** concentration (g/L) of glucose and ethanol.

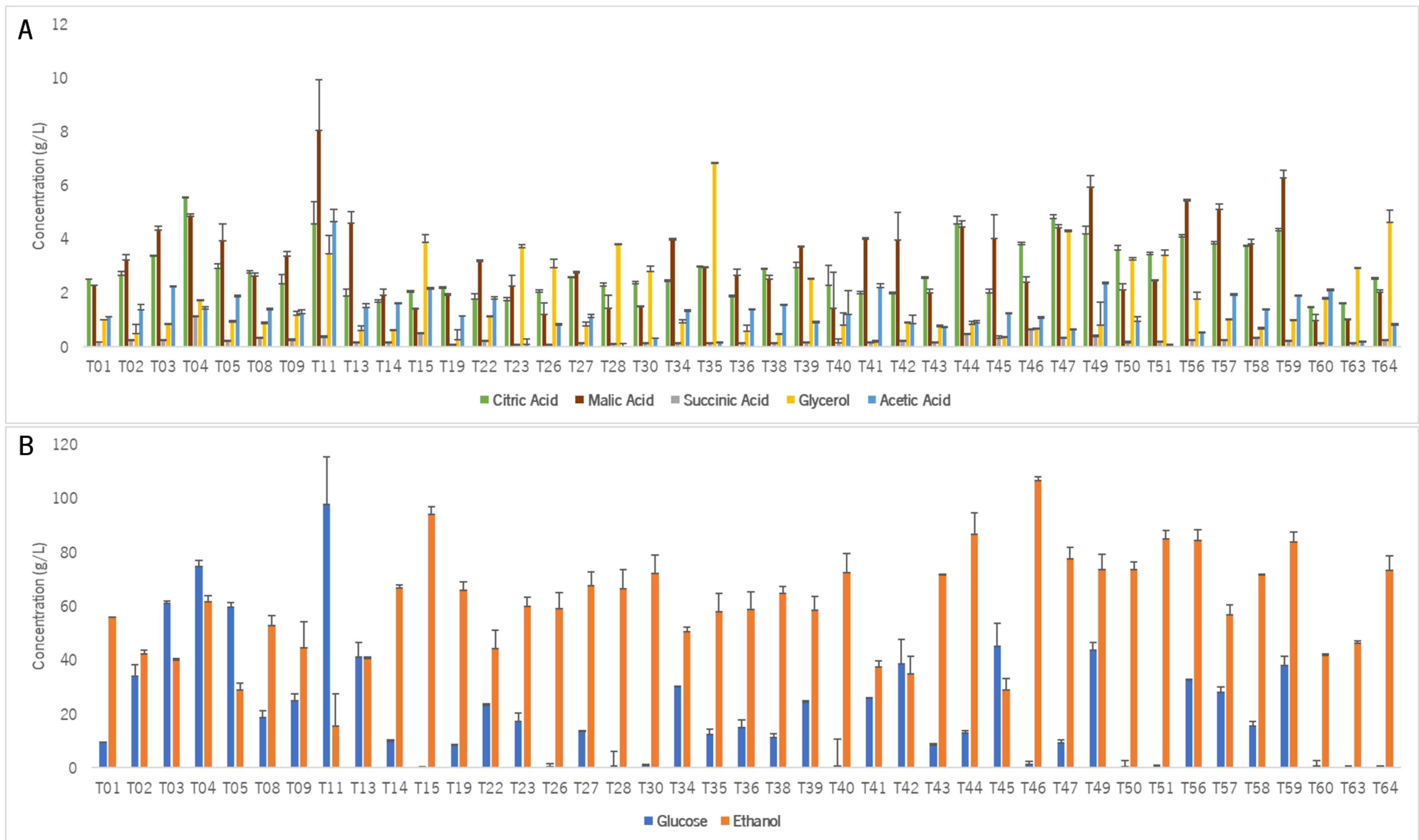

Figure 4- HPLC analysis results of the *T. delbrueckii* set after 192 h of incubation. **A:** concentration (g/L) of citric, malic, succinic, glycerol and acetic acids. **B:** concentration (g/L) of glucose and ethanol.

Table 4- Concentrations of glucose, ethanol, and organic acids (acetic, citric, malic, succinic) obtained by HPLC for each isolate. Range of values indicates the highest or smallest content detected. **A**: 120 h **B**:192 h

A

Highest [Acetic acid]			Highest [Citric acid]			Highest [Ethanol]			Highest [Glycerol]			Highest [Glucose]			Highest [Malic acid]			Highest [Succinic acid]		
Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.
T11	2.38	0.10	T04	4.92	0.14	T15	85.14	8.45	T15	3.55	0.29	T11	101.19	1.27	T39	4.81	0.34	T04	0.92	0.03
T03	2.12	0.04	T11	4.60	0.01	T51	75.68	2.91	T11	3.54	0.03	T04	85.72	2.51	T49	4.78	0.22	T15	0.45	0.02
T15	2.07	0.06	T59	4.14	0.07	T56	73.88	3.78	T28	3.42	0.10	T59	66.62	1.8	T34	4.70	0.75	T08	0.31	0.01
Smallest [Acetic acid]			**Smallest [Citric acid]**			**Smallest [Ethanol]**			**Smallest [Glycerol]**			**Smallest [Glucose]**			**Smallest [Malic acid]**			**Smallest [Succinic acid]**		
Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.
T63	0.03	0.03	T60	1.57	0.02	T11	17.37	0.36	T35	0	0	T50	4.02	2.93	T26	1.60	0.03	T39	0	0
T56	0.17	0.05	T09	1.62	0.10	T45	26.13	0.34	T41	0.21	0.06	T26	4.29	0.23	T60	1,86	0.03	T26	0.06	0.01
T44	0.50	0.24	T63	1.67	0.09	T09	27.42	4.16	T45	0.31	0.01	T15	5.90	0.69	T15	2.04	0.11	T64	0.09	0.08

B

Highest [Acetic acid]			Highest [Citric acid]			Highest [Ethanol]			Highest [Glycerol]			Highest [Glucose]			Highest [Malic acid]			Highest [Succinic acid]		
Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.
T11	4.64	0.14	T04	5.54	0.15	T46	106.61	4.35	T35	6.81	0.23	T11	97.72	5.43	T49	5.93	0.22	T04	1.12	0.05
T49	2.32	0.17	T47	4.75	0.27	T15	93.89	3.03	T64	4.61	0.49	T04	74.71	1.64	T55	5.12	0.18	T46	0.63	0.04
T03	2.22	0.09	T11	4.57	0.26	T44	86.48	4.28	T47	4.28	0.86	T03	61.02	2.31	T17	5.12	0.65	T44	0.45	0.08
Smallest [Acetic acid]			**Smallest [Citric acid]**			**Smallest [Ethanol]**			**Smallest [Glycerol]**			**Smallest [Glucose]**			**Smallest [Malic acid]**			**Smallest [Succinic acid]**		
Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.	Strain	g/L	Std. Dev.
§	0	0	T60	1.46	0.01	T11	15.41	0.72	T41	0.18	0.03	T15	0.24	0.07	T60	0.98	0.02	T23	0.049	0.035
T51	0.01	0.01	T63	1.61	0.06	T05	28.67	3.76	T19	0.25	0.02	T26	0.33	0.07	T63	1.01	0.08	T26	0.053	0.011
T23	0.08	0.06	T14	1.65	0.04	T45	28.68	1.36	T38	0.46	0.01	T50	0.45	0.03	T26	1.20	0.03	T19	0.068	0.003

Legend: §- represents strains T09, T23, T26, T28, T30, T35, T39, T47, T50, T51 and T64, in which a concentration of 0 g/L was obtained.

After 192 h of fermentation, the glucose concentrations ranged from 0.24 ± 0.07 g/L to 97.72 ± 5.43 g/L. The strains with the lowest concentrations of this compound at 120 h were the same strains that had the lowest concentrations at 192 h. Strain T15, of the wine category, is the one that consumes more glucose at a later stage of fermentation, with a concentration of 0.24 ± 0.07g/L. This result suggests that this strain likely has the highest ethanol concentration. At this time point, the other two strains, T26 and T50, registered a glucose concentration of 0.33 ± 0.07 g/L and 0.45 ± 0.03 g/L, respectively. The strains with the highest glucose concentration were T11, T04 and T03. T04, whose origin is unknown, presented the second highest concentration: 74.7 ± 1.6 g/L. T03, which falls into the other beverages category, had a concentration of 61.0 ± 2.3 g/L. The strain with the highest glucose concentration was, once again, T11. At 192 h, it showed a concentration of 97.7 ± 5.4 g/L, which is a little lower than the one obtained after 120 h, meaning that there is glucose degradation between these two time points. However, this process is apparently slow, as the concentration values remained quite high.

4.1.2. Ethanol

Regarding ethanol, which is produced by glucose metabolism, by analyzing the data of the lowest and highest concentration detected for this compound at 120 h (Table 4), it is possible to conclude that its concentration varied between 17.37 ± 0.36 g/L and 85.1 ± 8.5 g/L. The strain that showed the lowest concentration of this compound was strain T11, which is part of the food category. The other two strains with the lowest values were T45 and T09, with an ethanol concentration of 26.13 ± 0.34 g/L and 27.4 ± 4.2 g/L, respectively. These strains belong to the other beverages and bread categories, respectively. In turn, the strain with the highest ethanol concentration was T15, from the wine category, with a concentration of 85.1 ± 8.5 g/L. The other two strains that followed T15 with the highest concentration values were T51 and T56, both from the arboreal/soil category. These strains registered ethanol concentrations of 75.68 ± 2.91 g/L and 73.88 ± 3.78 g/L respectively, demonstrating their high capacity to produce ethanol.

Regarding the data obtained after 192 h of fermentation, the values ranged between 15.41 ± 0.72 g/L and 106.6 ± 4.4 g/L (table 4). The lowest concentration detected corresponded again to strain T11.This value is slightly lower than that detected at 120 h, which was not expected considering that ethanol was presumably the only compound being produced. An example of what would be expected is the behavior displayed by strain T45, which shows a concentration of 26.13

± 0.34 g/L at 120 h and a concentration of 28.68 ± 1.36 g/L at 192 h. Another strain that showed a low production of ethanol was T05, a strain belonging to the water category that showed an ethanol concentration of 28.67 ± 3.76 g/L. When it comes to the highest ethanol concentrations, at 192 h, strain T46 registered the highest value: 106.6 ± 4.4 g/L. This value is a little higher than expected. In fact, considering the 200 g/L of glucose of the culture medium, a maximum ethanol concentration of 100 g/L could have been anticipated. Regarding the other strains, the one that had the second highest concentration of this compound (93.89 ± 3.03 g/L) was strain T15, from the wine category. The third highest value was detected in strain T44, the only one with a clinical biotechnological origin, with a concentration of 86.48 ± 4.28 g/L.

4.1.3. Malic acid

Concerning the other compounds, we had to take into account the fact that malic and citric acids were present in the MS medium at a concentration of 6 g/L. In turn, after we subjected some samples of the various media used throughout the different fermentations by HPLC, it turned out to our surprise that the initial malic concentration was almost twice as high as intended, 14 g/L instead of 6 g/L. On the other hand, analyzing the peaks obtained for each compound in our HPLC samples, we found that this event occurred when high concentrations of glucose were present, meaning that the malic peak was not well defined and therefore some results were not used because they showed incorrect concentrations. Through the analysis of the data represented both in the graphs (figures 3A and 4A) and in table 4, it can be observed that citric and malic acids are both consumed by all strains.

The analyzed data represented in figure 3A allow us to conclude that all strains are able to consume this compound, as verified in the literature. At 120h, there were five strains that presented, concentrations higher than 6 g/L, but their malic peaks were not well defined. As far as their categories are concerned, three of them belong to the tree/soil category - T57, T58, and T59 - and the remaining two to the food category - T11 and T56. On the other hand, analyzing the peaks obtained for each compound in our HPLC samples, we found that this event occurred when high concentrations of glucose were present, meaning that the malic peak was not well defined and therefore some of the results were not used because they presented incorrect concentrations. According to the data in Table 4, the strain that showed the highest concentration, 4.81 ± 0.34 g/L, was T39. Then the two strains with the highest concentrations were T49 and T34, with 4.78

± 0.22 g/L and 4.70 ± 0.75 g/L, respectively. With regard to the lowest concentrations detected, strain T26, from the arboreal/soil category, registered the lowest value, 1.60 ± 0.03 g/L. The second lowest concentration, 1.86 ± 0.03 g/L, was also detected in a strain from the same category, T60. Strain T15, of the wine category, showed the third lowest concentration of this compound, 2.04 ± 0.11 g/L. At a later stage (192 h), as seen in table 3, the malic concentration ranged from 0.98 ± 0.02 g/L to 5.93 ± 0.22 g/L. In this case, only two strains showed concentrations higher than 6 g/L, strain T11, showed a content of 8.05 ± 0.43 g/L, while strain T59, from the tree/soil category, exhibited a content of 6.28 ± 0.23 g/L. As mentioned before, these concentrations were not assumed since they showed not well-defined peaks. The strain with the highest concentration was T49, a strain belonging to the food category, with a concentration of 5.93 ± 0.22 g/L, (table 4). This was followed by strains T55 and T17, both with a concentration of 5.12 g/L, the former with 5.12 ± 0.18 g/L and the latter with 5.12 ± 0.65 g/L. Regarding the lower concentrations, a decrease in their value was noticeable. The lowest concentration was 0.98 ± 0.02 g/L, detected in the fermentation of strain T60, followed by strain T63 with a concentration of 1.01 ± 0.08 g/L, and strain T26 with a concentration of 1.20 ± 0.03 g/L, all of which are part of the arboreal/soil category.

4.1.4. Citric acid

Regarding citric acid, at 120 h, the concentrations of the strains analyzed ranged from 1.57 ± 0.02 g/L to 4.92 ± 0.14 g/L (table 4). The highest concentration of this compound was detected for strain T04, a commercial strain of unknown origin. Then, a content of 4.60 ± 0.01 g/L was detected for strain T11 (food category), followed by a content of 4.14 ± 0.07 g/L for strain T59, a constituent of the Arboreal/Soil category. Interestingly, this category also includes strains that registered some of the lowest concentration values. In fact, the lowest levels of citric acid were detected in the soil-isolated T60 strain. This was followed by a concentration of 1.62 ± 0.10 g/L in strain T09, from the bread category, and 1.67 ± 0.09 g/L for a strain that also belongs to the Arboreal/Soil category, T63 (table 4). For the concentrations detected at 192 h, it was possible to verify that they varied in a range between 1.46 ± 0.01 g/L and 5.54 ± 0.15 g/L. Compared to the range detected at 120 h, it is possible to verify an increase in the value of the maximum concentration detected, as well as a decrease in the value of the minimum concentration (table 4). In fact, the citric acid content registered by T60 went from 1.57 ± 0.02 g/L to 1.46 ± 0.01 g/L, and from 1.67 ± 0.09 to 1.61 ± 0.06 g/L in the case of T63, with the former showing the greatest decrease. As for the strains that were still producing this compound between 120 h and 192 h,

the ones that showed the highest concentrations were T04 and T47. Another strain that also showed a high concentration of this compound was T11, though a decrease in its content was verified between 120 h and 192 h, from 4.60 ± 0.01 to 4.57 ± 0.26 g/L (table 4).

4.1.5. Formic acid

As for formic acid, few strains showed any concentration of this compound. In fact, at 120 h, formic acid levels were only detected in three strains, namely T03 with a content of 0.22 ± 0.02 g/L, T05 with 0.17 ± 0.03 g/L, and T08 with 0.13 ± 0.04 g/L. The categories to which these three strains belong are distinct, the first being from the other beverages category, the second from the water category, and the last one from the bread category. Regarding the second time point (192 h), in addition to T03 and T05, it is possible to verify the existence of other strains showing concentrations of this compound. In fact, the strain that showed the highest concentration was strain T44 (tree/soil category), showing a concentration of 0.44 ± 0.3 g/L. From the same category, T49 reported a concentration of 0.18 ± 0.07 g/L. Regarding the wine category, which is the one of greatest interest in this study, two strains exhibited levels of formic acid, T26 and T30, with concentrations of 0.15 ± 0.13 g/L and 0.16 ± 0.03 g/L, respectively. The strain that showed the lowest concentration of this compound after 192 h was T43, with a concentration of 0.04 ± 0.05 g/L.

4.1.6. Acetic acid

Another very important compound is acetic acid, which, at 120 h, showed a concentration range between 0.03 ± 0.03 g/L (detected in strain T63, from the Arboreal/Soil category) and 2.38 ± 0.10 g/L (found in strain T11 that belongs to the food category) (Table 4). The two following strains with the highest concentration of this compound were T03 and T15, strains from the other beverages and wine category, respectively. The former exhibited a concentration of 2.12 ± 0.04 g/L, while the latter registered a value of 2.07 ± 0.06 g/L. When it comes to the lowest concentrations, the other strains that followed strain T63 were T56 and T44. The first one belongs to the food category and had a concentration of 0.17 ± 0.05 g/L. The other is the only strain of the clinical category, and a concentration of 0.50 ± 0.24 g/L was observed. Based on the data obtained at the second time point, in strain T28, a constituent of the Arboreal/Soil category, there was no detection of acetic acid. Interestingly, another strain from the same category, T51, also

showed a reduced concentration of this compound, 0.01 ± 0.01 g/L. The third lowest concentration was detected in a strain of the bread category, T23, with a value of 0.08 ± 0.06 g/L. The strains that produced the highest acetic acid content were strains from the food category, T11 and T49, with concentrations of 4.64 ± 0.14 g/L and 2.32 ± 0.17 g/L, respectively. The third strain with the highest production rate, however, was from a different category (other beverages – T03), showing a concentration of 2.22 ± 0.09 g/L.

4.1.7. Succinic acid

When we observe the data from table 4, it is possible to note that succinic acid is the compound studied that overall presented the lowest concentrations. In the first time point, the concentrations varied between 0 g/L and 0.92 ± 0.03 g/L. Strain T39, isolated from wine, was the one that detected the lowest concentration, followed by strains T26 and T64, both from the Arboreal/Soil category. These strains had concentrations of 0.06 ± 0.01 g/L and 0.09 ± 0.08 g/L, respectively. The highest concentration, 0.92 ± 0.03 g/L, was detected for T04. The second and third highest concentrations were 0.45 ± 0.02 g/L (which was detected in a strain from the wine category, T15) and 0.31 ± 0.01 g/L (in a strain belonging to the bread category, T08), respectively.

With regard to the data obtained at 192 h, it can be seen that there is a slight increase in the concentrations detected, with the values ranging from 0.049 ± 0.035 g/L to 1.12 ± 0.05 g/L. As seen previously, strain T04, the commercial strain, was once again the strain with the highest concentration. Next, concentrations of 0.63 ± 0.04 g/L (T46) and 0.45 ± 0.08 g/L (T44) were detected. T46 belongs to the wine category, while T44 is from the clinical category. In terms of the strains associated to the lowest concentrations, T23 (bread category) had the lowest concentration, 0.049 ± 0.035 g/L, followed by T26 (tree/soil category), with a succinic acid content of 0.053 ± 0.011 g/L. The third lowest concentration detected was in strain T19, belonging to the category of greatest interest (wine), with a content of 0.068 ± 0.003 g/L.

4.1.8. Glycerol

Finally, it was also important to analyze the presence of glycerol, which is a compound that was not present in the medium. Comparing Figures 3A and 4A, it is possible to see that there is production of this compound by all the strains, even though not all of them produced glycerol in the first 120 h. This was the case for wine strain T35 (0 g/L). The following lower concentrations

observed were 0.21 ± 0.06 g/L and 0.31 ± 0.01 g/L. The former corresponds to a sample of strain T41, also from wine origin, while the second was detected in strain T45 (other beverages category). The three highest concentrations – 3.55 ± 0.29 g/L, 3.54 ± 0.03 g/L, and 3.42 ± 0.1 g/L (table 3) – were detected in strains T15, T11 and T28, respectively, all of which belonging to different categories. Strain T15 belongs to the wine category, whereas T11 is part of the food category and T28 is associated to the arboreal/soil category. By comparing the concentration ranges between the two time points (table 3), it can be seen that the concentrations varied between 0 g/L and 3.55 ± 0.29 g/L, at the first time point, and 0.18 ± 0.03 g/L and 6.81 ± 0.23 g/L, at the second time point (192 h). The concentration of 6.81 ± 0.23 g/L was observed for strain T35, the same strain that had a concentration of 0 g/L at 120 h. The following two highest concentrations were observed in a strain of the Arboreal/Soil category and in one from the water category. In the former strain, T64, a concentration of 4.61 ± 0.49 g/L was observed, while in the latter, T47, that value was 4.28 ± 0.86 g/L. At this time point, the lowest concentrations were all observed in strains from the wine category. Strain T41 had the lowest concentration, of only 0.18 ± 0.03 g/L. Next, in the sample from T19, the glycerol concentration was 0.25 ± 0.02 g/L, while in T38 a content of 0.46 ± 0.01 g/L was detected. Good reproducibility was observed between the three replicates of each strain. We observed that few strains produced formic acid and, in the cases that it was produced, the detected values were so low that we could be facing a certain error.

4.2 Analysis of fermentation parameters

With regard to the fermentation parameters, the yield of the fermentation was also calculated, taking into account the amount of sugar consumed during the fermentation process until the particular timepoint in analysis. For this purpose, all the values of the calculated yields for both timepoints (120 h and 192 h) were presented in table 5. The calculated yields showed some variability, ranging from 0.17 ± 0.02 to 0.48 ± 0.03 at 120 h and between 0.15 ± 0.02 and 0.55 ± 0.01 at 192 h. With these results it is possible to confirm the production of ethanol from glucose by all strains. Furthermore, by comparing the range of results between the two timepoints, it is possible to see that the ethanol production continues until a later timepoint. Importantly, an aspect that was occasionally observed was a decrease in yield between 120 h and 192 h, which could be due to evaporation or to the consumption of ethanol after glucose exhaustion, further studies being needed for its elucidation. Another question arises about the fact that yields higher than the theoretical yield occur in two strains. The maximum expected yield would be 0.51, the

value of the theoretical yield, so in the future it will be necessary to evaluate the behavior of these two strains, this were probably experimental errors.

