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Fermentation of fruit juices by the osmotolerant yeast *Candida magnoliae*

Dissertation for the degree of Master in Biotechnology

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Abstract

This study focuses on the assessment of the fermentation conditions required to modulate the metabolic flux in the osmotolerant yeast *Candida magnoliae* and evaluate its potential to produce low-alcoholic and low-caloric fermented beverages. For that purpose, two strains, PYCC 2903 and PYCC 3191, were used and fermentation conditions as oxygenation, sugar concentration and the ratio of glucose to fructose were studied using synthetic culture media. *Candida magnoliae* PYCC 2903 was subsequently used to ferment real industrial fructose-rich substrates such as fruit juices.

Sugar consumption profiles for *C.magnoliae* PYCC 2903 incubated aerobically in the presence of high fructose and glucose concentrations (15%, 10% and 5%) showed a selective utilization of fructose, denoting a preference for this sugar over glucose. The lower ratio between ethanol and sugar alcohols yield was obtained for both strains incubated under oxygen limitation simulating industrial fructose-rich substrates, confirming the ability of this yeast to direct fermentation towards alternative products.

Enzymatic assays for hexokinase activity in terms of capacity and affinity for glucose and fructose were performed, aiming to elucidate its contribution to the fructophilic behaviour of this yeast. Enzymatic assays for both strains showed that the V_{max} is two to threefold higher for fructose than for glucose but K_m is also 10-20-fold higher for this sugar than for glucose. Hence, hexokinase kinetic properties do not explain fructophily in *C.magnoliae.* This indicates that fructose transport is probably determining in this respect, as observed for other fructophilic yeasts.

Fruit juice fermentations with *C.magnoliae* PYCC 2903 revealed a potential for the production of beverages with interesting sensorial properties. Pear and peach fermentations exhibited the best results with the lowest ratio between ethanol and sugar alcohols yield and the most pleasant organoleptic features.

Keywords: *Candida magnoliae*; fructophily; sugar alcohols; hexokinase; fruit juices; low-alcoholic fermented beverages.

Resumo

O presente trabalho teve como objectivo avaliar as condições de fermentação necessárias para modular o fluxo metabólico na levedura osmotolerante *Candida magnoliae* e o seu potencial para produzir bebidas fermentadas com reduzido teor alcoólico e calórico. Para este propósito foram utilizadas duas estirpes, PYCC 2903 e PYCC 3191, e estudadas as condições de fermentação como a oxigenação, a concentração de açúcar e o rácio entre a glucose e a frutose utilizando um meio de cultura sintético. A estirpe *Candida magnoliae* PYCC 2903 foi posteriormente utilizada para fermentar substratos industriais reais ricos em frutose tais como os sumos de fruta.

Os perfis de consumo de açúcar para a *C.magnoliae* PYCC 2903 incubada em condições aeróbias e com concentrações elevadas de frutose e glucose (15%, 10% e 5%) mostraram uma utilização selectiva da frutose, evidenciando uma preferência por este açúcar relativamente à glucose. O menor rácio entre o rendimento do etanol e dos açúcares álcoois foi obtido para ambas as estirpes incubadas em condições de limitação de oxigénio simulando substratos industriais ricos em frutose, confirmando a capacidade desta levedura para direcionar a fermentação para produtos alternativos.

Foram realizados ensaios enzimáticos para a actividade da hexocinase em termos de capacidade e afinidade para a glucose e a frutose com o intuito de elucidar acerca do seu contributo para o comportamento frutofílico desta levedura. Os ensaios enzimáticos para ambas as estirpes mostraram que o V_{max} é duas a três vezes superior para a frutose do que para a glucose mas o K_m também é 10-20 vezes superior para este açúcar do que para a glucose. Portanto, as propriedades cinéticas da hexocinase não explicam a frutofilia em *C.magnoliae*. Isto indica que o transporte da frutose é provavelmente determinante neste contexto, como observado para outras leveduras frutofílicas.

As fermentações de sumos de fruta com a *C.magnoliae* PYCC 2903 revelaram um potencial para a produção de bebidas com propriedades sensoriais interessantes. As fermentações de pêra e pêssego exibiram os melhores resultados com o menor rácio entre o rendimento do etanol e dos açúcares álcoois e as características organolépticas mais agradáveis.

Palavras-chave: *Candida magnoliae*; frutofilia; açúcares álcoois; hexocinase; sumos de fruta; bebidas fermentadas com reduzido teor alcoólico.

Table of contents

List of Figures

List of Tables

List of Abbreviations

Abs- Absorbance

ATP- Adenosine Triphosphate

BSA- Bovine Serum Albumin

C.magnoliae- *Candida magnoliae* yeast

CBS- Centraalbureau voor Schimmelcultures Fungal Biodiversity Centre

CFU- Colony-Forming Units

CREM- Centre for Microbial Resources

DCV- Department of Life Sciences

DTT- Dithiothreitol

Fruc- Fructose

G-6-PDH- Glucose-6-Phosphate Dehydrogenase enzyme

Gluc- Glucose

HPLC- High-performance liquid chromatography

Hxk- Hexokinase enzyme

IR- Infrared

KCCM- Korean Culture Center of Microorganisms

KFCC- Korean Federation of Culture Collections

K_m- Michaelis-Menten constant

$MgCl_2$- Magnesium Chloride

$NADP^+$- Nicotinamide Adenine Dinucleotide Phosphate

NaOH- Sodium hydroxide

O.D- Optical density

^{o}Brix- Degrees Brix

PGI- Phosphoglucose Isomerase enzyme

PMSF- Phenylmethylsulfonyl Fluoride

PYCC- Portuguese Yeast Culture Collection

S.cerevisiae- *Saccharomyces cerevisiae* yeast

Sucr- Sucrose

TRIS- Triethanolamine Hydrochloride

TSS- Total soluble solids

TTA- Titratable acidity

UV- Ultraviolet

UV-Vis- Ultraviolet-Visible

V_{max}- Maximum velocity

YP- Yeast extract-Peptone

YPD- Yeast extract-Peptone-Dextrose

1. Introduction

1.1 Food fermentations

1.1.1 Historical perspective of food preservation and fermentation

Fermentation is a widely practiced and ancient technology dependent on the biological activity of microorganisms for production of a range of metabolites which can suppress the growth and survival of undesirable microflora in foodstuffs. Such an old process is used for food and beverages preservation and has been an effective form of extending the shelf-life of foods for millennia. Traditionally, foods were preserved through naturally occurring fermentations that ensure not only increased shelf-life and microbiological safety of a food but also made some foods more digestible. Nowadays due to modern industrialization, also known as large-scale production, there is an exploration of the use of defined strain starter systems to ensure consistency and quality in the final product. In addition to that, to ensure that food is maintained at a suitable level of quality from the time of manufacture through to the time of consumption, modern food processing is dependent on a range of preservative technologies (Caplice and Fitzgerald, 1999; Ross *et al*., 2002).