Table 6- Yields of all 41 fermentative processes (ratio between production of ethanol(g) and consumption of glucose(g))

	Yield of Fermentation	
Strain	**120 h**	**192h**
T01	0.27 ± 0.01	0.29 ± 0.01
T02	0.24 ± 0.01	0.25 ± 0
T03	0.29 ± 0.02	0.29 ± 0.01
T04	0.38 ± 0.01	0.49 ± 0.03
T05	0.21 ± 0.02	0.20 ± 0.02
T08	0.25 ± 0.02	0.29 ± 0.05
T09	0.17 ± 0.02	0.25 ± 0.06
T11	0.18 ± 0	0.15 ± 0.02
T13	0.22 ± 0	0.25 ± 0.01
T14	0.33 ± 0.02	0.35 ± 0.03
T15	0.34 ± 0.03	0.55 ± 0.01
T19	0.29 ± 0.03	0.34 ± 0.04
T22	0.22 ± 0.01	0.25 ± 0.02
T23	0.30 ± 0.01	0.33 ± 0.03
T26	0.28 ± 0.01	0.29 ± 0.03
T27	0.28 ± 0.04	0.36 ± 0.03
T28	0.34 ± 0.02	0.33 ± 0.03
T30	0.35 ± 0.01	0.36 ± 0.01
T34	0.25 ± 0.04	0.30 ± 0.04
T35	0.31 ± 0.03	0.31 ± 0.03
T36	0.31 ± 0.01	0.32 ± 0.01
T38	0.29 ± 0.04	0.34 ± 0.03
T39	0.40 ± 0.01	0.33 ± 0.03
T40	0.36 ± 0.02	0.36 ± 0.01
T41	0.19 ± 0.01	0.21 ± 0.03
T42	0.18 ± 0.01	0.21 ± 0
T43	0.26 ± 0.02	0.37 ± 0.04
T44	0.35 ± 0.02	0.46 ± 0.04
T45	0.19 ± 0	0.19 ± 0.01
T46	0.37 ± 0.05	0.54 ± 0.02
T47	0.37 ± 0.03	0.41 ± 0.03
T49	0.32 ± 0.01	0.47 ± 0.02
T50	0.33 ± 0.03	0.37 ± 0.02
T51	0.41 ± 0.02	0.43 ± 0.02
T56	0.48 ± 0.03	0.50 ± 0.02
T57	0.29 ± 0	0.33 ± 0
T58	0.34 ± 0	0.39 ± 0.01
T59	0.33 ± 0.01	0.52 ± 0
T60	0.22 ± 0.01	0.21 ± 0
T63	0.24 ± 0.03	0.23 ± 0.03
T64	0.29 ± 0.01	0.37± 0.04

4.3 Analysis of *T. delbrueckii* inter-strain diversity

In order to confirm the good reproducibility of this samples, PCA was performed in both situations – with and without formic acid – and we came to the conclusion that formic acid displayed little or no influence on the distribution of the strains. Therefore, the results presented did not take into consideration the detected values for this compound. Another compound that was not taken into account was malic acid since some questions arose regarding its data. With this type of analysis, it was possible to represent the segregation of all 41 isolates (figure 5A- scores) and the studied compounds (figure 5B- loadings). Regarding the fermentation data at 120 h, a PCA was performed indicating 69.9% of variability among the strains, considering the first two components (PC1 – 46.2% and PC2 – 23.7%). Through the analysis of the results obtained within the PC3 component, we visualized that it did not contribute with additional information to the overall results and, for this reason, PC3 was not considered in the further analysis. Observing the loadings represented in figure 5B we can verify how the dispersion of the studied compounds will affect the dispersion of the 41 isolates, the scores, represented in figure 5A. Taking this into account it is possible to state that at 120 h, the strains located in the left part of the PCA, may be more influenced by ethanol and glycerol, while the isolates in the right region will be more influenced by the other compounds. Also, strains on the right side of the PCA may be more influenced by citric and succinic acids when positioned in the upper part, while those in the lower part will be more influenced by acetic acid and glucose.

The arrangement of the different strains throughout the PCA visualization showed an individual behavior within isolates of the same category, evidencing particular characteristics associated to each isolate regardless of their category. This can be verified in the arboreal/soil category that presents isolates scattered throughout the PCA (figure 5).

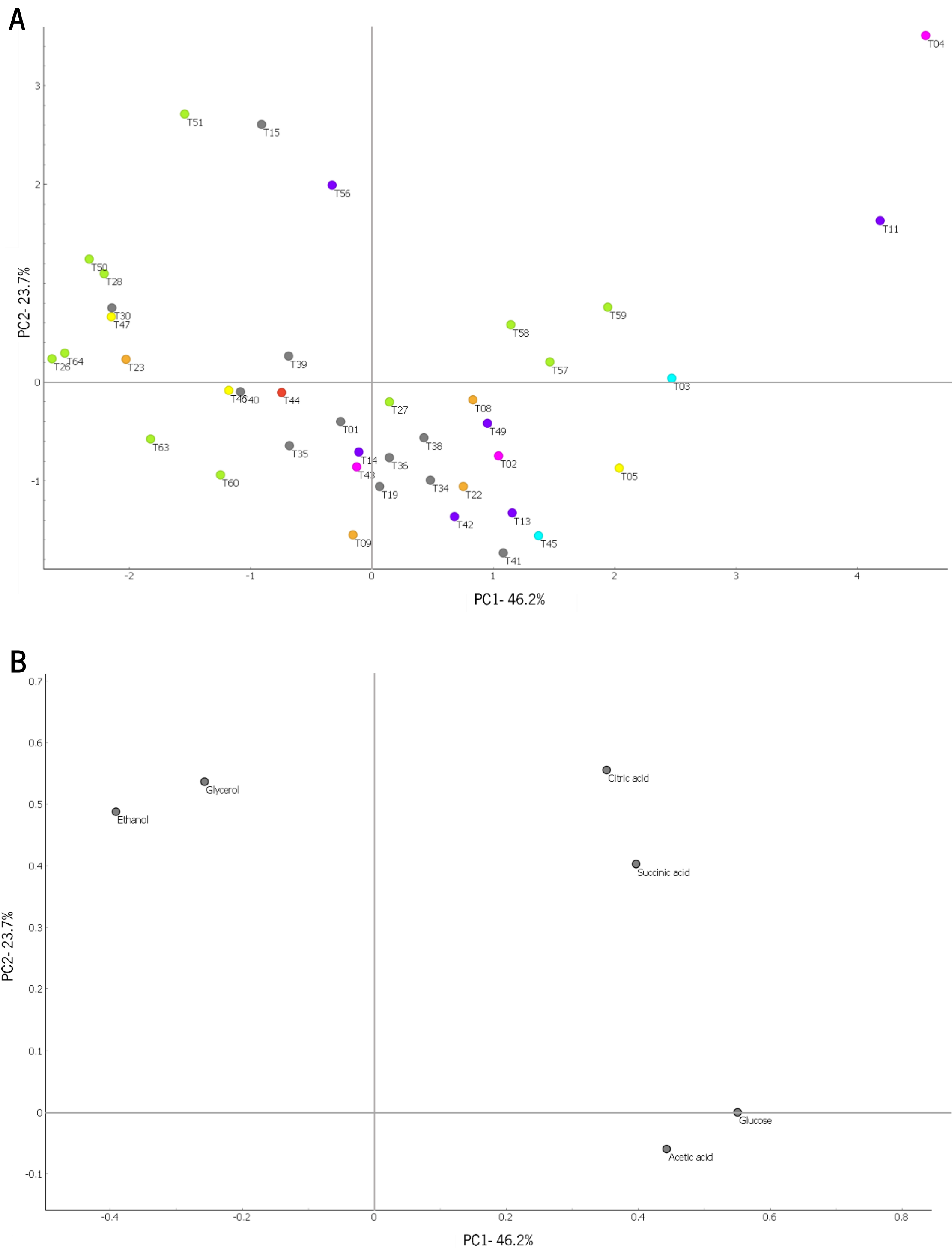

Figure 5- Principal Component Analysis (PCA) of 120 h HPLC data for 41 strains. The category of substrate are represent by different colors: –arboreal/soil; –bread; –clinical; –food; –other beverages; –unknown; –water and –wine. **A:** Scores – distribution of 41 analyzed strains **B:** Loadings - concentrations of 6 metabolites of interest.

Nevertheless, it is possible to divide this category into three subgroups, with a set of three strains distributed on the right part of the PCA, the T27 in a more central area, and the others on the left. As for the strains present in the wine category, they show a fairly close dispersion at the lower and right parts of the PCA. Of the group of strains in this category, T15, T30 and T39 are

dispersed in the upper part of the PCA, as shown by PC1 scrutiny. Due to its positioning in figure 5B, T15 shows high concentrations of glycerol and ethanol, alongside with low concentrations of glucose.

Concerning the other beverages group, the two strains that make up this category revealed being located in the lower part of the PCA, one on right and one in the left side. The bread category is formed by strains with a positioning in the lower part of the PCA, under the effect of the second component, with only T23 positioned in the upper part. Another category in which the strains showed distinct behavior was the water category, with one at the upper region of the PCA and two at the lower part. Regarding the food category, strains are mostly dispersed on the right side of the PCA, with only T14 positioned in a more central area and T56 in the upper part. The strains present on the right part of the PCA, with T11 standing out for being positioned the furthest to the right, correlate with a higher concentration of glucose. The three strains that constitute the unknown group showed distinct behaviors: two isolates located at the lower part of the PCA and one on the upper region, separated by the PC2 component (figure 5).

Another strain that exhibited a characteristic behavior was T04, the commercial strain, which was unexpectedly quite distant from the other strains. In fact, it was perhaps expected that it would be positioned closer to the central area of the PCAs, where a higher density of strains is located. Curiously, strain T44, which constitutes the clinical category, presents a rather neutral behavior, being dispersed to the left and showing little influence by the second component of the PCA. Strains T57, T58 and T59, part of the arboreal/soil group, are dispersed on the right and upper parts of the PCA, isolated from the other strains of the same category. This dispersion is correlated with a higher concentration of glucose, when compared to the others in their category. Also from this group, another strain that shows a peculiar behavior is strain T51, which disperses in the upper left part of the PCA, quite far from the other strains. This dispersion can be justified by a great concentration of ethanol and, in turn, low content of acetic acid. Furthermore, ethanol is more dispersed in the upper left region, whereas acetic acid is positioned in the lower right area.

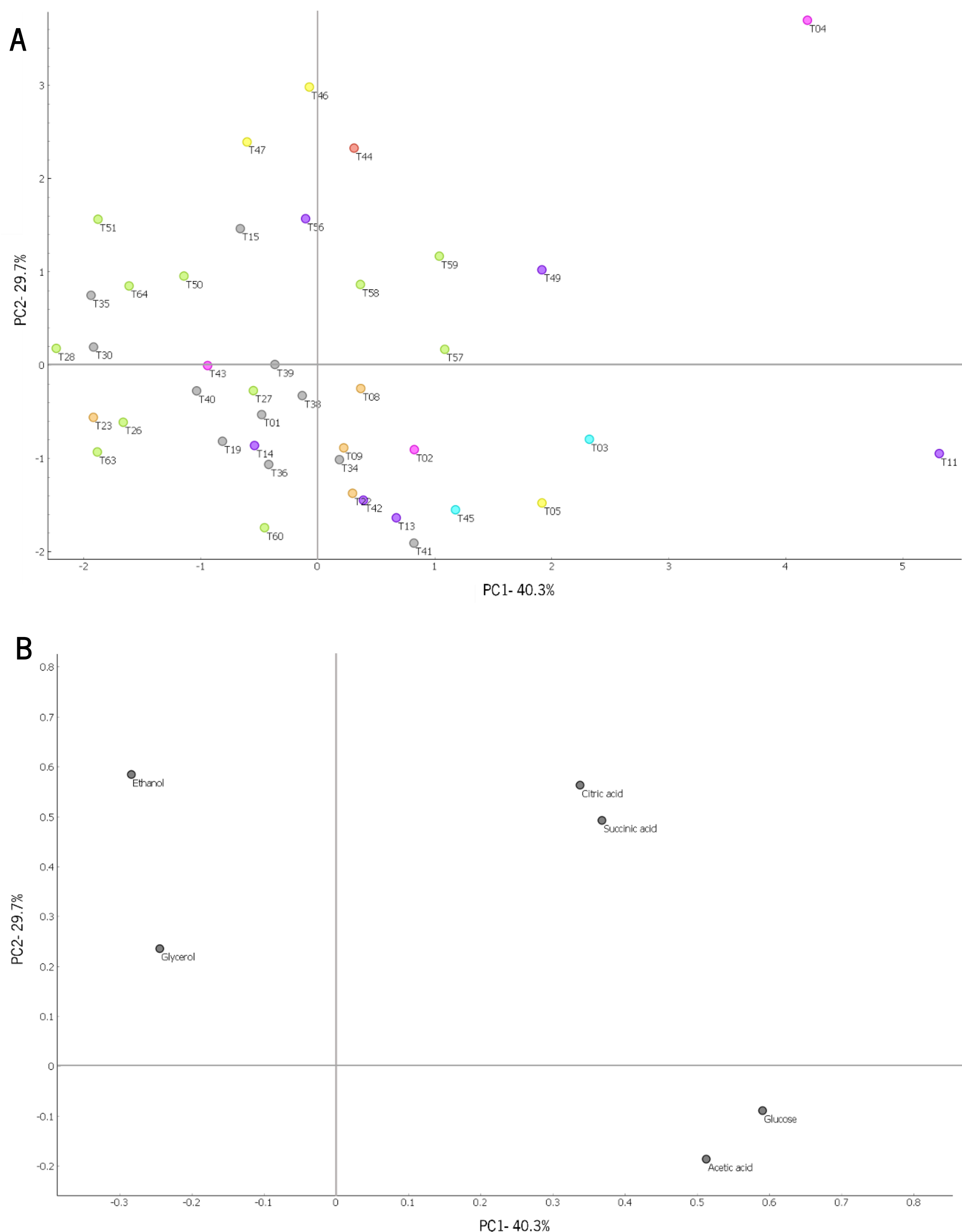

Figure 6- Principal Component Analysis (PCA) of HPLC data at 192 h for 41 strains. The categories of the substrates are represented by different colors: ■ –arboreal/soil; ■ –bread; ■ –clinical; ■ –food; ■ –other beverages; ■ –unknown; ■ –water and ■ –wine. **A:** Scores – distribution of 41 analyzed strains **B:** Loadings - concentrations of 6 metabolites of interest.

Given the dispersion of the compounds in Figure 6B, it can be seen that strains that are dispersed on the left side may be more influenced by the glycerol and ethanol compounds, while those on the right side are influenced by the other compounds. In turn, on the right side, the strains on the upper side may be influenced by citric and succinic acids, while those on the lower side may be more influenced by acetic acid and glucose compounds. The distribution by PCA of the

HPLC results obtained at the end of fermentation (192 h), detailed 70.0% of variability in the first two components (PC1 – 40.3% and PC2 – 29.7%). Regarding the strains that constitute the food category, the dispersion pattern was similar to the one observed at 120 h, with the strains dispersed throughout the PCA. However, the T11 strain, from that same category, placed in a more distant region from the others, in a lower part of the PCA and further to the right, which correlates with higher concentrations of acetic acid and glucose (figure 6). Also, in this final stage of fermentation, the wine strains were dispersed further to the left, both in the lower and upper areas of the PCA, as previously observed. Interestingly, strain T15 seemed to be closer together to the remaining strains of this category in this stage of fermentation than in the previous one, which translates into more identical glucose and ethanol concentrations between these strains. Nonetheless, T41 wine strain, which is in a lower right zone of the PCA, with T34, showed a different behavior from the others, correlating with the lowest concentration of glycerol. Regarding arboreal/soil strains, they remained dispersed throughout the PCA, with T57, T58 and T59 maintaining a considerable distance from the others, as previously observed at the first time point. In turn, strain T27 placed closer to the others in its category in this more advanced stage of the fermentation process. It is possible to observe that the strains that constitute the bread category showed a different behavior to that observed at 120 h, being dispersed in the lower area of the PCA, when analyzed by PC2. We can see that the set of three strains were located in the lower right zone, while T23 was dispersed to the left of the PCA. This behavioral difference may be due to the higher concentration of glycerol that this strain presents, compared to the others in its category. Regarding strain T44, it presented, at 192 h, a very different behavior from the one observed at 120 h, being dispersed to the upper and right area of the PCA (figure 6A). This difference in dispersion may be associated with an increase in ethanol concentration, as well as in citric acid, which was the compound in which the difference regarding the two time points was more noticeable.

Regarding the strains of unknown origin, they showed a small difference in the behavior previously verified, with the strain T43 being positioned in the left area of the PCA due to its low glucose concentration. The commercial strain, T04, was notably distant from the other strains, being dispersed in the upper right zone. In the other beverages category, an identical behavior to that of the previous time point was noted, with the strains dispersed to the right in the lower zone of the PCA, although the strains were found closer together after 192 h. As for strain T03, it was

located more to the right of the PCA, because it has a higher concentration of glucose, compared to strain T45.

Looking at the remaining groups, in the water category, two strains were dispersed in the upper and left zone - T46 and T47 -, while T05 was located in the lower right zone. The different dispersion patterns observed between the two timepoints for strain T05 may be justified by a lower concentration of ethanol and, consequently, a higher concentration of glucose, being therefore more influenced by this compound. Regarding T47, this strain showed a different behavior from T46, because even though both have a high concentration of ethanol and a low concentration of glucose, which place them in a higher zone of the PCA, T47 also has a high concentration of glycerol.

CHAPTER 5

DISCUSSION

One of the most raised questions in the winemaking field lies in the reason why there is an excessive increase in acetic acid in wines with a large presence of non-*Saccharomyces* yeasts (Jolly *et al.* 2014). Azzolini *et al.* (2012) showed in their studies that the use of *T. delbrueckii* in mixed fermentations does not cause an increase in acetic acid concentration. In our studies, it was possible to verify that the strains, at 192 h, showed variable acetic acid production, with concentrations ranging from 0 to 4.64 ± 0.14 g/L. At 120 h, the values detected for this compound varied between 0 g/L and 2.38 ± 0.10 g/L. Comparing the data between the two time points it was possible to see that strain T11 is the one with the highest production rate of this compound. Since this compound is not present in the MS medium, its concentration almost doubled between 120 h to 192 h of incubation. In contrast, in the literature, other authors have described *T. delbrueckii* as an inferior acetic acid producer compared to most non-*Saccharomyces* yeasts and even *S. cerevisiae* itself (Bely *et al.* 2008; Benito, 2019, Renault *et al.* 2009). Thus, taking into account that acetic acid and volatile acidity are two of the most important parameters for the quality of wine, our study focused attention on these aspects. Previous studies have shown that this compound is associated with a vinegary character that can easily be sensed in the wine's aroma if the acetic acid concentration is higher than 0.8 g/L (Benito, 2018). In general, our results appear to be superior to those in the literature. However, there are also strains that have concentrations lower than 0.8 g/L, which could be more suitable to use in wine production. With regard to the strains present in the wine category, the isolate that produced the lowest concentration of acetic acid was strain T35 with about 0.13 ± 0.01 g/L. This value is in agreement with the literature, but the same is not true for the others strains in this category. In fact, Prior *et al.* (2000) reported that, in hybrid strains of *S. cerevisiae*, an increase in glycerol production may result in an increase in acetic acid production. This suggests the possibility of a link between the metabolic pathways of these two compounds.

As far as glycerol is concerned, this compound is one of the most abundant metabolic products of the fermentation process, along with the CO_2 and ethanol. Moreover, it has been reported to be quite important for the wine's profile and thus one of the most important contributions of non-*Saccharomyces* yeasts, standing out for its smoothness and viscosity characteristics. In fact, wines fermented by non-*Saccharomyces* yeasts, such as *T. delbrueckii*, present higher levels of this compound compared to *S. cerevisiae* yeast, which has shown maximum levels of 9.1 g/L (Ivit *et al.* , 2020). In the literature it is reported that in *T. delbrueckii* the concentration of glycerol varies between 1 g/L and 10.5 g/L (Escribano *et al.* 2018; Ivit *et al.*

2020). With our results, it can be seen that glycerol production occurs at a lower rate than that depicted in the literature, and the concentration was observed to range from 0.18 ± 0.03 g/L to 6.81 ± 0.23 g/L at 192 h. The strain that showed the highest concentration of this compound was precisely from the wine category, which favors the data reported in the literature. On the other hand, the strain that had the lowest concentration of this compound was also from the wine category, strain T41, which raises the question whether or not this fermentation product contributes to produce a wine with a good organoleptic matrix. Other compounds that additionally contribute to the complexity of wines are malic, citric and succinic acid. These acids not only protect the wine against bacteria that may exist in the environment, but also contribute to the color, balance and flavor of the resulting product. Due to its low toxicity, high palatability and solubility, this compound has been used by various biotechnological industries. Previous studies have shown that its pleasant citrus flavor is highlighted as its major contribution to the aromatic profile of the wine (Ciriminna *et al.* , 2017; Vilela, 2019). The citric acid concentrations obtained in the study reveal that this compound was consumed by all the strains, which would be expected. However, there are also strains with high concentrations of this compound, even though an initial concentration of 6 g/L was used in the medium for fermentation. Previous studies with individual fermentations for *S. cerevisiae* and *T. delbrueckii* have shown that the former produces concentrations of 2.23 g/L, while the latter exhibits an average concentration of 2.27 g/L (Liu *et al.* , 2018). The strain that showed the closest value to the one reported in the literature was, in fact, T40, from the wine category, with a concentration of 2.26 ± 0.09 g/L. The two other strains with values in this range were strains T09 and T30, with a concentration of 2.32 g/L after 192 h of fermentation. Of note, it is not possible for us to guarantee that the initial concentration of this compound present in the medium (6 g/L) was completely consumed and only after that, did the strains produce these higher concentrations. While strain T30 belongs to the wine category, strain T09 is from the bread category. Therefore, the question arises as to whether this compound, in this concentration range, is important for both the bread and wine industries. As there was no major differences in the data observed between the two time points, it can be speculated that these strains showed a more intense consumption in the initial part of the fermentation. At 120 h, the concentrations varied between 1.57 ± 0.02 g/L and 4.92 ± 0.14 g/L, whereas at 192 h, the values ranged between 1.46 ± 0.01 g/L and 5.54 ± 0.15 g/L. These results suggest that some strains were able to consume this compound present in the medium and subsequently produce it in a more final stage of the process, as expected. The strains that exhibited the lowest concentration values of citric acid

were T60 and T63, both from the arboreal/soil category, showing a decrease between 120 h and 192 h.