Traditional fermentation resulting from a natural occurrence was used during thousands of years for food transformation and preservation by many different people, even before the entire microbiological and biochemical basis behind the process was known. As far back as 8000 years ago the art of cheese-making was developed at a time when plants and animals were just being domesticated, in the fertile Crescent between Tigris and the Euphrates rivers in Iraq (Figure 1.1).

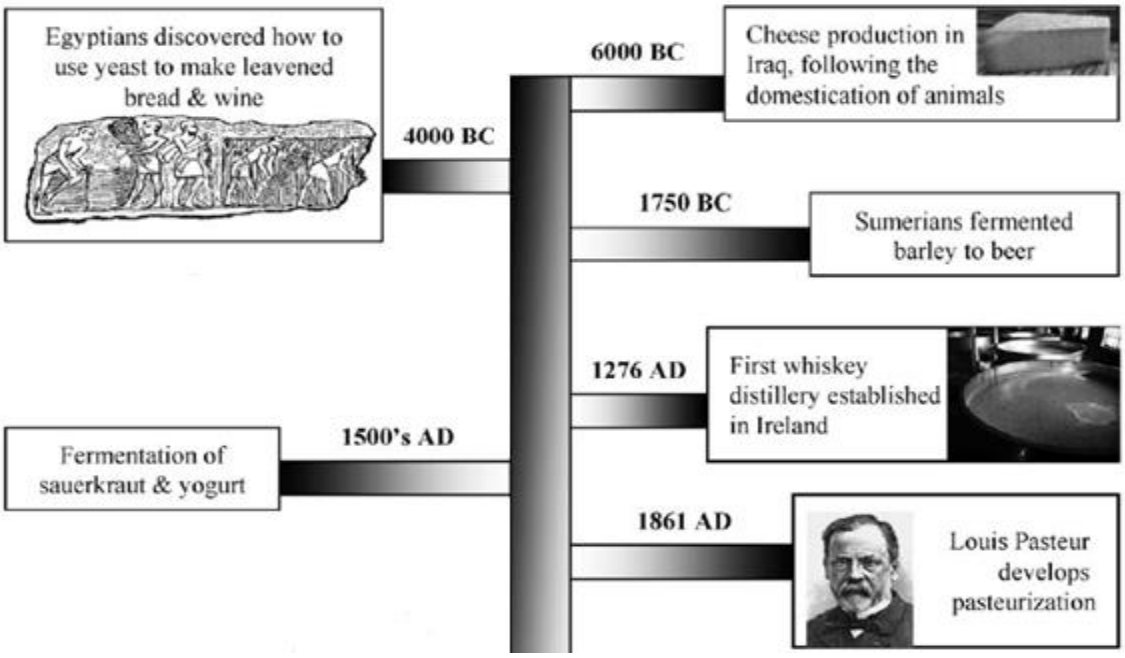


Figure 1.1- Some major events in food fermentation and preservation through the years (adapted from Ross *et al*., 2002).

Later, alcoholic fermentations involved in winemaking and brewing are thought to have been developed during the period 2000–4000 BC by the Egyptians and Sumerians (Figure 1.1). The Egyptians also developed dough fermentations used in the production of leavened bread. As mentioned, fermentations have been exploited as a preservation method of food and beverages for thousands of years however, microorganisms were recognized as being responsible for the fermentation process only in the most recent past when pasteurization was also developed. Coincident with this discovery, was the time of the industrial revolution (Figure 1.1) (Ross *et al*., 2002).

By the middle of the 19th century two key events occurred that had a very important impact on the manner in which food fermentations were performed and on our understanding about the process. Firstly, the industrial revolution that resulted in the concomitant concentration of large masses of population in towns making impractical the traditional method of food supplying within local communities. So, after this historical event, a dramatic shift from food production for local communities to large-scale food production occurred. This allowed the development of large scale fermentation processes for commercial production of fermented foods and alcoholic beverages. Beyond the requirement to produce in large amounts, there was a need to industrialize the manufacturing process to service these new markets. Secondly, from the 1850s onwards, the developing of microbiology as a science resulted on the understanding the biological basis of fermentation. Thus, the essential role of bacteria, yeasts and moulds in the generation of fermented foods became understood and such knowledge resulted in more controlled and efficient fermentations (Caplice and Fitzgerald, 1999).

The coincidence between industrialization of fermented foods and scientific advances at a microbiological level was fortunate. The beginning of retailing and mass marketing required the availability of products with consistent quality and safety (Caplice and Fitzgerald, 1999; Ross *et al*., 2002). Towards the end of the 19th century, characterization of the microorganisms responsible for fermentation led to the isolation of starter cultures for many fermented foods and particularly milk-derived products. These cultures could be produced on a large scale and are required to supply factories involved in the manufacture of products in large amounts. Although the world has evolved towards industrialization using sophisticated technologies which are capable of producing large amounts in a short time, there are regions, even in Europe, where fermented foods remain manufactured in a traditional way. For some cheeses and fermented meats and vegetables the concept of backslopping, which consists in the insertion of a small portion of a previous batch of fermented food into the start of new batch of food to be fermented, was kept. Most of the products that result from this process retain flavour and aroma characteristics that the industrialized fermented foods have lost and thus are considered of better quality. However, considering the emerging popularity and consequent rising demand of these products, it appears to be inevitable that the only way for this expanding market to be satisfied is to upscale the manufacturing process (Ross *et al*., 2002).

One of the most interesting challenges about this issue regards allowing the large-scale production of fermented foods without losing the particular traits associated with products made in a traditional manner, taking advantage of the benefits produced by both methods. Initially, fermenting food substrates had, as its main purpose, preservation of final product, however, increasing and continuous development of several alternative techniques for food preservation replaced this essential role of fermentation. Thus, the majority of fermented foods began to be produced because their particular characteristics such as aroma, flavour and texture, which are very appreciated by the consumer. Nonetheless the environment generated by the fermentation is crucial in ensuring the shelf-life and microbiological safety of the products but this aim is modulated depending on the world region and the way in which the fermentation process is carried out. In certain parts of the world where the fermentation process continues performed on an artisanal manner, the preservation still the major purpose.

During the fermentation process, end-products or by-products such as acids, alcohols and carbon dioxide are normally produced resulting from carbohydrates metabolism. These compounds play an important role in modifying the organoleptic features of the initial substrate, providing nutritional benefit to consumer.