Regarding malic acid it was not possible to conclude correctly how it varies throughout the fermentation. At 120 h, the strains showed concentrations in a range between 1.60 ± 0.03 g/L and 4.81 ± 0.34 g/L. As reported in the literature, the *T. delbrueckii* is able to metabolize this compound (Belda *et al. ,* 2015; Escribano *et al.* , 2018), and, as such, it would be expected that the maximum value would be less than its initial concentration of 6 g/L. In turn, it was found that in the presence of high glucose concentration, as in our MS medium, the peak detected in our HPLC results for this compound showed some overlap in some strains, resulting in the non-utilization of some results. In turn, it was possible to verify that all strains were able to metabolize this compound, as expected. By analyzing the concentrations present at 120 h, it is possible to observe that there were 5 strains that presented concentrations higher than 6 g/L: three of them from the arboreal/soil category – T57, T58 and T59 – and the remaining two from the food category – T11 and T56. These results might suggest that malic acid degradation occurs at a later stage, or even after fermentation occurs, since we obtained lower results at 192 h. Thus, further studies regarding malic acid should be performed.

In the context of winemaking, succinic acid contributes positively to the analytical composition of wine since it is responsible for increasing the acidity and antibacterial activity of the wine. However, this acid is also associated with a 'salt-bitter-acid' taste, which at high concentrations would contribute negatively to the wine quality (Jolly et al. , 2014). Several studies have been looking at *T. delbrueckii* and comparing it to the ability of a well-studied yeast, *S. cerevisiae*, to produce this compound. In their studies, Puertas *et al.* (2016) reported that the *S. cerevisiae* strain reached maximum values of 0.65 g/L of this compound, while the *T. delbrueckii* strain was reportedly a better producer of this compound, registering concentrations between 0.84 g/L and 1.11 g/L. In our results, at 120 h, the strains showed concentrations between 0 g/L and 0.92 ± 0.03 g/L. At 192 h, the maximum concentration was observed again in strain T04. That value was 1.12 ± 0.05 g/L which corresponds to the one in the literature. Another compound analyzed was formic acid. As pointed out in the previous section, this compound was not added to the PCA analysis since the concentration of this compound is quite low, which may be associated to an error.

Finally, the two other compounds that were also analyzed were glucose and ethanol. These compounds were represented separately from the results obtained for the other compounds, in order to achieve a better analysis of each compound under study since these two compounds presented values in different ranges from the others. Both glucose and ethanol are essential to evaluate the fermentative profile of the *T. delbrueckii* strain. In regard to glucose, this is the compound that is mostly present in the medium, with a concentration of about 200 g/L. In the results obtained at 120 h, it is possible to verify that the glucose concentrations varied between 4.02 ± 2,93 g/L and 101.19 ± 1.27 g/L. Focusing on the strains that have the lowest glucose concentration, these can be considered as strains with good fermentative power, since they degrade glucose faster.

Nevertheless, the fermentative power is not only associated with glucose degradation, but also with ethanol production. Thus, information related to glucose alone is not enough to conclude about the fermentative power of the strains analyzed. The strain that showed the lowest glucose concentration, of 4.02 ± 2.93 g/L, was T50, part of the arboreal/soil category. Regarding the strains with higher glucose concentrations, there are two possibilities: either they have a lower ability to degrade glucose, or they may take more time to degrade this compound. For the samples obtained at a later stage of fermentation, the range of variation in glucose concentration is relatively smaller than before. The T15 wine strain is the one that consumes more glucose at a later stage of fermentation, with a concentration of 0.24 ± 0.07 g/L. This result suggests that this strain is associated with a high fermentative power. Moreover, it would be expected that this strain would that have the highest ethanol concentration, since glucose degradation is related to ethanol production. The fermentation profile of this strain shows great variability, which is confirmed by the previous results for each strain. In turn, in order to allow more final conclusions about this fermentative power, it is necessary to analyze the data obtained related to the ethanol compound.

In this way, observing the data obtained at 120 h, it is possible to verify that this compound, which is not present in the first stages of the fermentation process, is produced by all strains. In fact, the lowest value of ethanol concentration detected was 17.37 ± 0.36 g/L and, as expected, it belongs to the strain with the lowest fermentative power – T11 – with biotechnological origin in food. The concentrations of ethanol in the first fermentation phase ranged from 17.37 ± 0.36 g/L to 85.1 ± 8.5 g/L, with strain T15 having the highest concentration of this compound. This result was expected since, as observed earlier, this strain would have shown optimal fermentative power with respect to glucose consumption. As seen previously, the strain with the lowest ethanol

concentration was strain T11, but at 192 h with a lower concentration than at 120 h, which was not expected. Interestingly, the same was verified for other strains. This data leads us to hypothesize that perhaps an error has occurred regarding the results of some strains. These results allowed us to conclude that T15 presents a good fermentative power, since its high glucose degradation is associated with a high ethanol production, as can be seen in the data presented at both time points.

CHAPTER 6

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Wine is a beverage whose search has intensified over the years. This great increase in its demand has led to the search for new ways to contribute to the production of wines with better organoleptic profiles. In this sense, several studies have been conducted in order to investigate the possibility of using non-*Saccharomyces* yeasts for alcoholic fermentation. Several studies involving the yeast *T. delbrueckii* have shown that this species has a high potential for winemaking. Taking this information into account, the aim of the present study was to evaluate the metabolic profile of a diverse range of *T. delbrueckii* strains, in order to allow a better evaluation of the biotechnological potential of this species. Regarding the results obtained, contrary to what is represented in the literature, *T. delbrueckii* showed a higher production of acetic acid, which raises some issues. In turn, regarding its fermentative power, this yeast, as indicated in previous studies, showed an optimal capacity to ferment glucose into ethanol. With regard to the final yields of the fermentation process, it was found that the maximum value reached was 0.55 ± 0.01 belonging to a strain of wine origin. It is also possible to assess the interest of *T. delbrueckii* for wine production, since it is a yeast, in general, with low fermentative yields, not leading to the production of very high concentrations of ethanol, which would not be favorable.

In order to obtain greater reproducibility in the data obtained during our tests, we used a synthetic medium to mimic grape must, which may have some impact on the results obtained. Thus, in future studies, it would be advantageous to use a real must since it is a much more complex medium, with respect to the compounds present, which would allow obtaining results with greater similarity to what occurs in real wines, since the compounds evaluated can be influenced by the conditions of the environments where the grapes are found. It would also be very interesting to perform this study using a denser *T. delbrueckii* library, with a wider number of strains in use and covering a wider variety of geographic regions. Finally, in order to complement the results obtained, it would be of great interest to carry out this study evaluating the fermentation of mixed inoculations of *T. delbrueckii* and *S. cerevisiae*, in order to understand how these two strains are related and in what aspects the mixed inoculation affects our obtained results.

CHAPTER 7

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CHAPTER 7

APPENDIX

Appendix 1 – Composition of the different mother solutions–amino acids, trace elements, vitamins, and anaerobic factors–that compose the used MS medium.

Table A1.1. Amino acid mother solutions (MSA) composition.

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-Glutamine	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

Table A1.2. Mother Solution of Trace elements (MST) composition

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_2BO_2	1
$(NH_4)_6.\ Mo_7O_{24}$	1

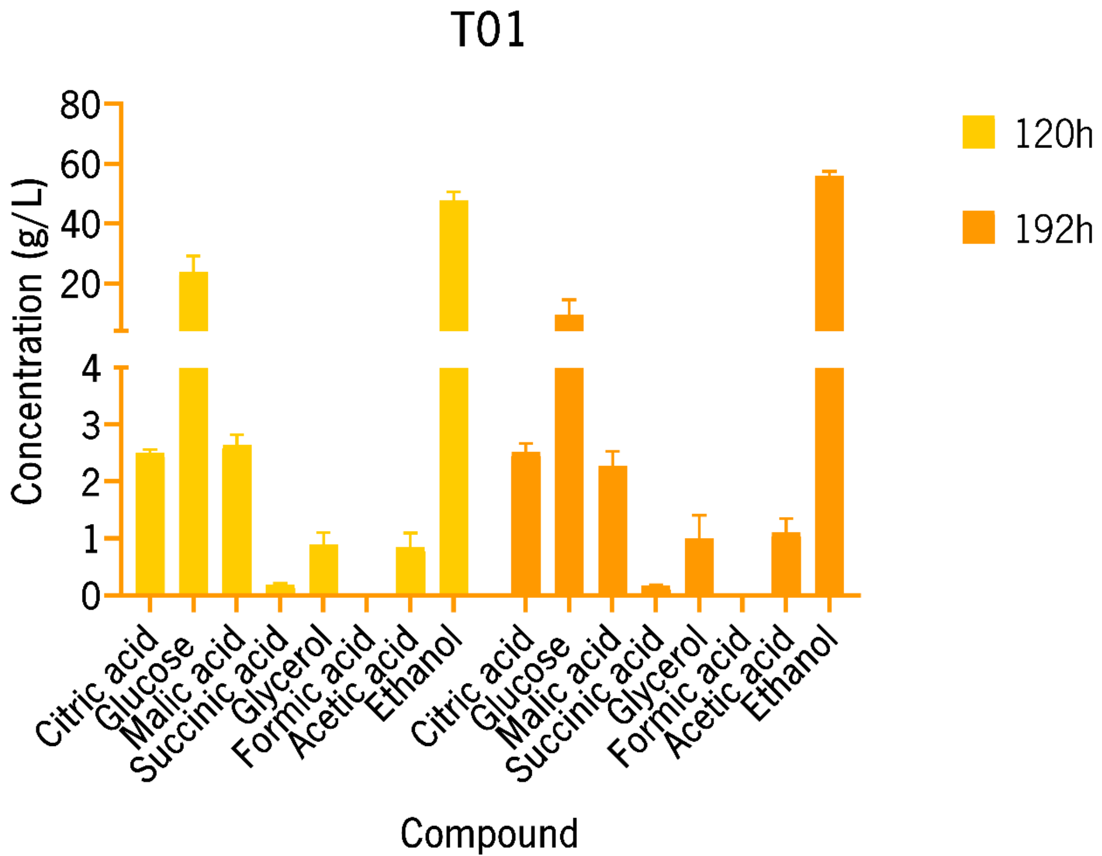
Table A1.3. Mother Solution of Vitamins (MSV) composition.

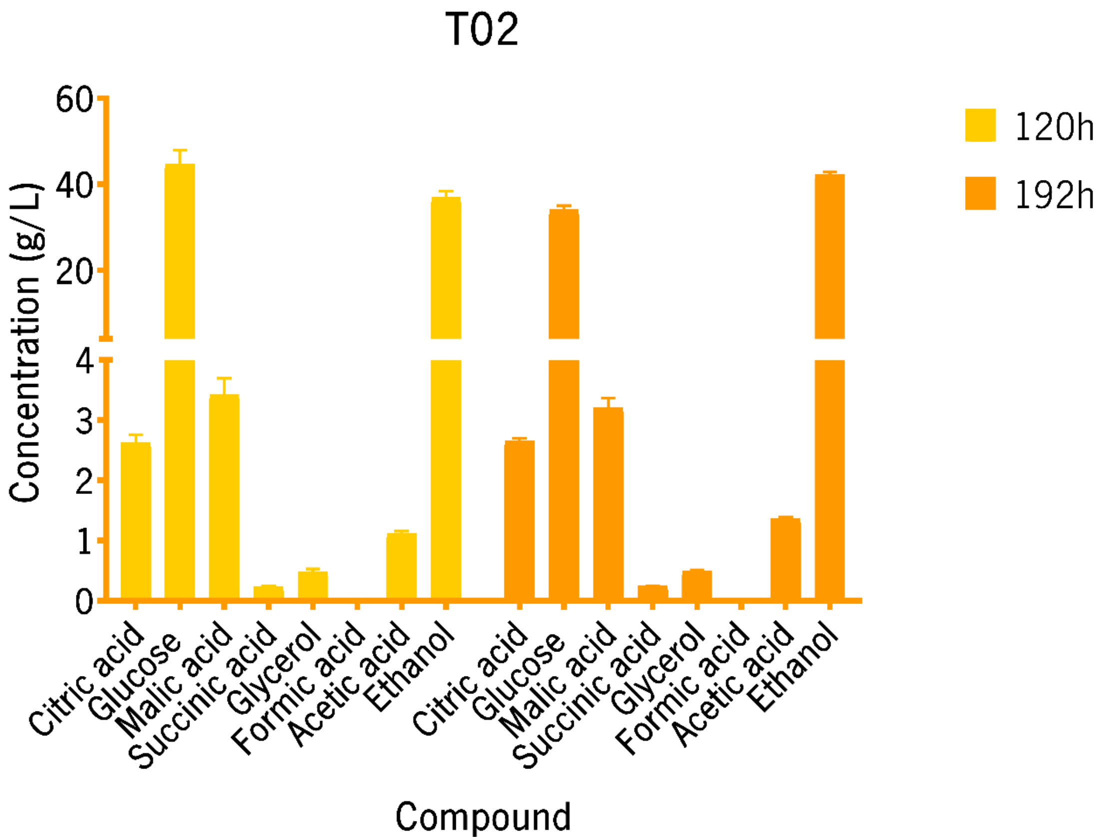
Elements	Amount in grams (1 L)
Myo-Inositol	2.0
Pantothenic acid	0.15
Thiamine, hydrochloride	0.025
Nicotinic acid	0.2
Pyridoxine	0.025
Biotin	0.0003

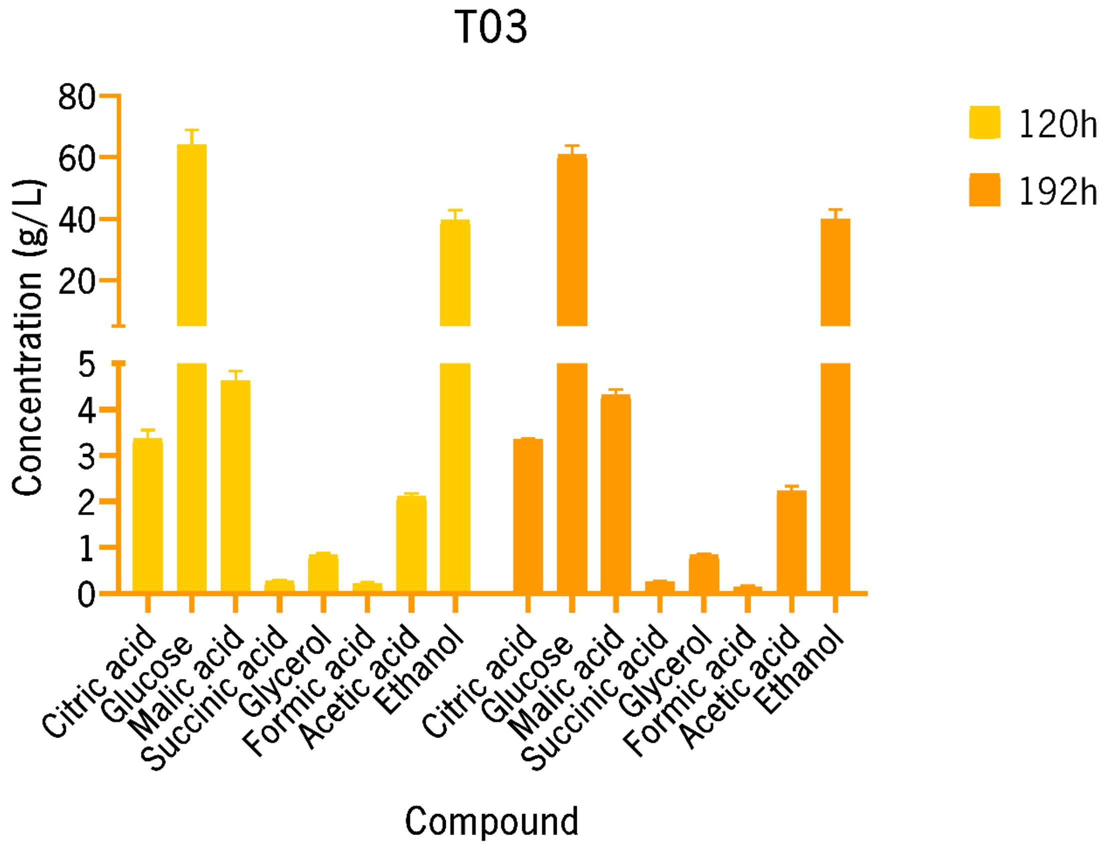
Table A1.4. Mother Solution of Anaerobic Factors (MSF) composition.

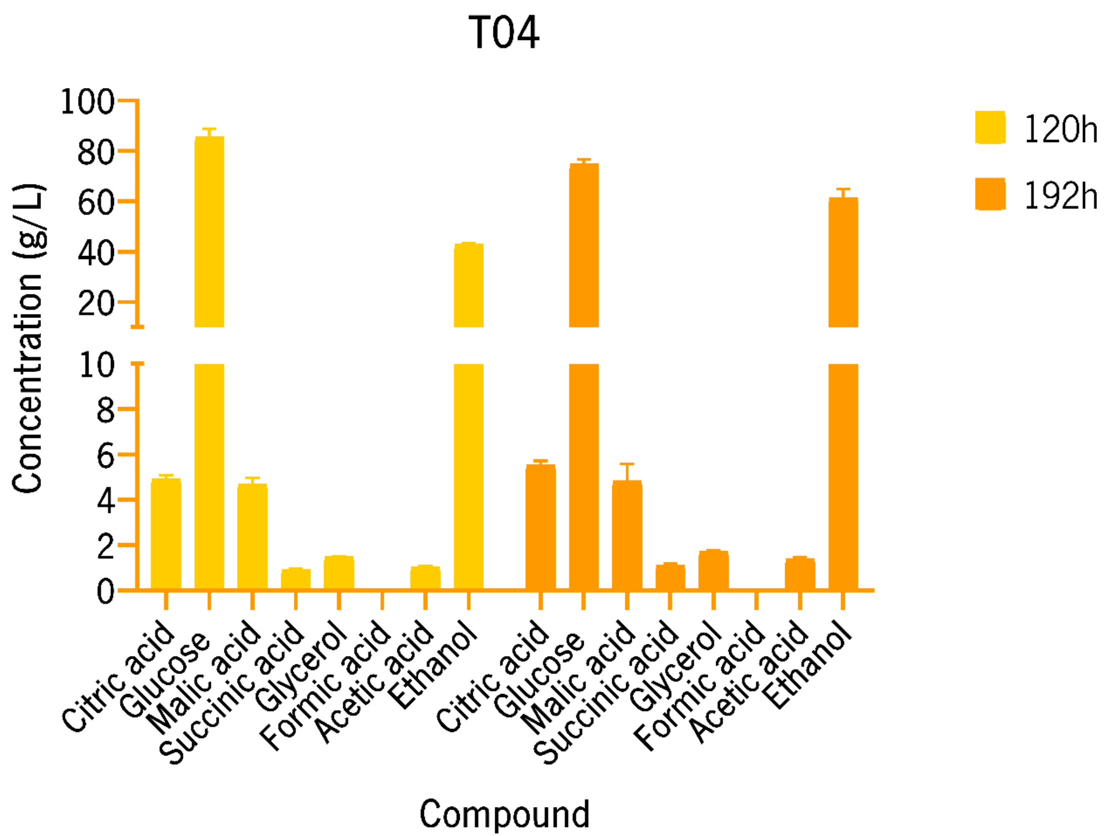
Elements	Amount in grams (1 L)
Ergosterol	1.5
Oleic acid	0.5
Pure ethanol + Tween 80	50 mL +50 mL