Since the dawn of civilization methods have been described for the fermentation of different substrates such as plant and animal products. Fermented foods enriches human dietary through a wide diversity of flavors, textures and aromas and different compounds as vitamins, proteins, amino acids and fatty acids (Blandino *et al*., 2003; Caplice and Fitzgerald, 1999; Steinkraus, 2002).

The chemical definition of fermentation describe this process as strictly anaerobic, nonetheless, the general understanding of the process involve both aerobic and anaerobic carbohydrate breakdown (Caplice and Fitzgerald, 1999).

1.1.2 Role of microorganisms responsible for the fermented foods

One of the oldest food processing technologies known to man is the production of fermented foods. Nowadays, the numerous microorganisms (living components) that are responsible from biochemical transformation in the fermentation process are well known and the vast majority are filamentous or unicellular fungi and bacteria. Table 1.1 illustrates the most common fermented foods produced worldwide from different raw materials by biological activity of different microorganisms. Wild fermentation bacteria and yeast cover the continents and permeate ecosystems, in the air, soil, water, plants and animals being a natural resource available to people all over the world. Thus, there are two kingdoms of life in fermentation ecosystems which comprises fungi and bacteria. Fungi includes yeasts (unicellular) which are mainly associated with the production of alcoholic beverages and molds (multicellular) used for instances for cheese production. Bacteria are responsible for pickles, cheese and cured sausages production (Bennett, 1998).

There are different ways to classify food fermentations and one of them is concerning the raw material from which fermented food is produced. Considering the most common fermented foods illustrated in Table 1.1 is possible distinguish two major categories: (1) Plant products that includes substrates as cereals, vegetables and fruits and (2) Animal products as milk and meat (Scott and Sullivan, 2008).

There are many different types of commercial fermentations from vegetables substrates including the most economically profitable: olives, cucumbers (pickles) and cabbage (sauerkraut, Korean kimchi). Most vegetable fermentations occur by providing specific conditions for the growth of microorganisms already present in the raw material. In some cases, microorganism selection are accomplished by added salt thereby favouring the lactic acid bacteria. Those bacteria convert vegetable fermentable sugars into lactic acid, and are mainly *Lactobacillus* (*Lb. plantarum*, *Lb. brevis* and *Lb. bulgaricus*), *Leuconostoc* (*Lc. mesenteroides* and *Lc. plantarum*) and *Lactococcus spp.* (Caplice and Fitzgerald, 1999; Steinkraus, 2002). Another vegetable that is also widely used to produce fermented foods is soy bean. This raw material is able to produce different types of Asian foods such as soy sauce, tempeh and miso in which fermentation process is conducted by *Aspergillus oryzae* or *Rhyzopus oligosporus* (Bennett, 1998; Blandino *et al*., 2003). Concerning fruits and fruit juices, the fermented products more spread worldwide are wines, wine vinegars, cider and perry. Wines are produced from grapes and is the result of alcoholic fermentation by the yeast *Saccharomyces* (*S.cerevisiae*, *S.pastorianus*, *S.bayanus*). Wine vinegar production requires two stages, the first one is an alcoholic

Table 1.1- Several common fermented foods and some of the most well-known players in the fermentation ecosystem (from Scott and Sullivan, 2008).

Food	Raw Material	Fermentors (selected species)
Beer	Grain malt	*Saccharomyces cerevisiae*
Bread	Grain flour	*Saccharomyces cerevisiae*
Butter	Milk	*Streptococcus* spp.
		Leuconostoc spp.
Cheese	Milk	*Lactobacillus* spp. (primary)
		Lactococcus spp. (primary)
		Pediococcus spp. (primary)
		Streptococcus spp. (primary)
		Leuconostoc spp. (secondary)
		Propionibacter spp. (secondary)
Chocolate	Cacao bean	*Saccharomyces cerevisiae*
		Candida rugosa
		Kluyveromyces marxianus
Coffee	Coffee bean	*Erwinia dissolvens*
Kefir	Milk	*Saccharomyces kefir*
		Torula kefir
Kimchi	Napa, Daikon	*Lactobacillus plantarum*
		Lactobacillus brevis
		Streptococcus faecalis
		Leuconostoc mesenteroides
		Pediococcus pentosaceus
Miso	Soy bean	*Aspergillus oryzae*
Olives	Olives	*Candida spp.*
		Cryptococcus spp.
		Debaryomyces hanseii
		Lactobacillus spp.
		Saccharomyces spp.
Pickles	Cucumber	*Leuconostoc mesenteroides*
		Lactobacillus spp.
Sauerkraut	Cabbage	*Coliform spp.*
		Leuconostoc plantarum
		Lactobacillus spp.
Sausages	Meat and spices	*Pediococcus spp.*
		Lactobacillus spp.
Soy Sauce	Soy bean	*Aspergillus oryzae*
Tempeh	Soy bean	*Rhyzopus oligosporus*
Vinegar	Fruit juice	*Saccharomyces cerevisae*
		Gluconobacter spp.
		Acetobacter spp.
Wine	Grapes	*Saccharomyces cerevisiae*
		Saccharomyces bayanus
Yogurt	Milk	*Lactobacillus bulgaricus*
		Streptococcus thermophilus

fermentation performed by the yeast *S.cerevisiae* capable to produce ethanol which is subsequently transformed in acetic acid during the second stage (acetic fermentation) by acetic acid bacteria (AAB) such *Gluconobacter spp. and Acetobacter spp.* Cider (not shown in Table 1.1) is produced from apple juice and alcoholic fermentation is mainly carried by *Saccharomyces* yeasts (*S.cerevisiae* and *S.bayanus*). Perry (not shown in Table 1.1), as well as wine and cider, is produced using the same alcoholic fermentation process with the difference that starting material are pears instead of grapes and apples (Ghorai *et al*., 2009).

Plant products such as malt and flour grains are used as raw material for the production of cereal-based fermented foods. Although cereals are deficient for example in essential aminoacids, fermentation could be the most simple and economical method of improving their nutritional value, sensorial properties and functional qualities (Blandino *et al*., 2003). One of the most manufactured cereal-based fermented alcoholic beverages is beer which results from alcoholic fermentation carried out mostly by *S.cerevisiae* (Bennett, 1998; Blandino *et al*., 2003). Another fermented alcoholic beverage produced worldwide that is traditional of Japan and China is sake, also known as rice wine (not shown in Table 1.1). Sake is produced from polished and steamed rice rich in starch (Blandino *et al*., 2003). A fungus, *Aspergillus oryzae*, which is capable of converting the starch into simple sugars assimilable by yeasts is inoculated to grow on the surface of the rice. Afterwards, rice mash is fermented through lactic acid fermentation using some bacteria and yeasts (Ghorai *et al*., 2009). Grain flour is used for bread manufacturing and in this case alcoholic fermentation conducted by *S.cerevisiae* has as main purpose carbon dioxide formation instead of ethanol (Ghorai *et al*., 2009).