Appendix 2 – Individual HPLC analysis results of the *T. delbrueckii* set






T03
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h


T04
Concentration (g/L)
100
80
60
40
20
10
8
6
4
2
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
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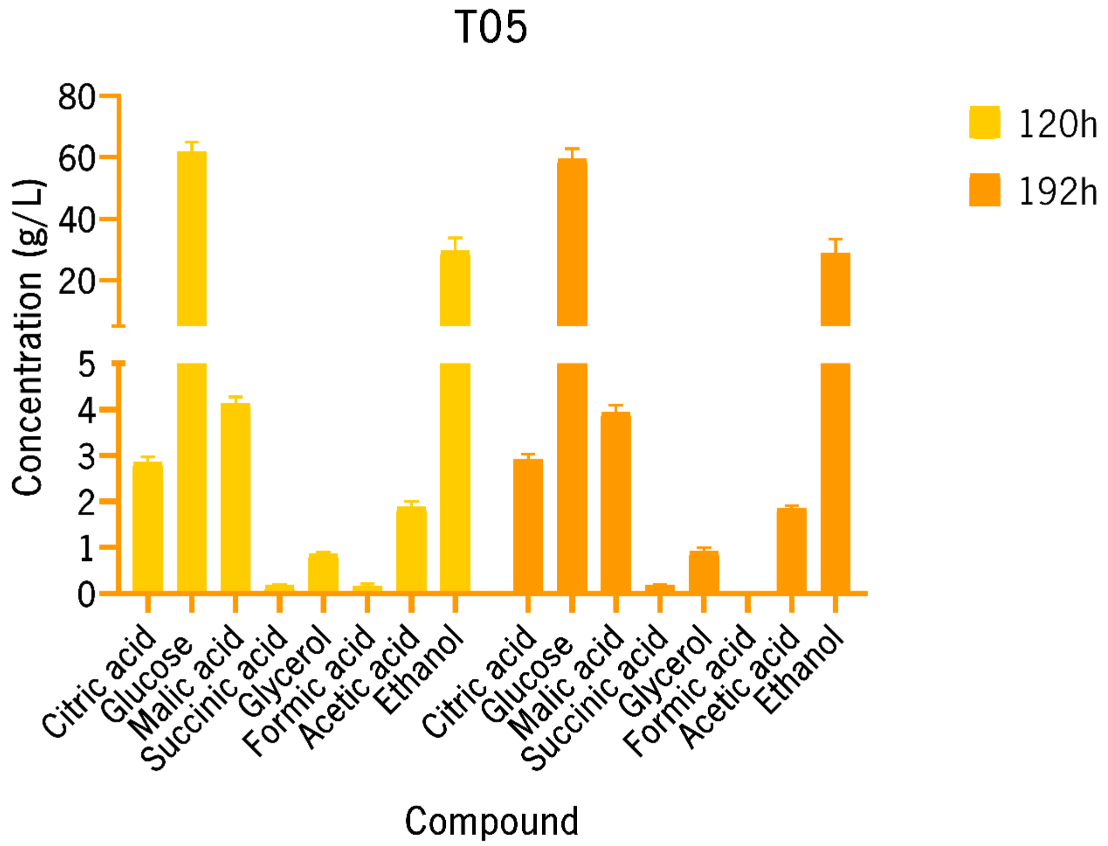
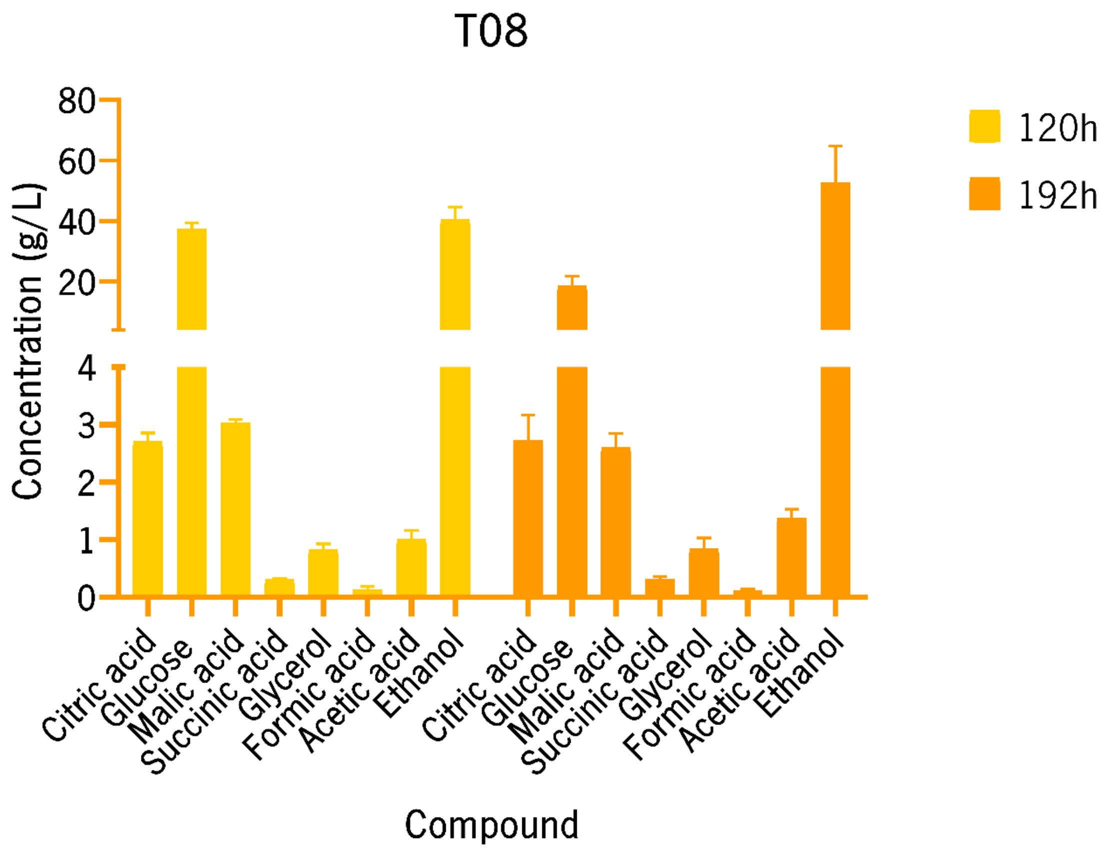
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h


T05
80
60
40
20
5
4
3
2
1
0
Concentration (g/L)
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound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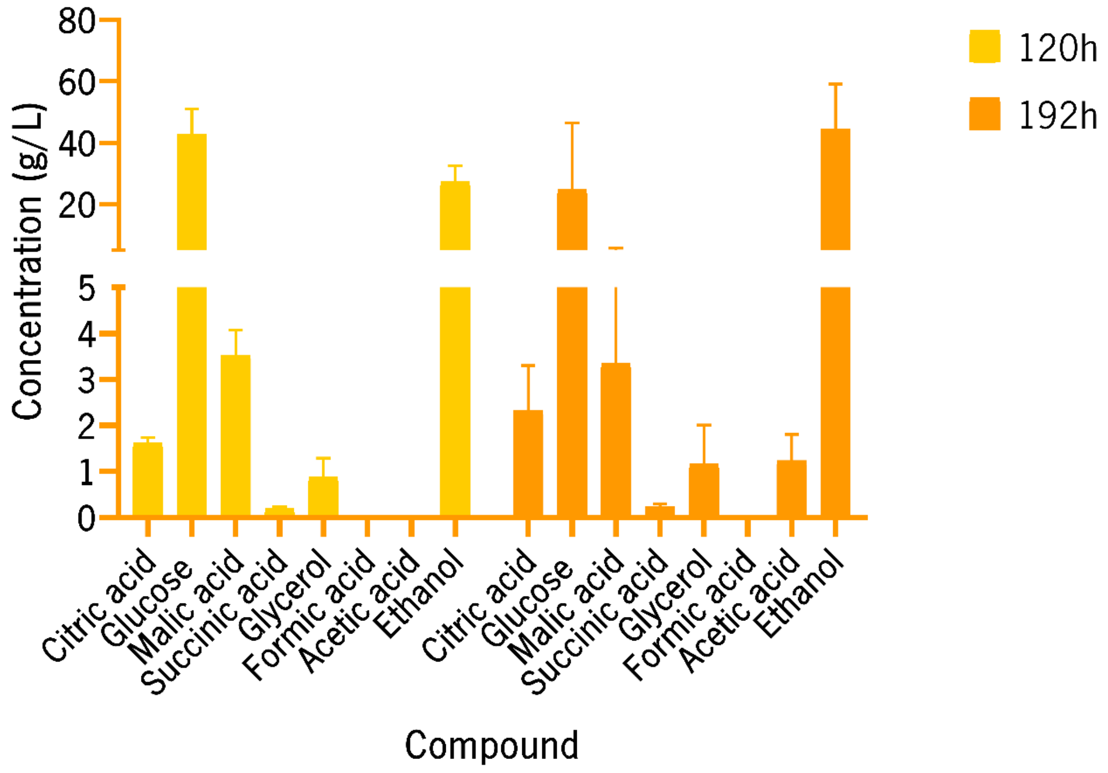
60
40
20
4
3
2
1
0
Concentration (g/L)
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound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Concentration (g/L)
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40
20
5
4
3
2
1
0
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Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
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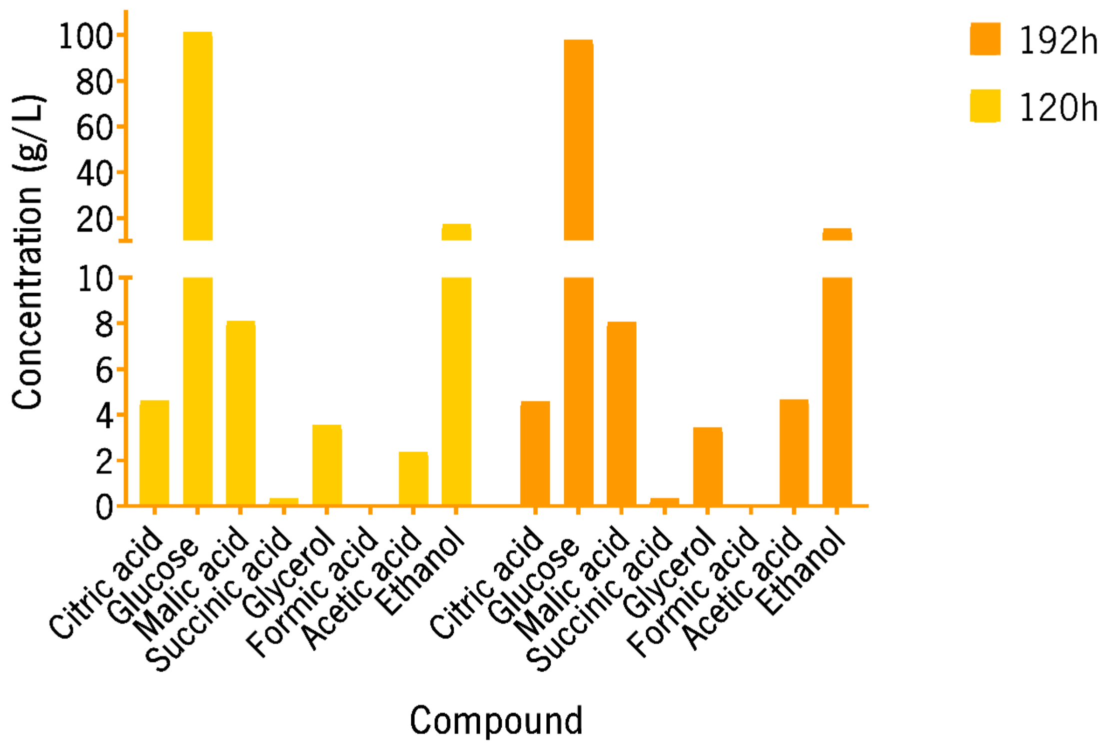
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h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Concentration (g/L)
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20
10
8
6
4
2
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Glucose
Malic acid
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Glycerol
Formic acid
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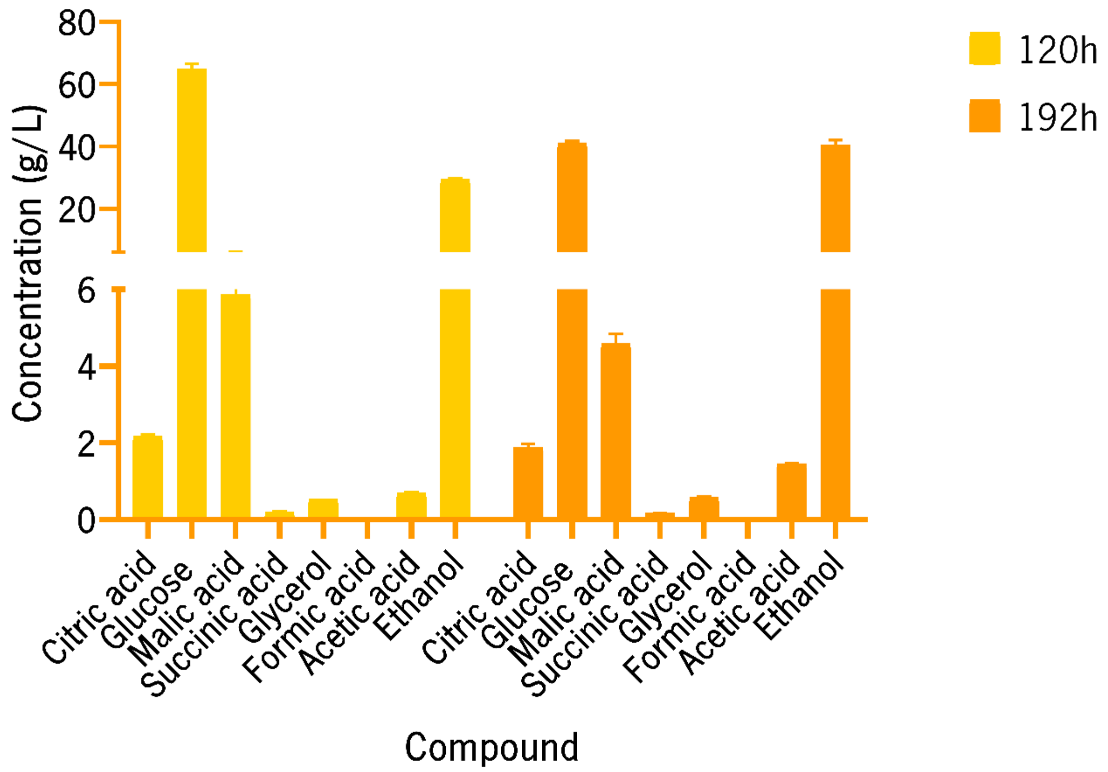
Formic acid
Acetic acid
Ethanol
Compound
192h
120h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60
40
20
6
4
2
0
Concentration (g/L)
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Glucose
Malic acid
Succinic acid
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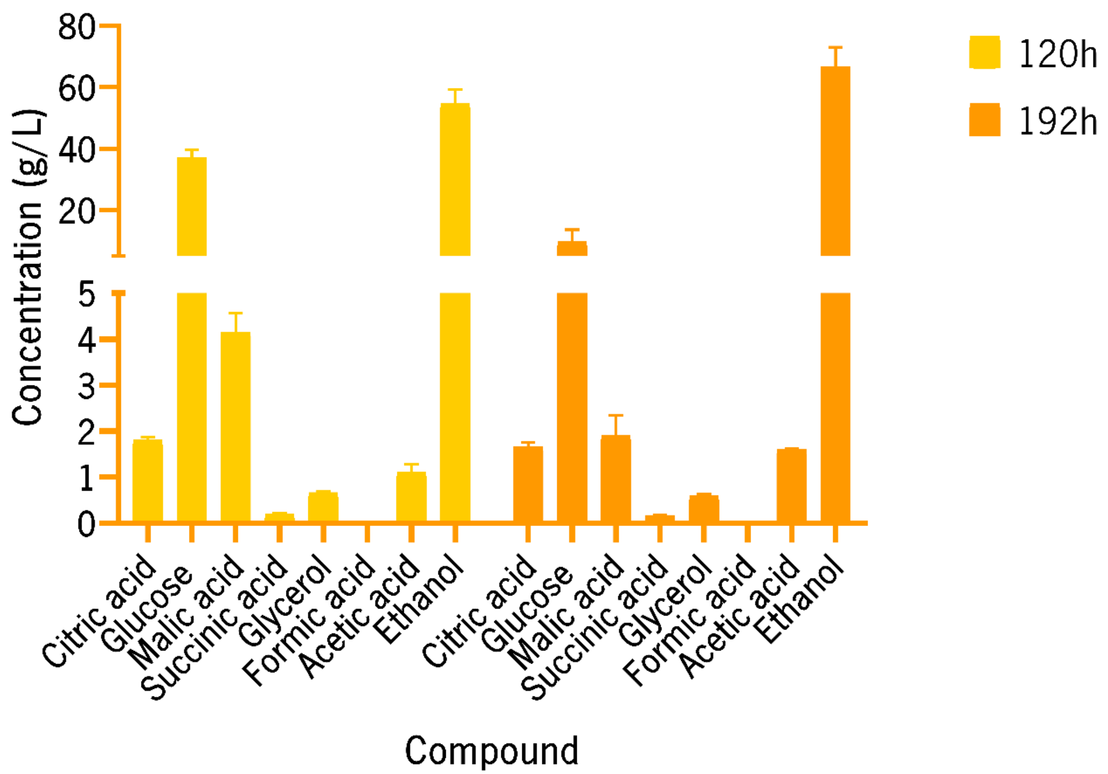
120h
192h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40
20
5
4
3
2
1
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Concentration (g/L)
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Glucose
Malic acid
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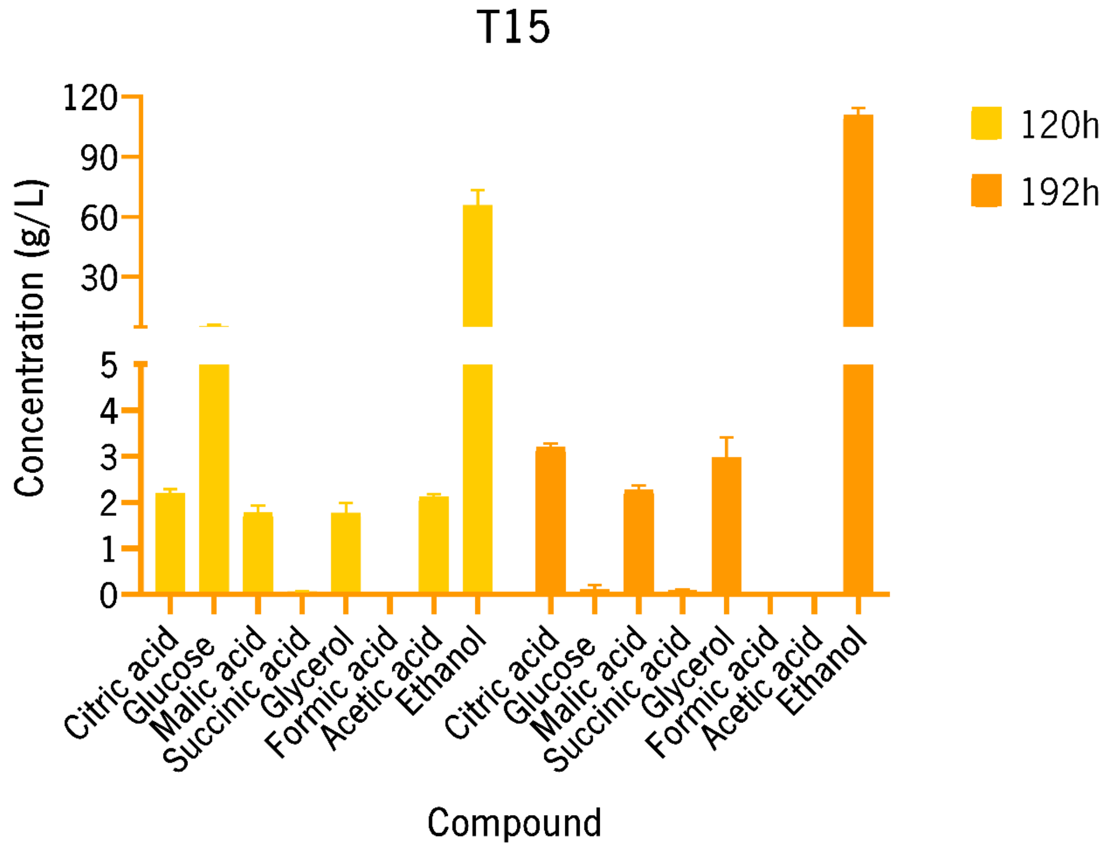
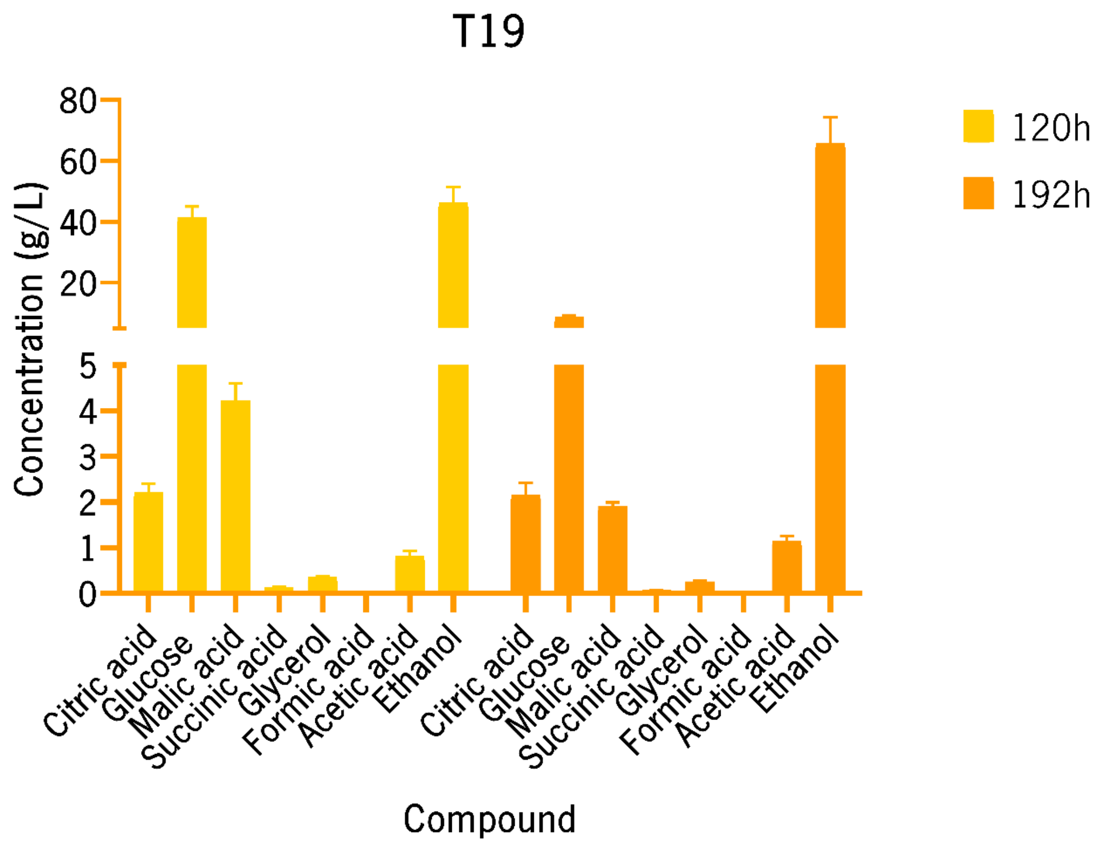
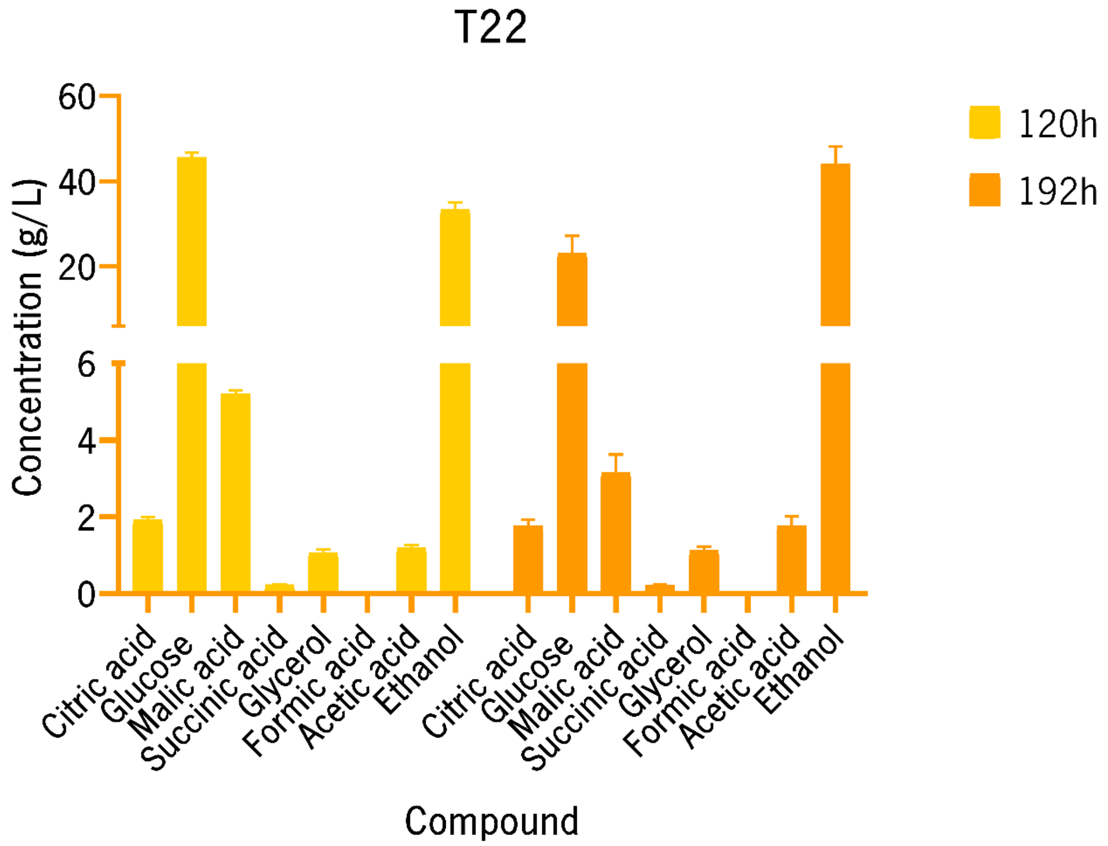
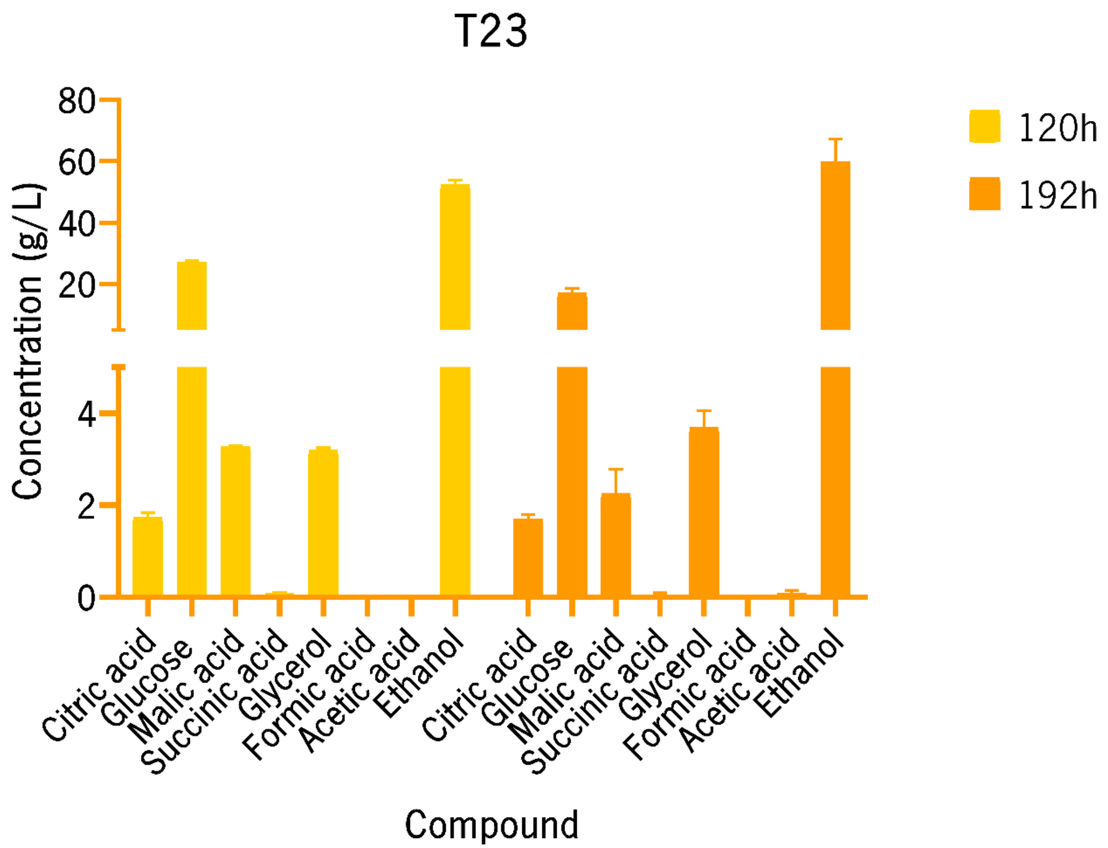
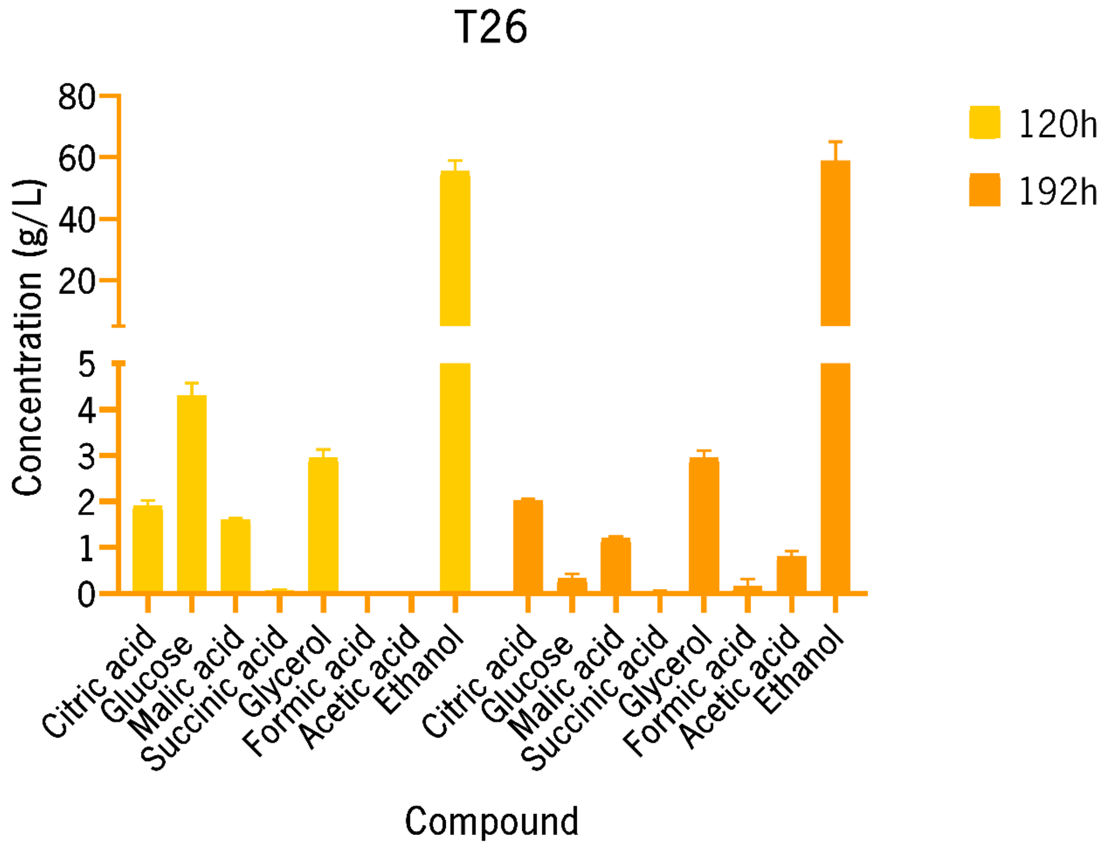
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