Fermented foods from animal products include predominantly cheeses, yogurts and sausages. Cheeses are produced from milk and in spite of the fact that some of these products depend on the natural lactic flora present in this raw material, large scale production uses specific starter cultures. Lactic acid bacteria present in unpasteurized milk are responsible for lactose fermentation (milk sugar) into lactic acid (Steinkraus, 2002). Cheese production results from lactic acid fermentation carried by lactic acid bacteria such *Lactobacillus* (*Lb. bulgaricus*), *Lactococcus spp. and Streptococcus thermophilus* (Ross *et al*., 2002). In some processes, depending on the end product, a secondary microorganism is added (*Propionibacter spp*.) which is able to affect texture. Besides lactic acid bacteria other microorganisms such as moulds mainly *Penicillium* (*P. roqueforti* and *P. camemberti*) that can influence the flavor, yeasts and bacteria can be added (Bennett, 1998). Like cheeses, yogurts are produced from milk and result from lactic acid fermentation. Starter cultures used for yogurt production consists in an equal mixture of two lactic acid bacteria, *Lb. bulgaricus* and *S. thermophilus*, which are able to grow in different stages of production since they tolerate distinct pH ranges (Caplice and Fitzgerald, 1999).

Another fermented food produced from animal sources, in particular the meat, are the sausages. Fermented sausages are produced as a result of lactic acid fermentation of a mixture of minced meat, fat, salt, curing agents (nitrate/nitrite), sugar and spices. Starter cultures used for fermented sausage production consists in a mixture of lactic acid bacteria such as *Lactobacillus*

spp. and *Pediococcus spp.* In addition to bacteria, starter cultures with yeasts (*Debaryomyces hansenii* known as *Candida famata*) and moulds (*Penicillium nalgiovense and Penicillium chrysogenum*) are available for the production of these fermented foods (Caplice and Fitzgerald, 1999).

1.1.3 Alcoholic fermentation carried out by yeasts

Many years ago, alcoholic fermentation was accidentally discovered and afterwards yeasts were found to be the driving force behind it. Briefly, ethanol fermentation is a biological process that occurs under anaerobic conditions, i.e. independent of oxygen and consists in the direct conversion of sugars such as glucose and fructose into cellular energy producing as by-products carbon dioxide and ethanol. Fermentable sugars that are rapidly converted into ethanol and CO_2 are present in different types of substrates such as fruit juices, diluted honey, sugarcane juice, palm sap, germinated cereal grains or hydrolyzed starch, which are used for alcoholic fermentation process. Ethanol and carbon dioxide are produced nearly in equimolar amounts and CO_2 is responsible for flushing out the residual oxygen present, maintaining fermentation under anaerobic conditions (Steinkraus, 2002).

Several reports have been published about production of ethanol through fermentation by microorganisms, and various bacteria and yeasts have been reportedly used for this production. Therefore, there are many microorganisms capable of accumulating high ethanol concentrations, yielding this as the major product. However, *Saccharomyces cerevisiae* still remains the most commonly used and preferred microorganism for alcoholic fermentation. This typical yeast is also generally recognized as safe (GRAS) as a food additive for human consumption (Lin and Tanaka, 2006).

The main metabolic pathway involved in ethanol fermentation is glycolysis, which consists in the metabolism of one molecule of glucose with a final production of two molecules of pyruvate. Under anaerobic conditions or sugar excess, the pyruvate can be further reduced to ethanol with the release of carbon dioxide (Figure 1.2).

To drive biosynthesis, which involves a variety of energy-requiring reactions, and the maintenance of the yeast viability, yeast cells used the two ATPs produced in glycolysis. If ATPs are not continuously consumed, the glycolytic metabolism of glucose will be interrupted due to intracellular accumulation of ATP, which inhibits one of the most important enzymes in this process (phosphofructokinase).

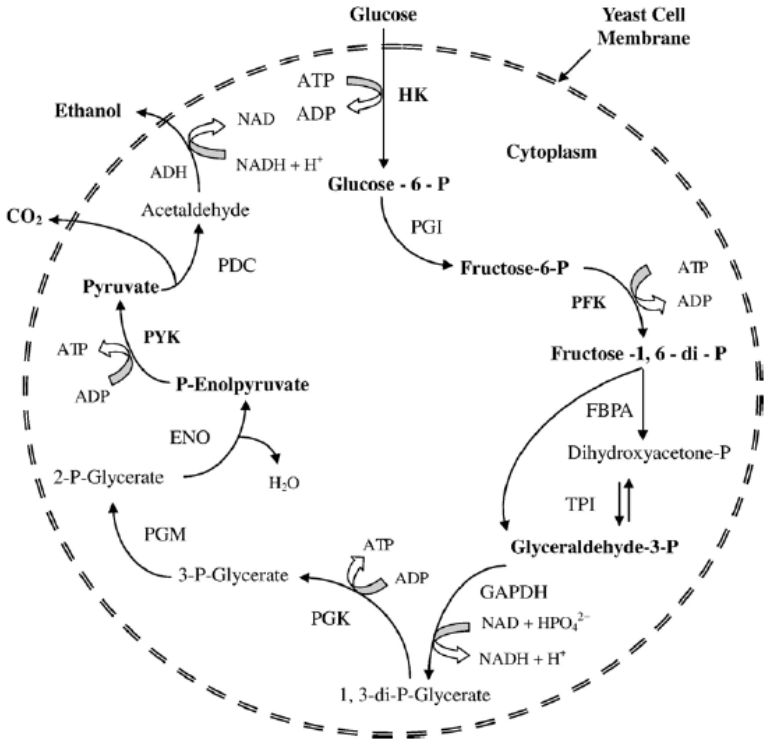


Figure 1.2- Metabolic pathway of alcoholic fermentation in *S.cerevisiae*. Abbreviations: HK (hexokinase), PGI (phosphoglucose isomerase), PFK (phosphofructokinase), FBPA (fructose bisphosphate aldolase), TPI (triose phosphate isomerase), GAPDH (glyceraldehyde-3-phosphate dehydrogenase), PGK (phosphoglycerate kinase), PGM (phosphoglyceromutase), ENO (enolase), PYK (pyruvate kinase), PDC (pyruvate decarboxylase) and ADH (alcohol dehydrogenase) (from Bai *et al*., 2008).