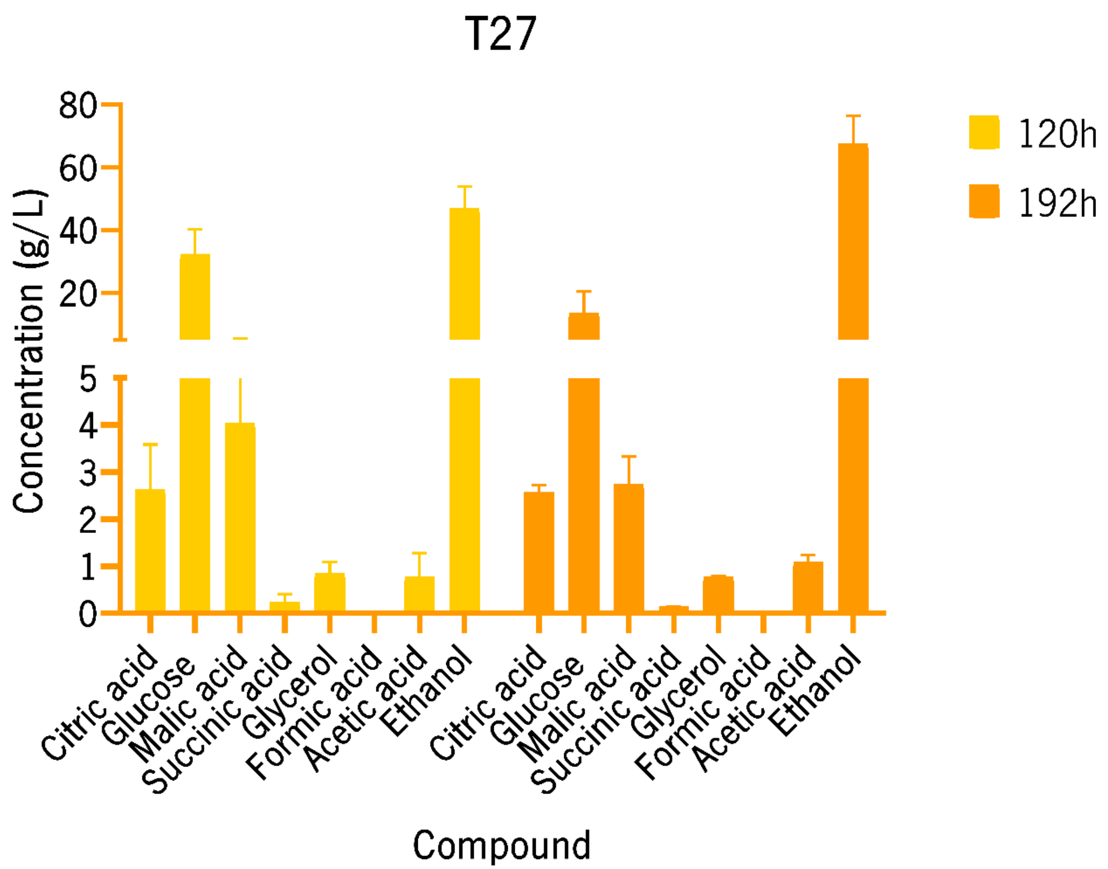

T15
120
90
60
30
5
4
3
2
1
0
Concentration (g/L)
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound

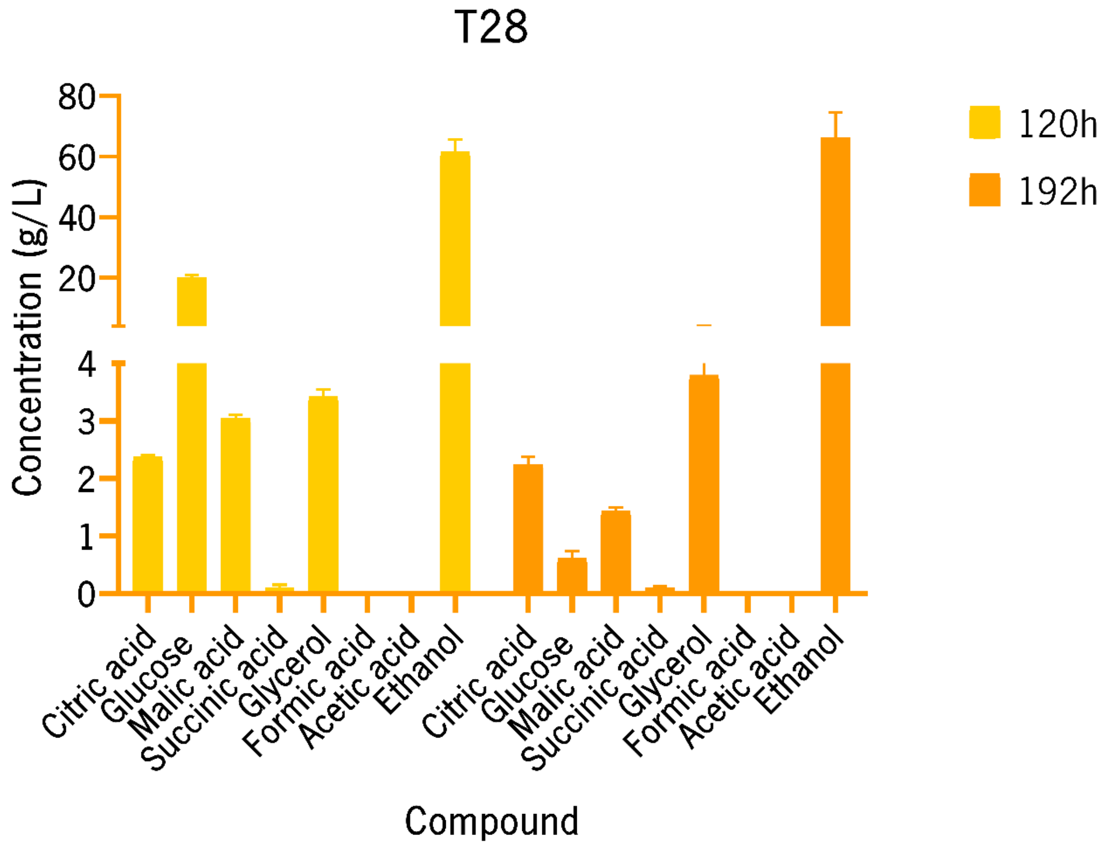

T19
80
60
40
20
5
4
3
2
1
0
Concentration (g/L)
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound

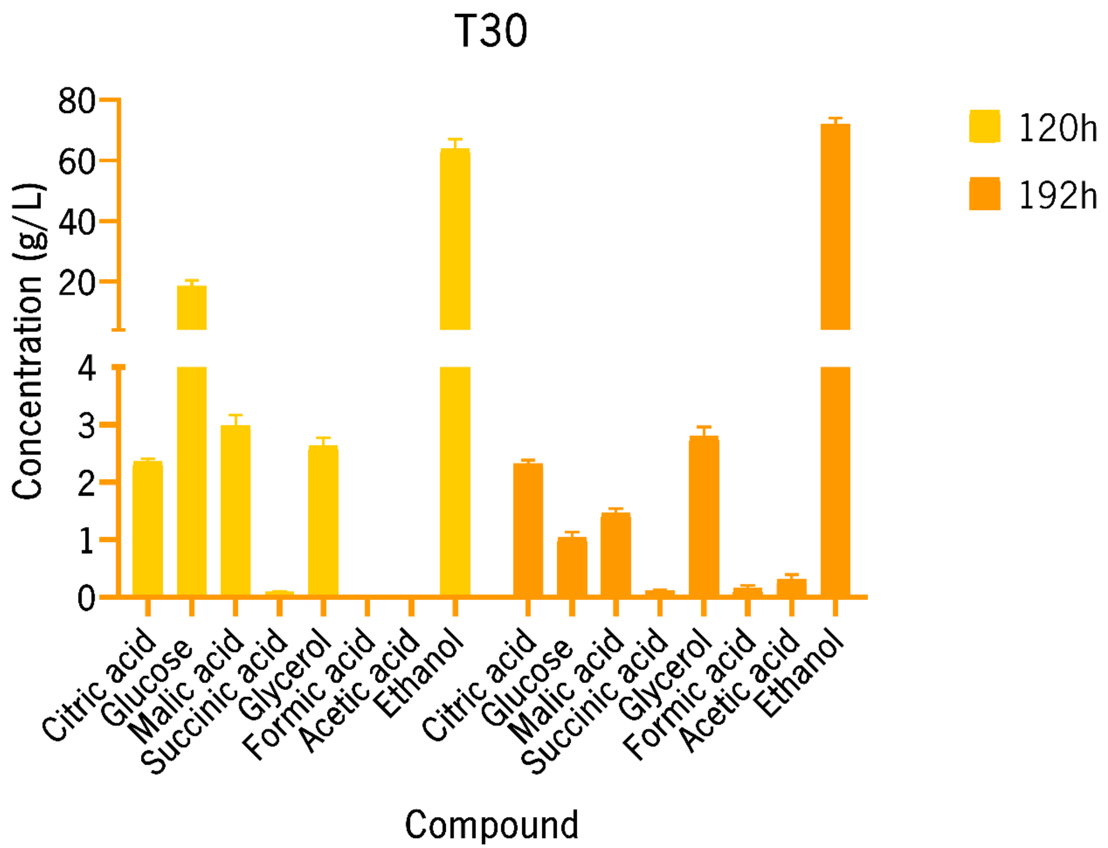

T22
Concentration (g/L)
60
40
20
6
4
2
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

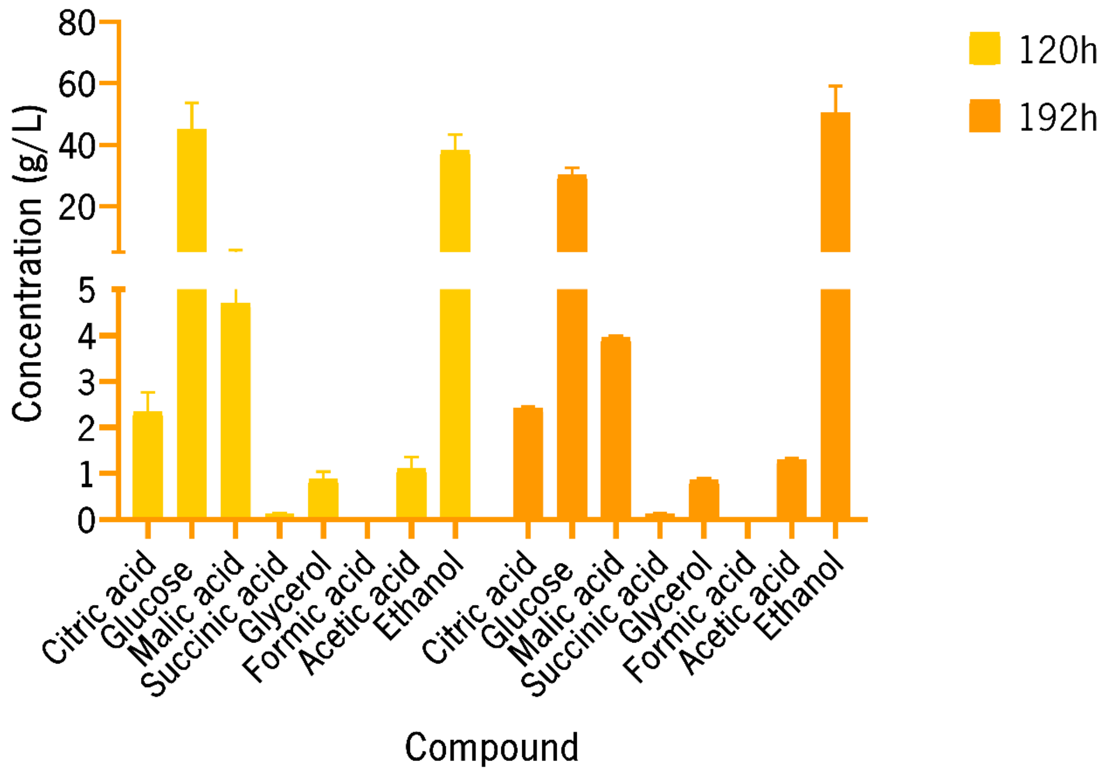

T23
Concentration (g/L)
80
60
40
20
4
2
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