Various by-products are also produced during alcoholic fermentation besides ethanol and CO_2 (Ross *et al*., 2002). The main one is glycerol produced from dehydroxyacetone phosphate (DHAP) conversion resulting in the release of oxidized NAD. Glycerol biosynthesis is a consequence of the utilization of glycolytic intermediates to produce DHAP decreasing the flux of pyruvate formation. In addition to ethanol, CO_2 and glycerol other by-products such as organic acids and higher alcohols are produced at a much lower levels. This by-product production as well as the growth of yeast cells direct some glycolytic intermediates to the corresponding metabolic pathways, decreasing the ethanol yield (Bai *et al*., 2008).

1.2 Beverages industry

1.2.1 Alcoholic, low- and non-alcoholic fermented beverages

Alcoholic fermented beverages dominate the market of fermented beverages since industrialization of the process. The market for alcoholic fermented beverages is enormous and is mostly controlled by sales of wine and beer followed by cider and sake. Nowadays there is a huge variety of these products that mainly depends on the type and quality of substrate used, fermentation conditions, region of the world and manufacturing process. Over recent years, alcoholic fermented beverages-consumption has faced a duality. On the one hand, consumption

tend to increase due to social events and ever earlier consumption by adolescents, and on the other hand, consumption tends to decrease due to health concerns in modern society and low consumer purchasing power. The decline in consumption, relative to health concerns, is mainly caused by the scientific advances about the effects of alcohol and prevention campaigns to educate the population. The harmful effects of alcohol are much better known, however, recent findings regarding this subject showed that low and moderate alcohol intake enhanced health and well-being (Brányik *et al*., 2012). The major harmful effects of alcohol consumption are mostly accidents, violence and chronic alcohol abuse leading to chronic health and nutritional problems (Brányik *et al*., 2012; Room *et al*., 2005). Despite all these negative effects on the human body, alcohol continues to be consumed throughout the world and still dominates the market of fermented beverages.

The production of low-alcoholic fermented beverages has different historical reasons. During World Wars (1914-1918 and 1939-1945) there was a shortage of raw materials forcing the use of adjuncts and, such blends of substrates, led to the production of beverages with low alcohol content. Furthermore, in the years between 1919 and 1933 the prohibition to manufacture, sell and consume alcohol increased the production of this low- alcoholic kind of beverage.

In recent years, a new concept of low- and non-alcoholic fermented beverages arose, typically defined as containing an alcoholic strength greatly reduced or even inexistent when compared with alcoholic beverages. The production of low- and non-alcoholic fermented beverages is an alternative to soft drinks and alcoholic beverages in food industry and in spite of the fact that these type of beverages are a small percentage of the output of food industry, a significant growth of these products recently occurred, revealing the global trend for a healthier lifestyle (Brányik *et al*., 2012).

Low- and non-alcoholic fermented beverages market was based on the creation of healthier versions with reduced alcohol content from a variety of beers and wines. These versions of alcoholic fermented beverages claim beneficial effects on health with a simultaneous effect of the lower energy intake and minimization of negative impacts of alcohol consumption. In addition to historical reasons there are many other factors that contribute to the increase in demand for low-alcohol and alcohol-free beverages such health, safety, diet or even prohibition of alcohol consumption due to labor protection laws. In addition, these beverages are recommend for specific groups of people as pregnant woman, people with cardiovascular and hepatic pathologies, sporting professionals and medicated people (Brányik *et al*., 2012; Francesco *et al*., 2014; Pickering, 2000). The legal definition of low- and non-alcoholic beverage varies from one country to another and the final content of ethanol influences this distinct classification (Brányik *et al*., 2012; Francesco *et al*., 2014; Pickering, 2000). In Europe, a non-alcoholic or alcohol-free wine and beer will usually have a final alcohol by volume content lower than 0.5% v/v, whereas a low-alcohol wine and beer ethanol content is between 0.5 and 1.2% v/v. (Francesco *et al*., 2014; Pickering, 2000) Wines can also be classified as reduced-alcohol in which ethanol content is between 1.2 and 6.5% v/v (Pickering, 2000).

The commercialization of beverages with reduced or absent ethanol content have to overcome some technical and marketing challenges. Since this is a relatively recent market still exist many limitations at the quality and economic level which have to be evaluated and improved (Pickering, 2000).

1.2.2 Biotechnological application of specific yeasts to yield low-alcoholic and low-caloric fermented beverages

So far, two main strategies to produce reduced alcohol beverages have been proposed (see Table 1.2). The first relies on physical methods such as thermal, membrane, adsorption and extraction to remove alcohol from alcoholic beverage whereas the second involves biological methods such as controlled (suppressed) alcohol production and use of specific low-alcohol producing yeasts (Brányik *et al*., 2012; Francesco *et al*., 2014; Pickering, 2000). This number of techniques, within these two basic strategies, varies in performance, efficiency and usability (Pickering, 2000).

Table 1.2- Examples of strategies and methods used in low-alcoholic fermented beverages production (based on Brányik *et al*., 2012; Francesco *et al*., 2014; Pickering, 2000).

Strategy- Principle	Method
Physical - Removal of alcohol from alcoholic beverage	Thermal: distillation under vacuum or atmospheric pressure; evaporation; freeze concentration. Membrane: dialysis, reverse osmosis. Adsorption: resins, silica gel. Extraction: organic solvents, supercritical carbon dioxide
Biological - Low alcohol production	Controlled (suppressed) alcohol production Specific low-alcohol producing yeasts
Other- Reduction of fermentable sugar concentration in fruit or juice	Use of unripe fruit Juice dilution Freeze concentration and fractionation Enzymes (e.g. glucose oxidase)
Other- Reduction of alcohol content	Dilution of alcoholic beverage

All these strategies illustrated in Table 1.2 have advantages and disadvantages.

The most important advantages from physical methods regards the possibility of reducing ethanol content to very low values (≈0.05% v/v) however, these methods have high operating costs, loss of volatile compounds (important factors that contribute to taste and aroma of the final product) and capital spending on equipment (Francesco *et al*., 2014). Although dealcoholization constitute one of the most applied strategies to produce low-alcoholic beverages this is not by far

the one that produces the best results, at least in terms of costs and end product organoleptic characteristics (Pickering, 2000).

Other common way to make low- and non-alcoholic fermented beverages consists in monitoring alcohol formation at very low values by arrest of fermentation. The fermentation activity can be arrested (stopped or checked) quickly by temperature inactivation (cooling to 0ºC or pasteurization) and/or by removal of yeast from fermenting must (filtration or centrifugation). Fermentation arrest is a simple and widespread method, without additional costs because it uses the same resources as for standard alcoholic fermentation. Nonetheless, this suppression of fermentation also prevents formation of essential compounds important for flavour, affecting final product quality (Bránayik *et al*., 2012; Francesco *et al*., 2014; Pickering, 2000).