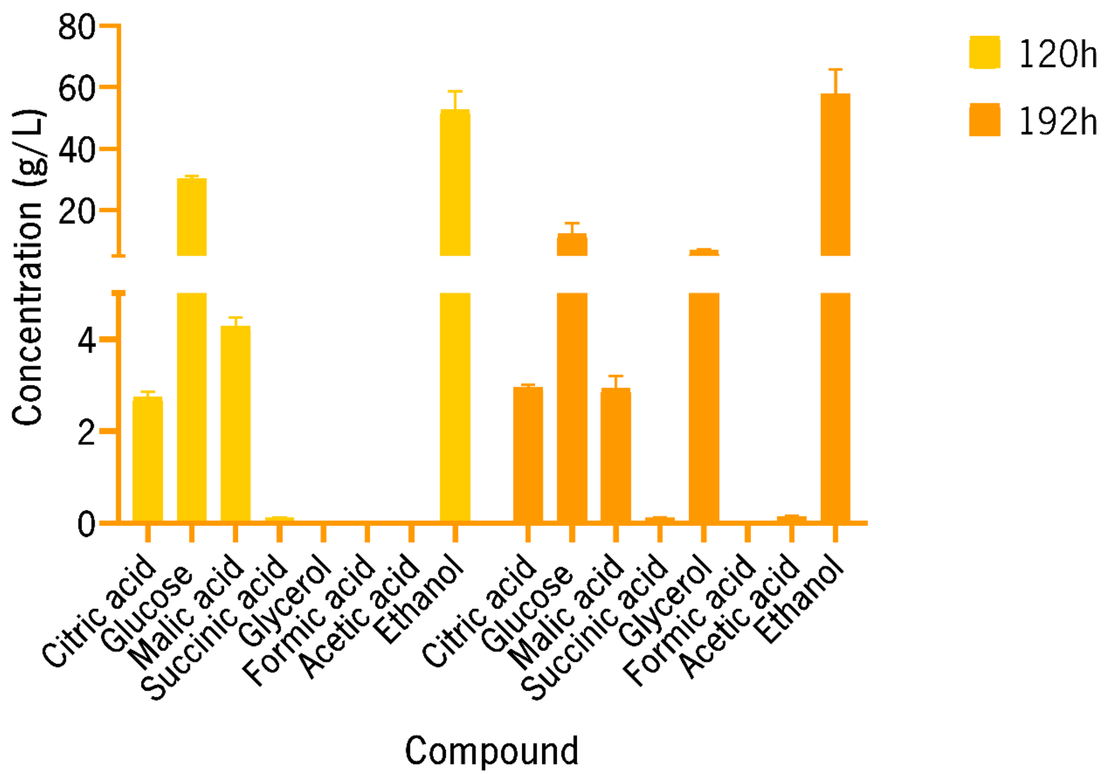

T26
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

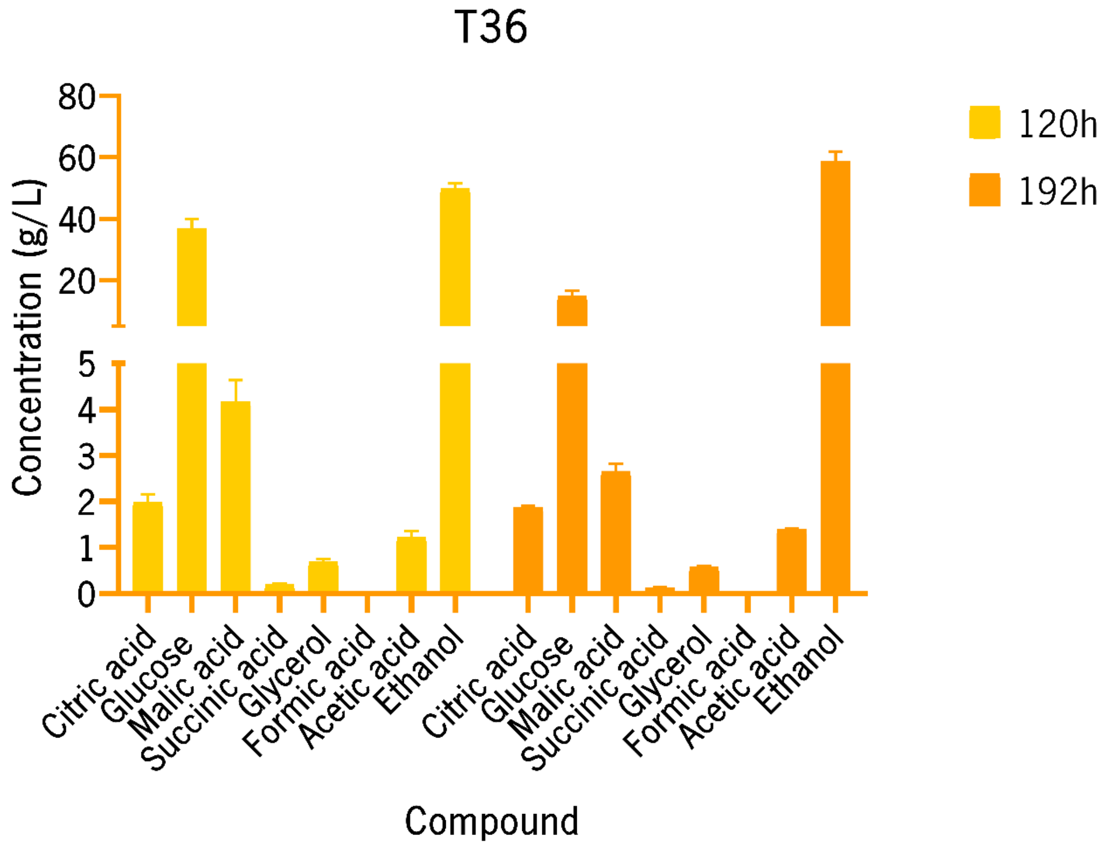

T27
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

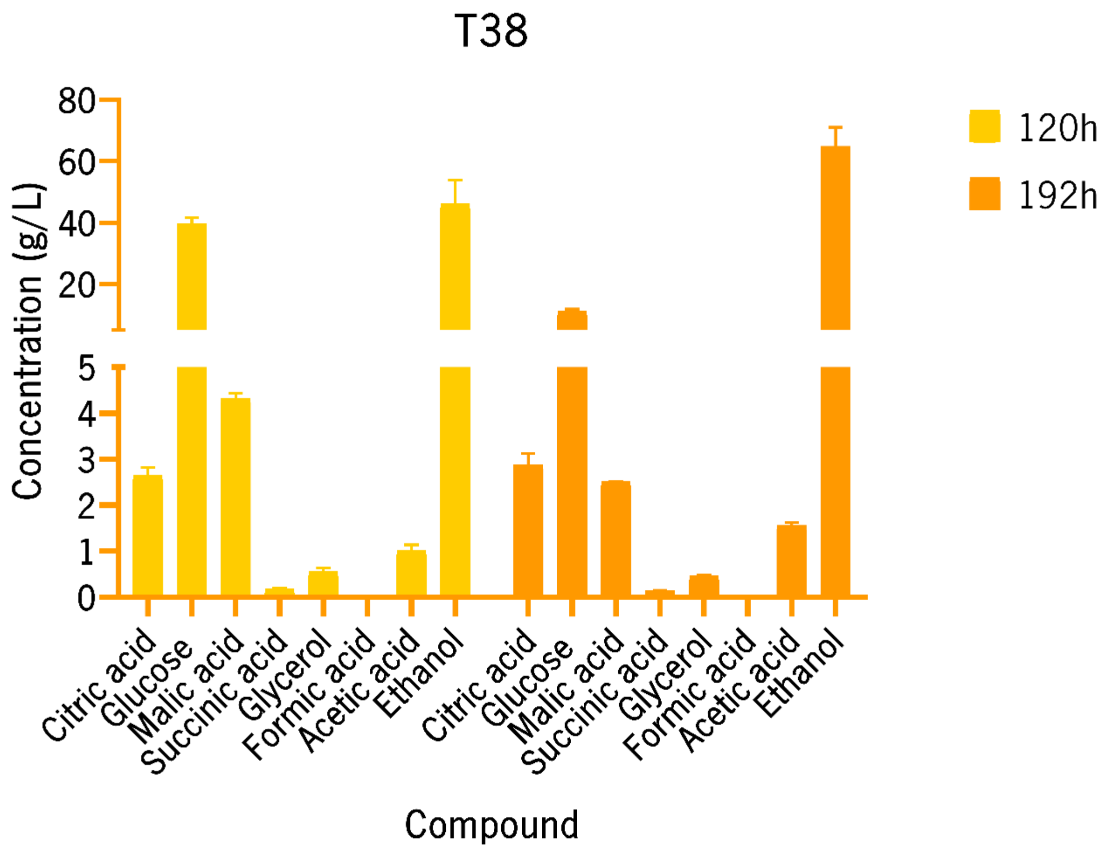

T28
Concentration (g/L)
80
60
40
20
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

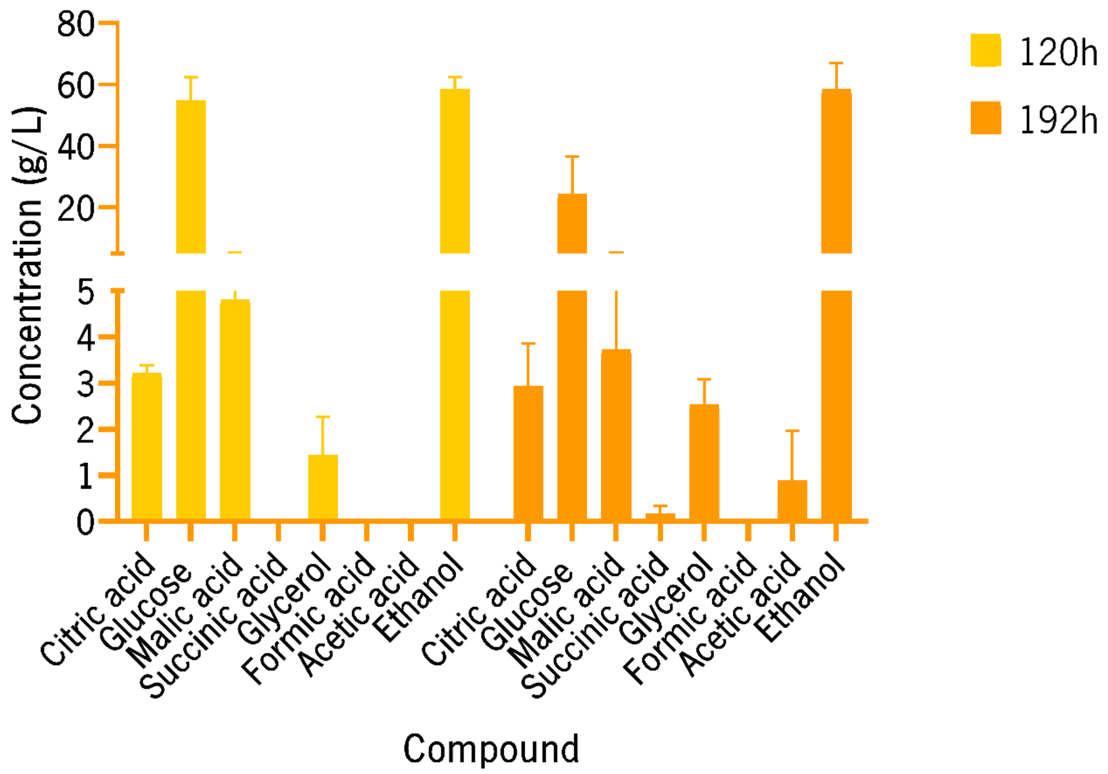

T30
Concentration (g/L)
80
60
40
20
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

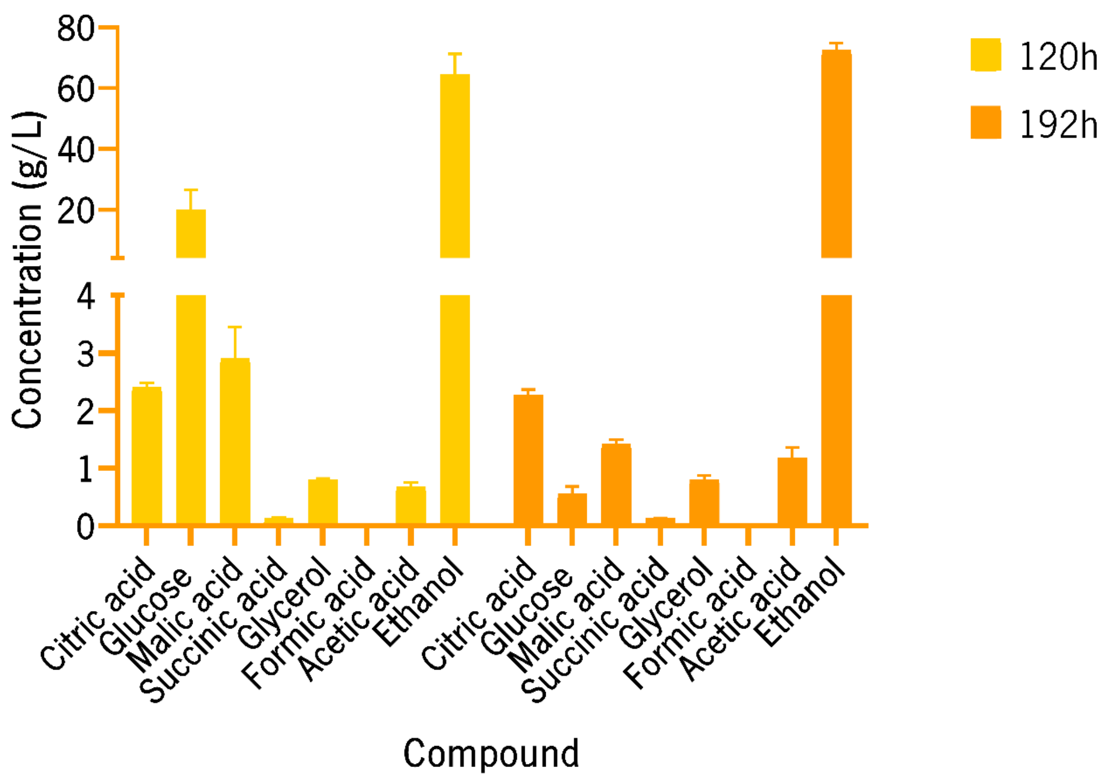

T34
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

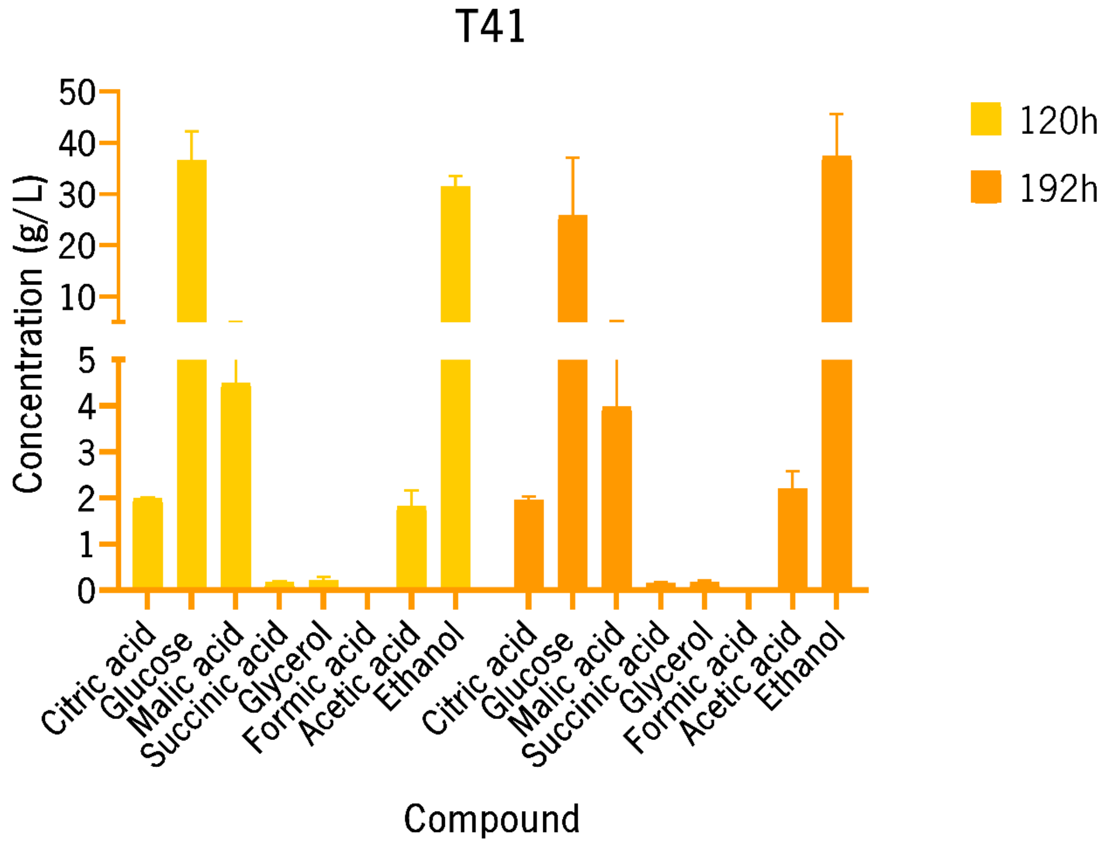

T35
Concentration (g/L)
80
60
40
20
4
2
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