Another strategy to reduce alcohol content in beverages regards the reduction of fermentable sugar (glucose, fructose and sucrose) in fruit or fruit juice. Harvesting fruit at an early stage of maturation result in a beverage with low-alcohol content since unripe fruit have much lower sugar concentration. However, fermenting unripe fruit has its drawbacks, particularly with respect to the aromas, because it originates a product with high acid levels (Pickering, 2000). A method also used for reduction of fermentable sugar is freeze, concentration and fractionation which involves the separation of fruit juice into a high-sugar and low-sugar fraction by freezing, forming a slush. Low-sugar fraction supplemented with high-sugar fraction volatile compounds are fermented to produce low-alcohol beverages (Pickering, 2000). This method also implies specific equipment investment. Other methods used for alcohol content reduction involves dilution with water, reduced-alcohol or partially fermented beverage to correct sensory imbalances (Pickering, 2000).

Last strategy (biological) capable of producing fermented beverages with low-alcoholic and low-caloric content is the use of specific low-alcohol producing yeasts. This kind of approach is still under development and so it can be quite explored as a possibility for the future. This process requires a specific yeast able to convert sugars into other end-products reducing ethanol production. Over the past years several studies have been made regarding this particular subject to screen yeast strains that might be used to yield this type of beverages. One approach included *S.cerevisiae* genetic manipulation by diverting sugar metabolism into glycerol production reducing ethanol formation (Pickering, 2000). However, genetic modified yeasts generates controversy among consumers who have a negative attitude towards the use of these microorganisms in the food industry. Additionally to ethical obstacles, improvements in typical yeasts like *S.cerevisiae* increase the process costs due to the construction of intentional modified microorganisms capable of producing low alcohol content. Therefore, the screening of specific yeast strains capable of consuming fermentable sugars and naturally producing lower amounts of ethanol could be an excellent option to overcome deadlocks associated with microbial improvement (Bránayik *et al*., 2012). Although it is a relatively recent strategy, it is deemed a great alternative compared with the other methods because it is a biological technique that takes advantage of microbial natural fermentative activity and does not require any additional investment in specific

equipment. In addition to that, depending on the yeast it is possible to guide the fermentation process towards low ethanol production using sugars to yield other fermentation products such as sugar alcohols (glycerol, mannitol and erythritol) enriching the organoleptic properties of the final low–alcoholic and low–caloric beverage.

Pichia stipitis proved to be able to remove more than 50% of juice sugar with no need to add nutrients and with practically no adverse effects on sensorial qualities. It has also been reported that *Pichia stipitis* and *Candida tropicalis* when incubated under aerobic conditions produce 25-30% less alcohol compared with typical alcoholic fermentation yeast (*S.cerevisiae*) and the end product displays an acceptable taste (Pickering, 2000).

Due to greater information about the benefits and risks of certain foods, nowadays consumers are more concerned about health issues that may result from a poor diet. For this reason, they try to reconcile a healthy product, preferably without added preservatives, with high sensorial quality (Renuka *et al*., 2009). To try to satisfy this demand of modern society, besides grape juice there is a possibility of fermenting other fruits aiming to produce healthier versions of alcoholic beverages, taking advantage of those natural substrates for a healthy diet. Such beverages can offer to consumers excellent alternatives, satisfying nutritional and sensorial needs.

1.3 Fructophily phenomenon

1.3.1 Fructophilic behaviour basis and role of fructophilic yeasts

Fruits used to produce alcoholic or other beverages are composed by different types and concentration of sugars. Usually, in the production of these beverages, typical yeasts preferentially consume glucose compared with the other sugars.

The basis of the phenomenon of fructophily in yeasts was first investigated by Sols in 1956 (Sousa-dias *et al*., 1996). While most yeasts show a glucophilic behaviour such as *Saccharomyces cerevisiae* (a typical wine and beer yeast) preferentially fermenting glucose compared to other sugars, there are other yeasts which have an opposite behaviour (Leandro *et al*., 2013; Yu *et al*., 2008). For those microorganisms, when glucose and fructose are both available in the medium, fructose is utilized more rapidly than glucose. Such fructophilic behaviour is characteristic of specific yeasts which are called fructophilic yeasts (Sousa-dias *et al*., 1996).

Fructophilic character of these microorganisms might prove to be important since the fruits normally have higher content of fructose than glucose. This peculiar characteristic has been investigated and is believed to be mainly associated with membrane transporters specific for fructose. These transporters in the yeast membrane, increase cellular input of this sugar. In addition, fructophily can also be linked to hexokinase enzymatic activity. This enzyme is responsible for the phosphorylation of glucose into glucose-6-phosphate and fructose into

fructose-6-phosphate and different kinetic parameters for glucose and fructose may also explain fructose preference.

The preference of one sugar over the other appears to be related to the hexose transport and/or phosphorylation steps, since the metabolism of glucose and fructose from fructose-6-phosphate is exactly the same for these two sugars, as illustrated in Figure 1.3 (Liccioli *et al*., 2011). This figure represents the central sugar metabolism carried by yeast cells and highlights the differences during glucose and fructose metabolism.