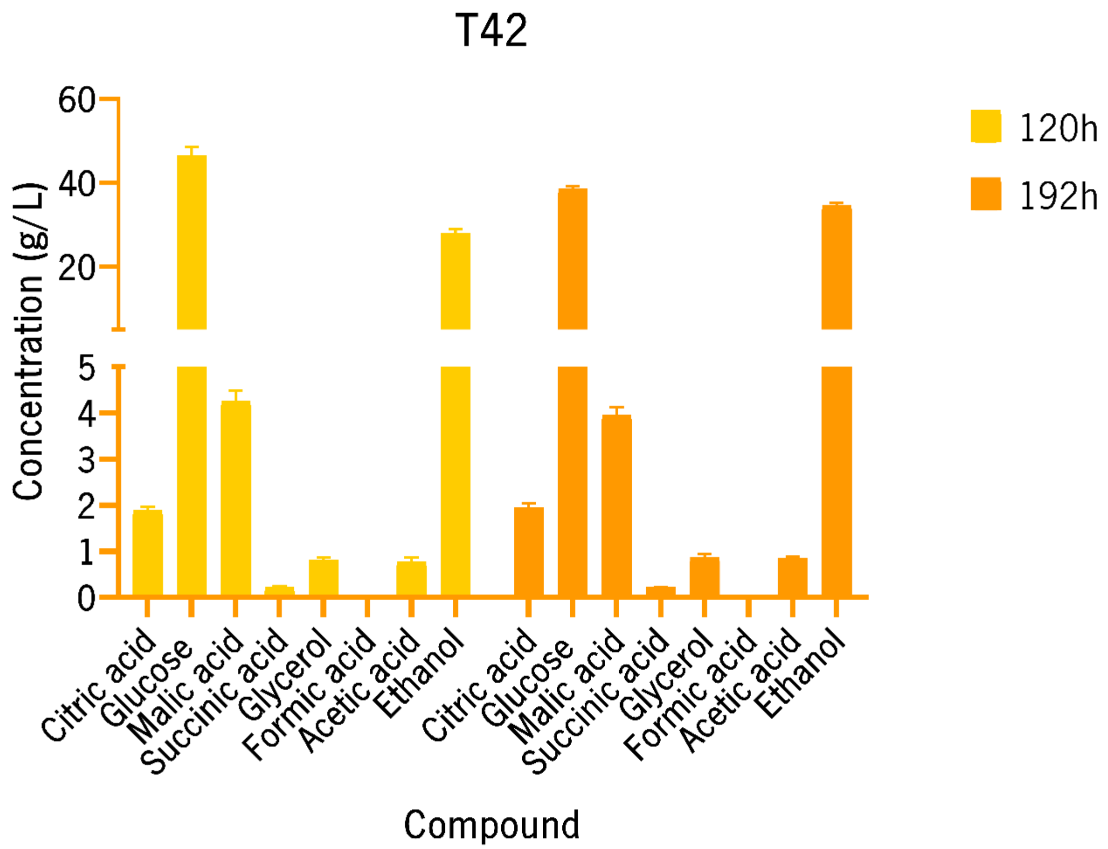

T36
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

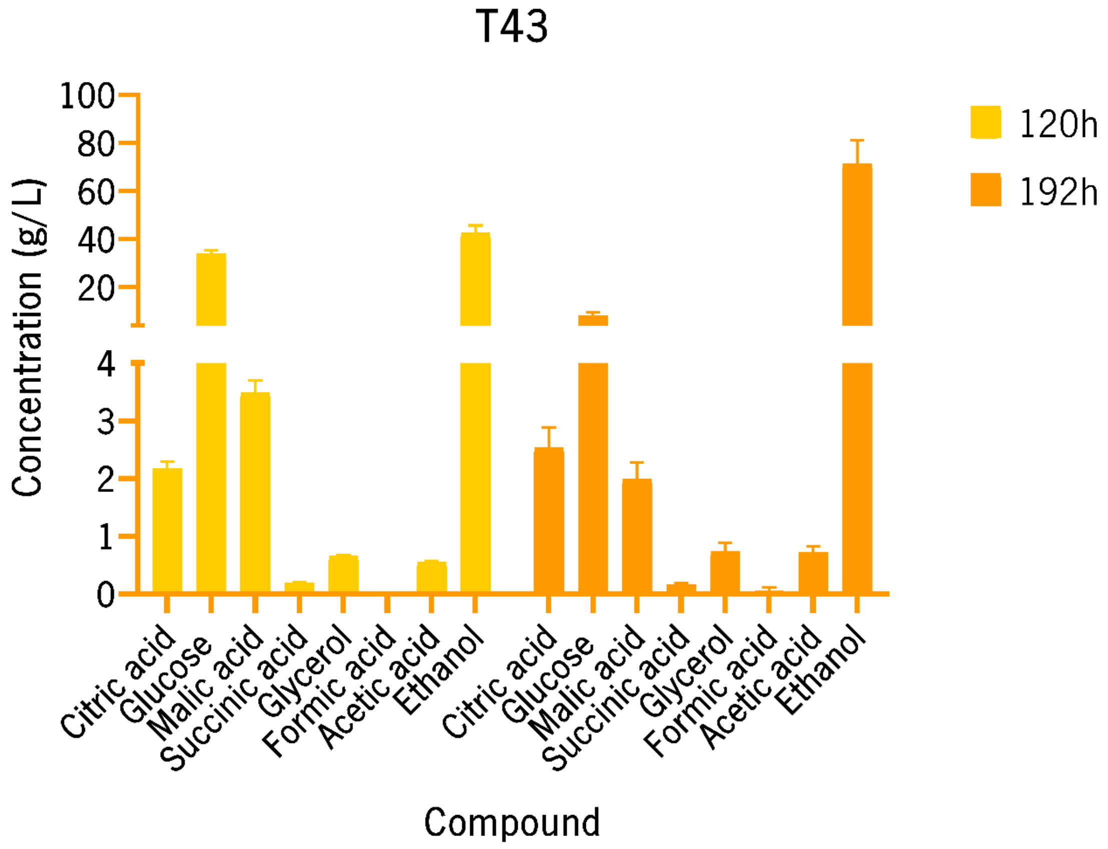

T38
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

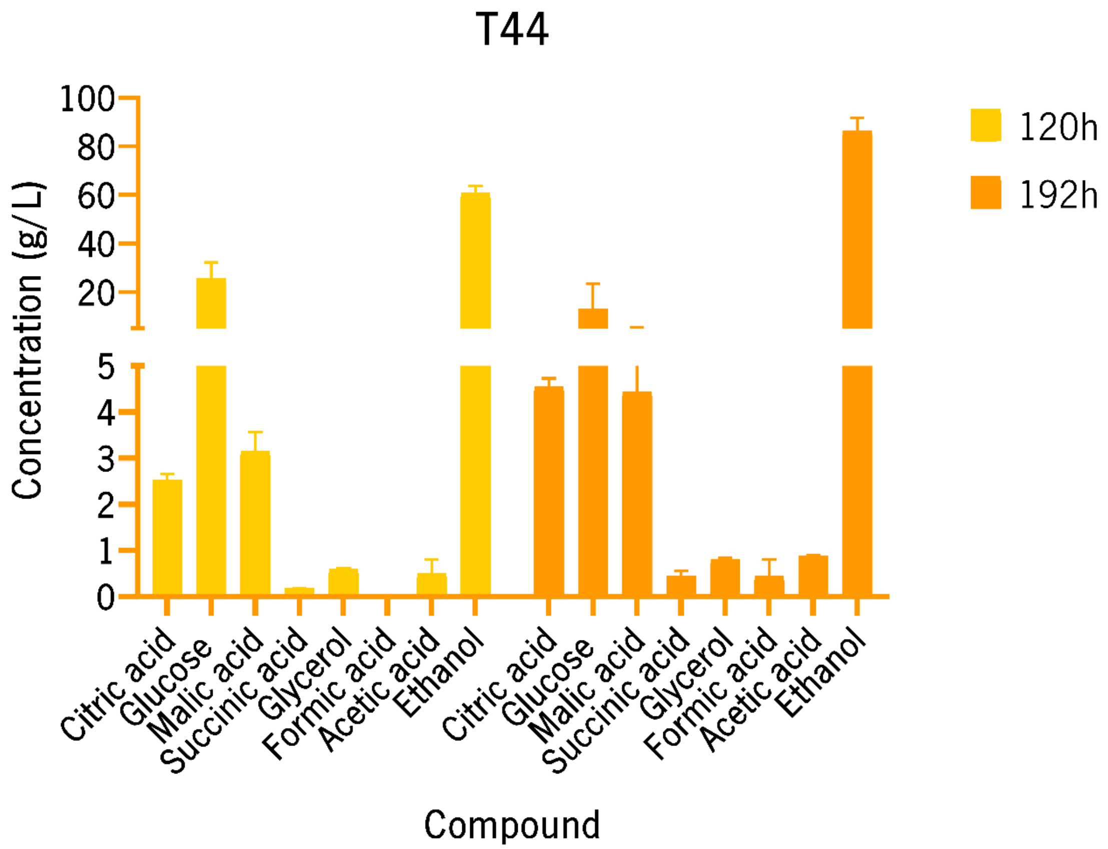

T39
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

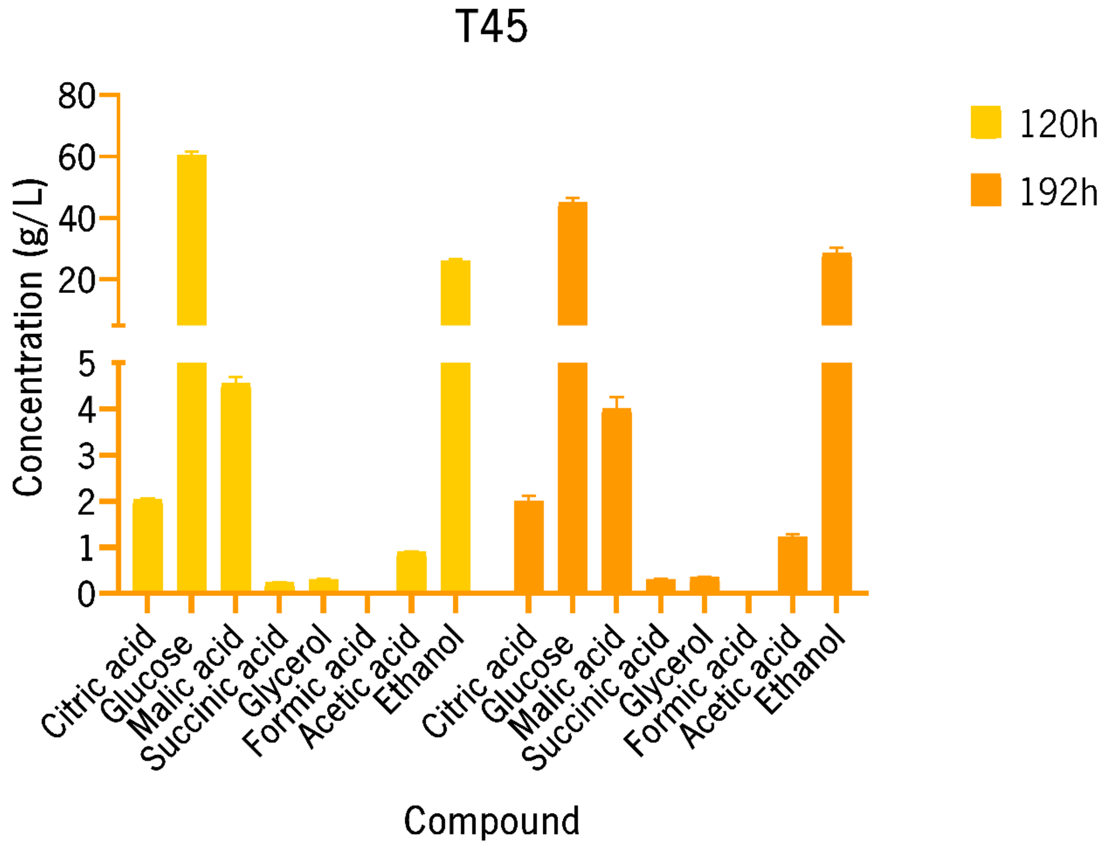

T40
Concentration (g/L)
80
60
40
20
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

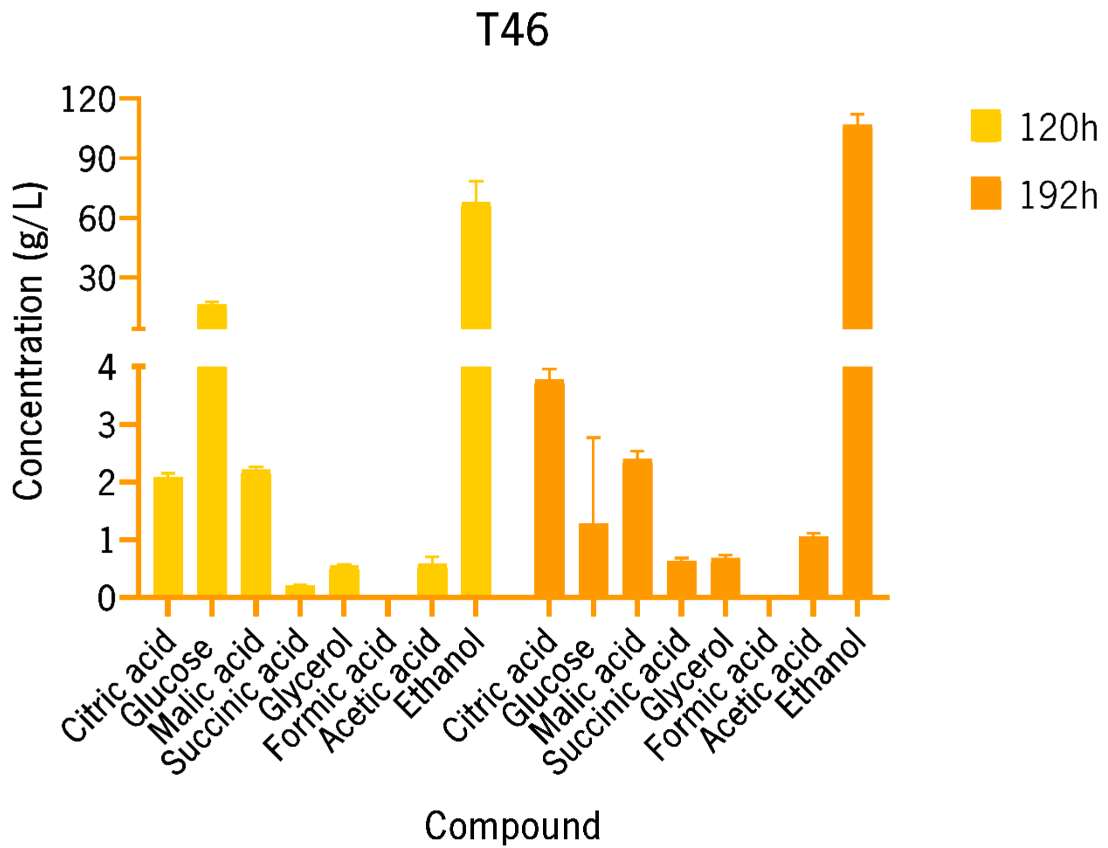

T41
Concentration (g/L)
50
40
30
20
10
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

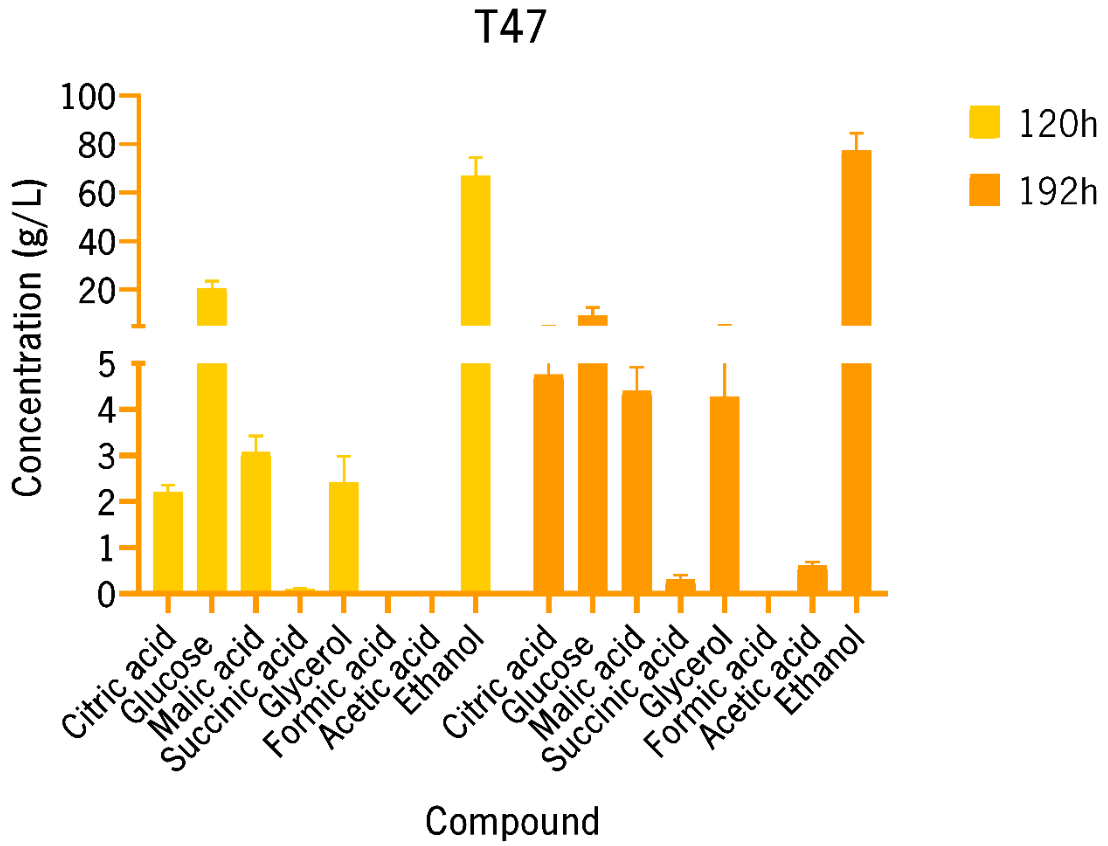

T42
Concentration (g/L)
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

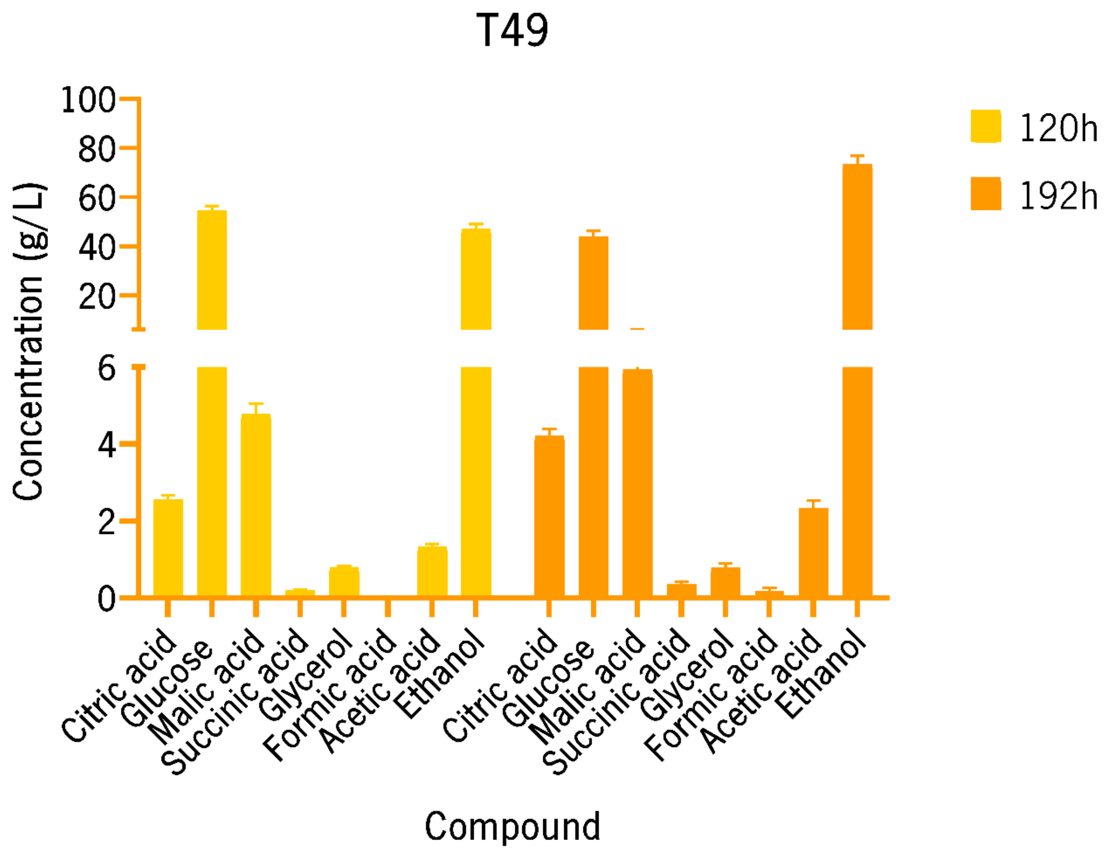

T43
100
80
60
40
20
4
3
2
1
0
Concentration (g/L)
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

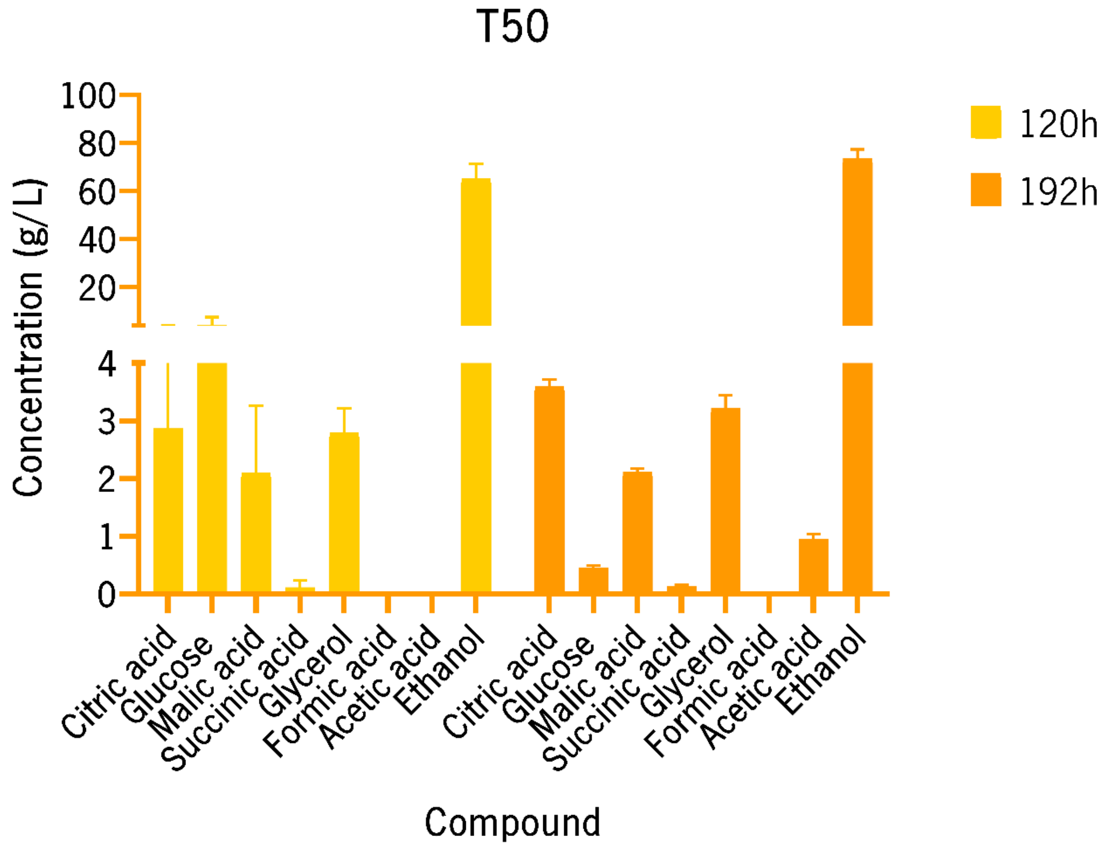

T44
100
80
60
40
20
5
4
3
2
1
0
Concentration (g/L)
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