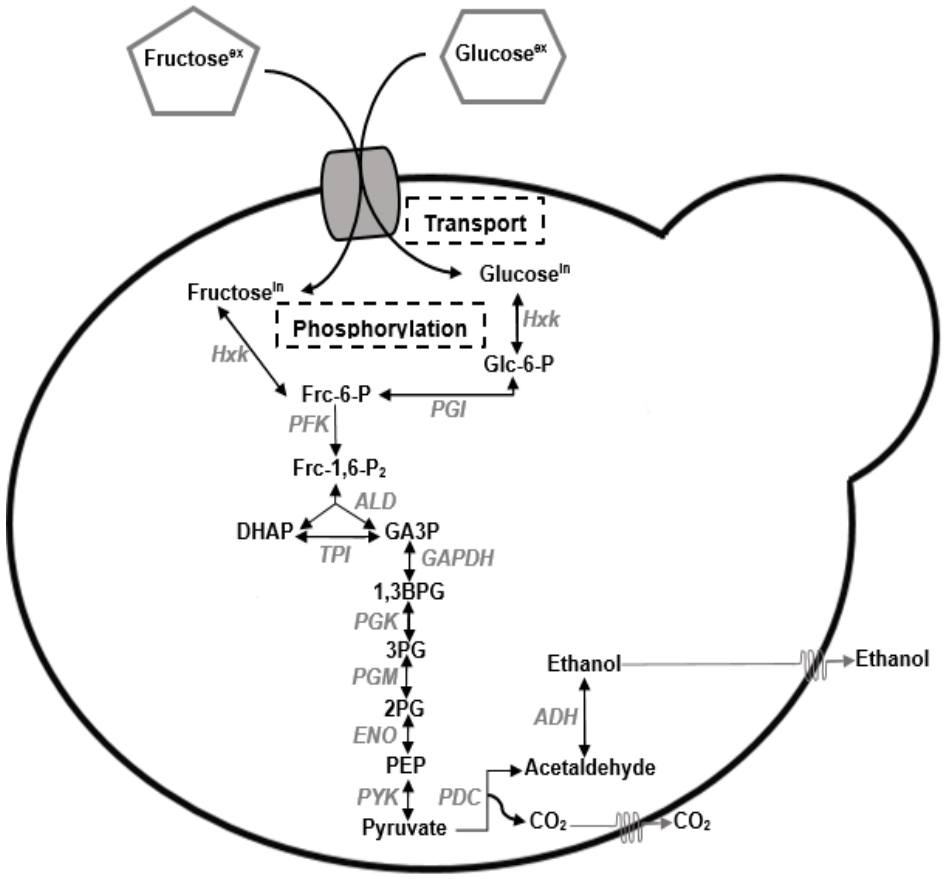


Figure 1.3- Representation of central sugar metabolism in yeast cells (typical microorganism *S.cerevisiae*) evidencing main steps that differs between glucose and fructose metabolism. Abbreviations: *Hxk* (hexokinase), Glc-6-P (Glucose-6-phosphate), Frc-6-P (Fructose-6-phosphate), *PGI* (Phosphoglucose isomerase), *PFK* (Phosphofructokinase), Frc-1,6-P_2 (Fructose-1,6-biphosphate), *ALD* (Aldolase), DHAP (Dihydroxyacetone phosphate), GA3P (Glyceraldehyde 3-phosphate), *TPI* (Triosephosphate isomerase), *GAPDH* (Glyceraldehyde-3-phosphate dehydrogenase), 1,3BPG (1,3-biphosphoglycerate), *PGK* (Phosphoglycerate kinase), 3PG (3-Phosphoglycerate), *PGM* (Phosphoglycerate mutase), 2PG (2-Phosphoglycerate), *ENO* (Enolase), PEP (Phosphoenolpyruvate), *PYK* (Pyruvate kinase), *PDC* (pyruvate decarboxylase), CO_2 (carbon dioxide) and *ADH* (Alcohol dehydrogenase) (based on Meier *et al*., 2011).

The group of fructophilic yeasts is relatively restricted and comprises *Zygosaccharomyces bailii*, *Zygosaccharomyces rouxii*, *Candida magnoliae*, *Starmerella bombicola* (or *Candida stellata*), *Candida zemplinina* (or *Starmerella bacillaris*) and *Hanseniaspora guilliermondii* (Yu *et al*., 2008). Yeasts with a fructophilic phenotype can be

isolated from different natural environments characterized by high sugar concentrations, where the main sugar present is fructose. Some examples of these environments are fruit juices and honeycombs. The discovery of these yeasts allowed a large number of studies to try to overcome one of the major problems in wine fermentation. *Saccharomyces cerevisiae* preference for consuming glucose before fructose can sometimes generate incomplete or stuck and slow or sluggish fermentations. Stuck fermentations are characterized by having higher than desired residual sugar content at the end of alcoholic fermentation and sluggish fermentations are defined as low utilization sugar rate fermentations (Liccioli *et al*., 2011).

Thus, during alcoholic fermentation carried by *S.cerevisiae* the consumption of both sugars follows a predetermined pattern with glucose content decreasing faster than fructose. This effect is reflected more clearly in the end of the fermentation where the discrepancy between glucose and fructose concentration is too high. Consequently, during the late stages of alcoholic fermentation fructose becomes the main sugar present since glucose was practically all consumed (Liccioli *et al*., 2011). The stress created from these conditions result in sluggish fermentations since yeasts, may became unable to ferment this sugar in the presence of large amounts of ethanol causing the termination of the fermentation.

Therefore, as a possible strategy to overcome this issue, a combination between glucophilic and fructophilic yeasts could be used, where the first ones dominate the early stage, consuming preferentially glucose, and the second takes over the later stage of fermentation process, depleting remaining fructose from the medium. Furthermore, non-*Saccharomyces* species might use fructose to produce other secondary metabolites that contribute to increased complexity of organoleptic features in end product.

1.3.2 Fructose membrane transporters

One of the main targets for fructose preference in some yeasts regards fructose transport carried by specific membrane transporters. These transporters are responsible for the rate limiting step of glycolytic flux and are capable of transporting the sugar from the outside to inside the cell. Hence, the fructophilic phenotype basis might reside in the performance of transport systems for hexose (Lee *et al*., 2013).

In most organisms, including yeasts, sugar transporters are crucial for supplying cells with energy and a source of carbon. Hexose transporters (Hxt) identified in *S.cerevisiae*, which mediate hexose (glucose and fructose) import, are membrane-spanning transport proteins (permeases) that transport sugar through passive, energy-independent facilitated diffusion down a concentration gradient. These transporters belong to the diverse Major Facilitator Superfamily. Sugar transporters, other than Hxt, that are members of this superfamily can operate via active proton symport mechanisms (Leandro *et al*., 2011). The kinetic characterization of *S.cerevisiae* low and high affinity transport systems revealed that affinity is always five or ten times higher for glucose than fructose. This affinity difference for glucose compared to fructose would support a

link between glucose preference and a glucophilic behaviour shown by *S.cerevisiae* (Liccioli *et al*., 2011; Pina *et al.*, 2004; Sousa-dias *et al*., 1996).