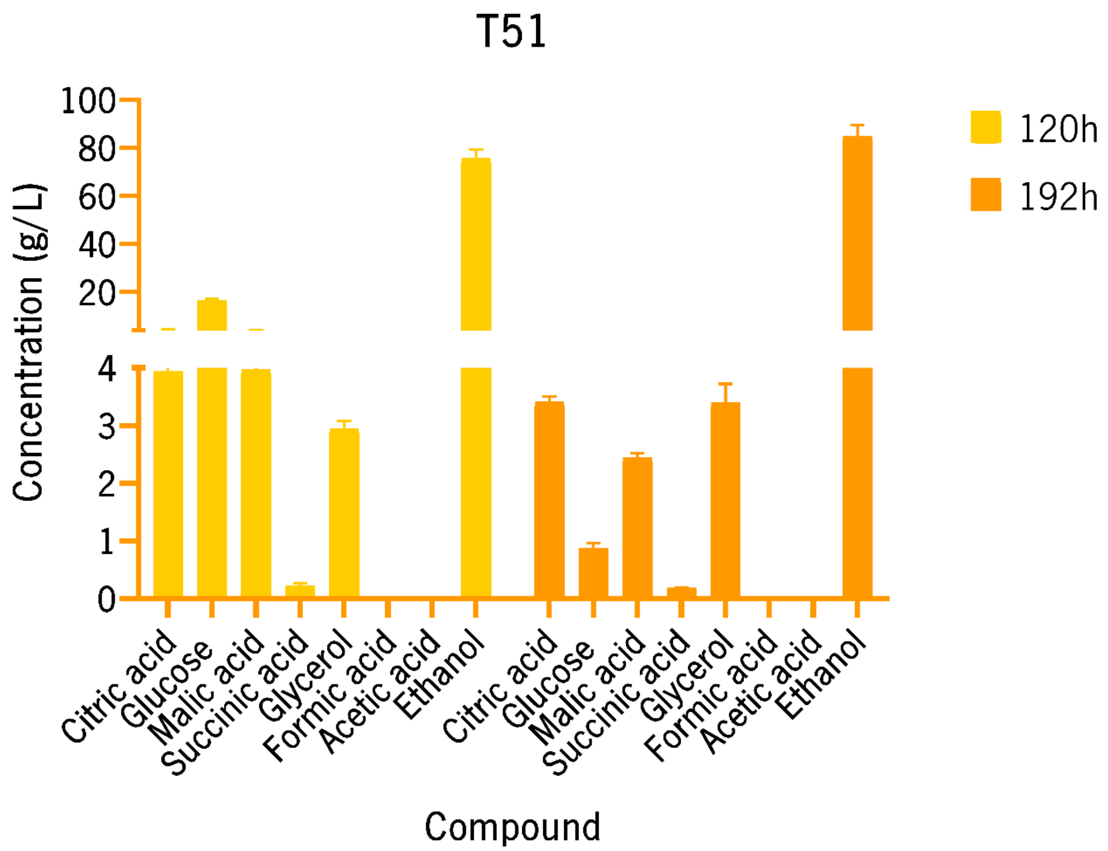

T45
Concentration (g/L)
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

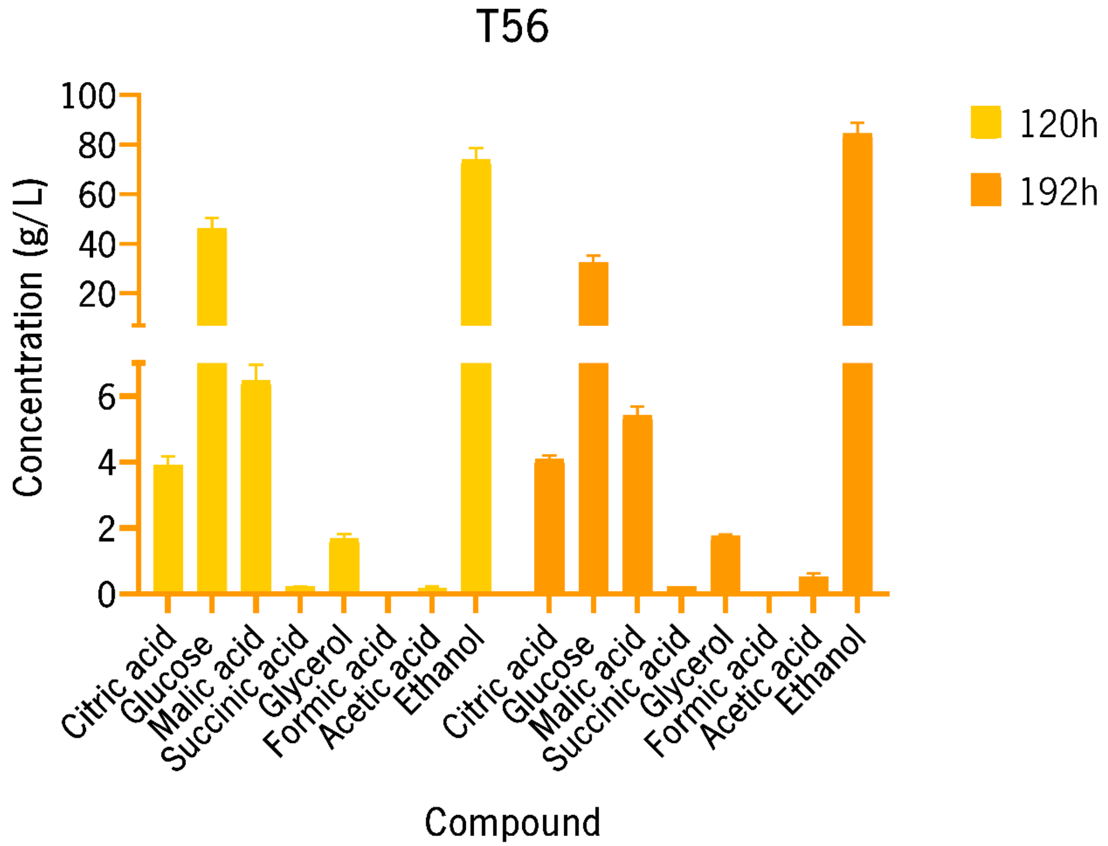

T46
Concentration (g/L)
120
90
60
30
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

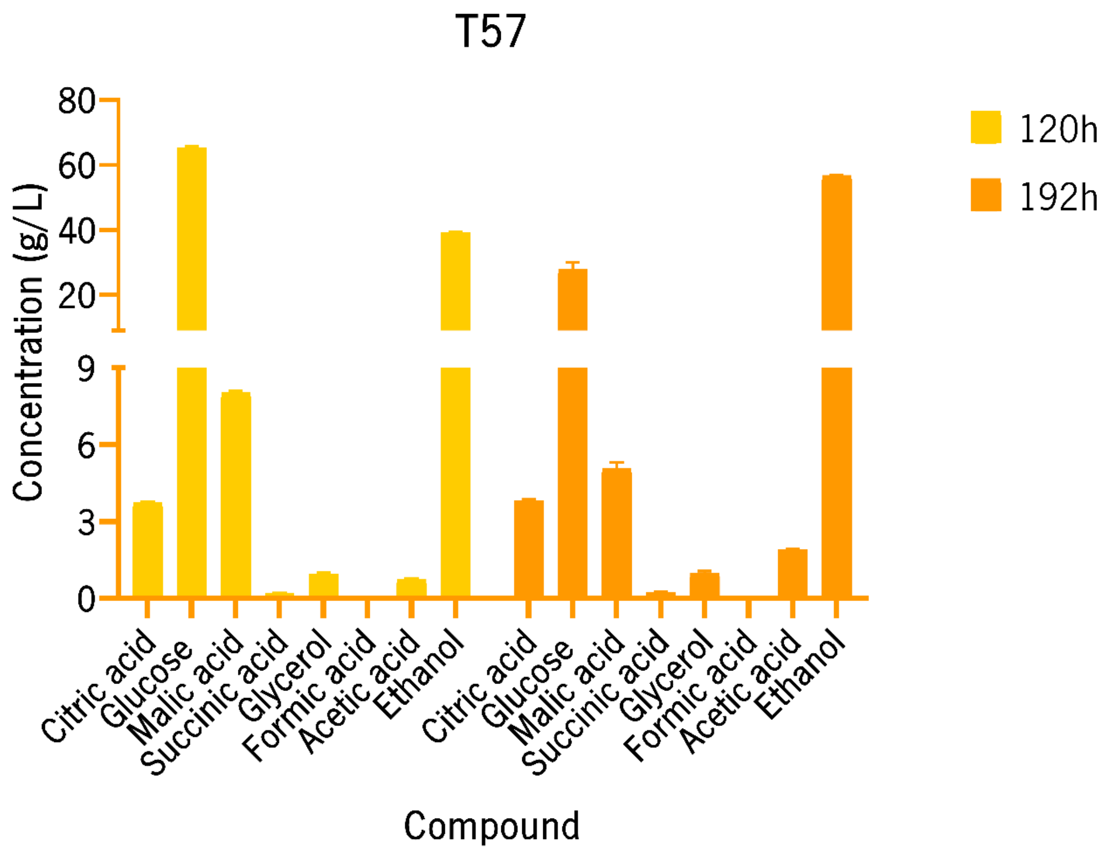

T47
Concentration (g/L)
100
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

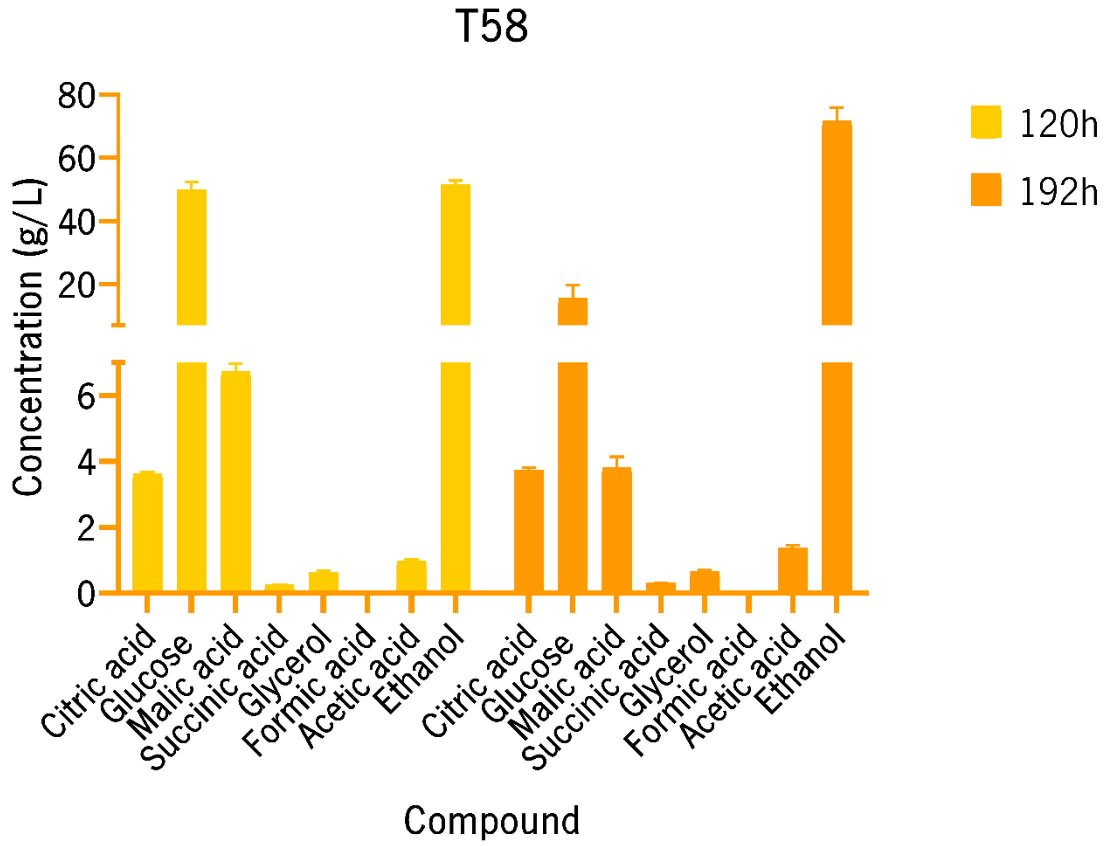

T49
Concentration (g/L)
100
80
60
40
20
6
4
2
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

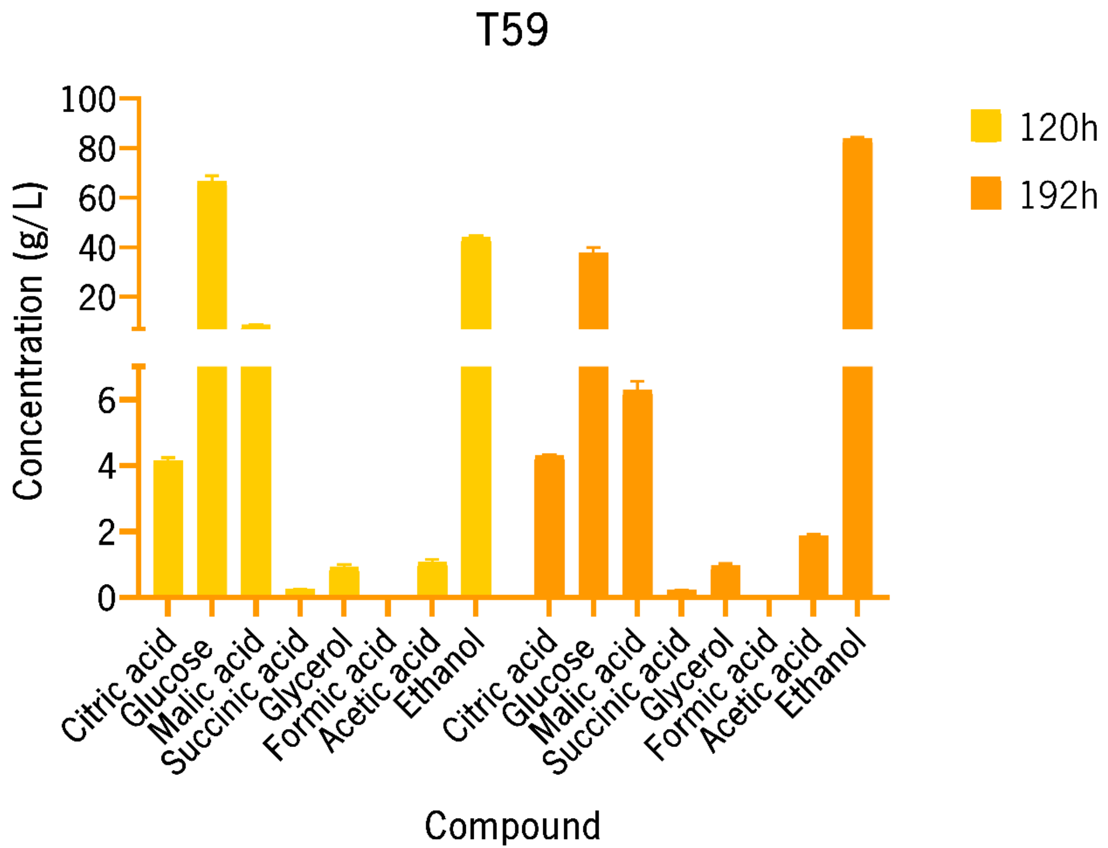

T50
Concentration (g/L)
100
80
60
40
20
4
3
2
1
0
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound

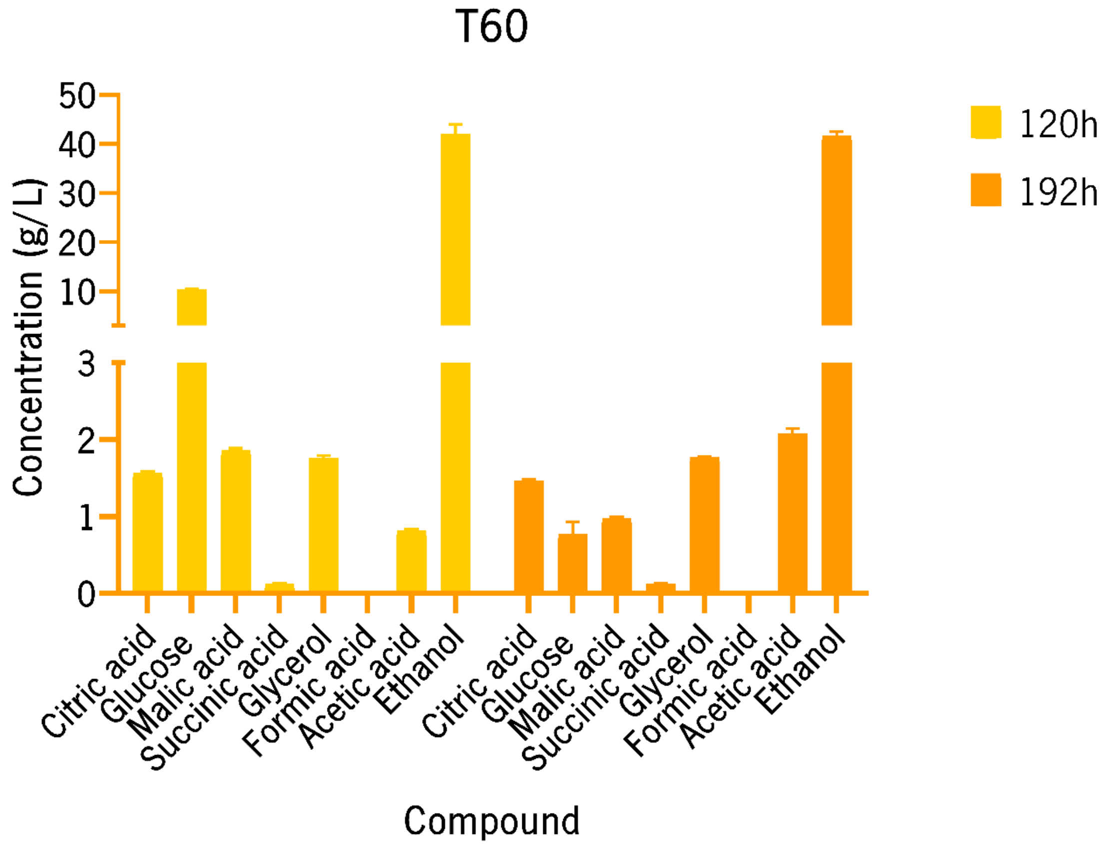

T51
Concentration (g/L)
100
80
60
40
20
4
3
2
1
0
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound

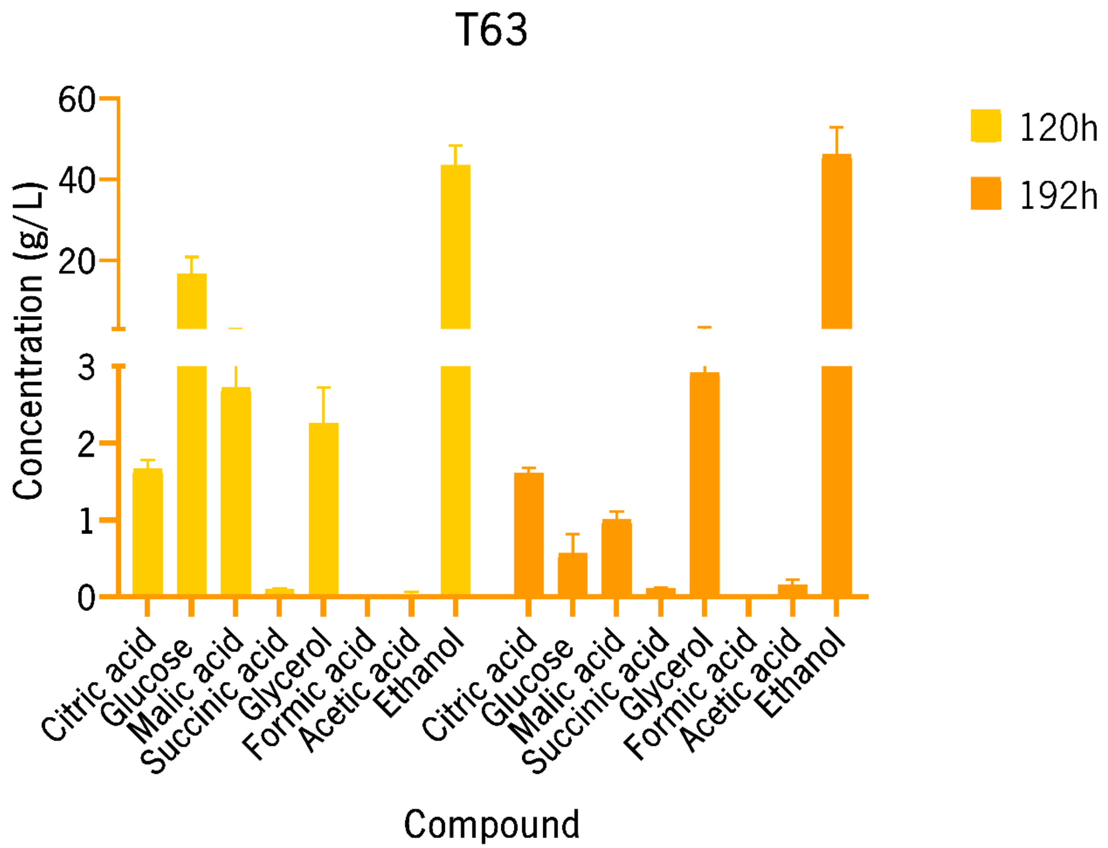

T56
Concentration (g/L)
100
80
60
40
20
6
4
2
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h

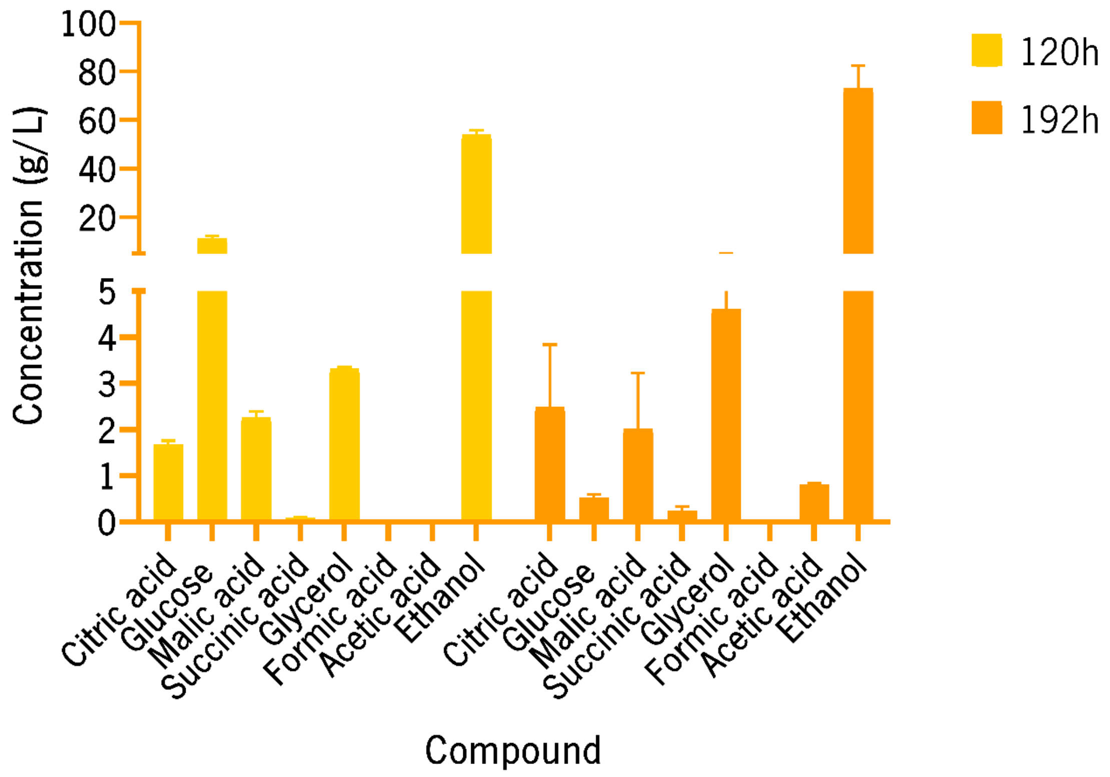

T57
Concentration (g/L)
80
60
40
20
9
6
3
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h


T58
Concentration (g/L)
80
60
40
20
6
4
2
0
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound


T59
Concentration (g/L)
100
80
60
40
20
6
4
2
0
120h
192h
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound


T60
50
40
30
20
10
3
2
1
0
Concentration (g/L)
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h


T63
60
40
20
3
2
1
0
Concentration (g/L)
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h


T64
Concentration (g/L)
100
80
60
40
20
5
4
3
2
1
0
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Citric acid
Glucose
Malic acid
Succinic acid
Glycerol
Formic acid
Acetic acid
Ethanol
Compound
120h
192h