However, in some non-*Saccharomyces cerevisiae* yeasts such as *Zygosaccharomyces bailii* (*Z.bailii*), *Zygosaccharomyces rouxii* (*Z.rouxii*) and *Candida magnoliae* (*C.magnoliae*) transport systems with a clear preference for fructose have been recently characterized, suggesting the emergence of a new family of sugar transporters (Leandro *et al*., 2011; Leandro *et al*., 2013; Lee *et al*., 2013). Curiously, or not, these yeasts who have fructose-specific transporters exhibit a fructophilic behaviour, consuming fructose faster than glucose, and are called fructophilic yeasts (Pina *et al.*, 2004; Sousa-dias *et al*., 1996). This particular behaviour in these two *Zygosaccharomyces* species, mainly at high sugar content, is based on three different mechanisms: The high capacity of the specific fructose transporter; the competition of fructose and glucose for the hexose transport system; and the inactivation of the glucose facilitator by high fructose concentrations (Sousa-dias *et al*., 1996). The two main fructose-specific transport systems identified and characterized in these three fructophilic yeasts are Fsy1, a specific high-affinity, low-capacity energy-dependent H^+ symporter that mediates fructose transport, and Ffz1, a low-affinity, high-capacity facilitated diffusion system specific for fructose with a poor homology to other facilitated diffusion systems like Hxt family. Usually, sugar-proton symporters only operate when relatively low sugar content is available, where facilitated diffusion would not be efficient enough due to their low affinity, and are able to transport sugar against its concentration gradient simultaneously with the movement of protons. Facilitators are employed when sufficient amount of sugar is present, so the transported molecules are rapidly metabolized inside the cells and an efficient facilitated diffusion occurs due to maintenance of the inward gradient of sugar (Leandro *et al*., 2013).

Nevertheless, the first fructose-specific transporters were identified and characterized in non-fructophilic yeasts and fungi. The first high-affinity fructose-specific symporter (*Sp*Fsy1) characterized was from *Saccharomyces pastorianus* PYCC 4457, the type strain of *S. carlsbergensis*. Later, other high-affinity fructose symporters were found in the aerobic milk yeast *Kluyveromyces lactis* (*Kl*Frt1) and the gray mold *Botrytis cinerea* (*Bc*Frt1). More recently, a high-affinity fructose symporter (*Sc*Fsy1) from the commercial wine yeast *S.cerevisiae* EC 1118 was reported. Moreover, three other fructose-specific transporters were characterized from *Zygosaccharomyces bailii* (*Zb*Ffz1) *and Zygosaccharomyces rouxii* (*Zr*Fsy1 and *Zr*Ffz1), which in contrast with the previous these two are fructophilic yeasts. Recently, two fructose-specific transporters from the fructophilic yeast *Candida magnoliae* JH110 (*Cm*Fsy1 and *Cm*Ffz1) were also identified and characterized (Lee *et al*., 2013). The kinetic parameters of these yeasts and fungi fructose-specific transporters are illustrated in Table 1.3.

Table 1.3- Kinetic parameters of fructose-specific transporters characterized in yeasts and fungi (based on Lee *et al*., 2013).

Species	Transporter	Function	K_m (mM)	V_{max} (mmol.h^{-1}.(gdw)$^{-1}$)	Reference
Saccharomyces pastorianus PYCC 4457	*Sp*Fsy1	H^+ symporter	0.16±0.02	3.8±0.2	(Gonçalves *et al*., 2000).
Kluyveromyces lactis	*Kl*Frt1	H^+ symporter	0.16±0.02	0.10±0.02	(Diezemann and Bloes, 2003)
Botrytis cinerea	*Bc*Frt1	H^+ symporter	1.1	0.66	(Doehleman n *et al*., 2005).
S.cerevisiae EC 1118	*Sc*Fsy1	H^+ symporter	0.24±0.04	0.93±0.08	(Galeote *et al*., 2010).
Zygosaccharomyces bailii	*Zb*Ffz1	Facilitator	80.4	3.3	(Pina *et al.*, 2004)
Zygosaccharomyces rouxii	*Zr*Fsy1	H^+ symporter	0.45±0.07	0.57±0.02	(Leandro *et al*., 2013)
Zygosaccharomyces rouxii	*Zr*Ffz1	Facilitator	424.2±16 3.1	12.7±3.3	(Leandro *et al*., 2011)
Candida magnoliae JH110	*Cm*Fsy1	H^+ symporter	0.13±0.01	2.1±0.3	(Lee *et al*., 2013).
Candida magnoliae JH110	*Cm*Ffz1	Facilitator	105±12	8.6±0.4	(Lee *et al*., 2013).

Despite these fructose transporters were first found in non-fructophilic yeasts, the majority of yeasts harboring such transporters exhibit fructophilic behaviour and the focus will be about them.

Zygosaccharomyces spp. has extreme osmotolerance properties and that is why they can be isolated from high-sugar and high-salt content environments such as sugar syrups, honey, fruit juices, carbonated soft drinks, sauces, salad dressing and ketchup. In some studies, *Z. bailii* and *Z.rouxii* are characterized as food-spoilage yeasts since they are able to survive and grow under harsh conditions such as those present in preserved food and beverages: low water activity, low pH and tolerate high temperatures that are restrictive for most yeasts (Leandro *et al*., 2011). These two *Zygosaccharomyces* species exhibit an abnormal resistance to common preservatives like sulphur dioxide, ethanol and acetic acid and have a high potential for the synthesis of

undesired by-products and off-flavours like acetic acid or acetaldehyde (Leandro *et al*., 2013; Pina *et al.*, 2004). On the other hand, *Candida spp*. are a heterogeneous genus of yeasts, containing endosymbionts of animal hosts, commensals of the skin, the gastrointestinal and the genitourinary tracts, plant pathogens as well as species utilized in the refinement of food and beverages. *Candida magnoliae* is a species from this genus that is able to grow over a wide range of pH values, under high sugar concentrations and also have a different fermentative profile, producing sugar alcohols instead ethanol (Lee *et al*., 2013).

In *Z. bailii* and *Z.rouxii*, sugar transporters, designed as Ffz (**F**ructose **F**acilitator of **Z**ygosaccharomyces) have been identified and characterized. *Zr*Ffz1 and *Zr*Ffz 2 transporters in *Z.rouxii* are two similar low-affinity high-capacity facilitators that transport specifically fructose or both fructose and glucose, respectively (Leandro *et al*., 2013). *Zr*Ffz1 transports fructose with a K_m of 400mM and a V_{max} of 13 mmol.h^{-1}.g^{-1}, while *Zr*Ffz2 transports glucose and fructose with similar affinity, K_m of 200mM and capacity, V_{max} of 4 mmol.h^{-1}.g^{-1}, values (Leandro *et al*., 2011). A study about the transport systems in *Z. bailii* demonstrated that fructose was transported by a specific low-affinity, high-capacity transport system with a K_m of 65.6mM and a V_{max} of 6.7mmol.g^{-1}.h^{-1} (Sousa-dias *et al*., 1996). Another investigation, showed for this yeast that fructose uptake involve facilitated diffusion mechanisms and is carried by a high-capacity, low-affinity transporter specific for this sugar and a second system that transports with low-capacity and high-affinity sugars like glucose, fructose and 2-deoxyglucose. The fructose-specific transporter Ffz1 (permease) exhibited a K_m of 80.4mM and V_{max} of 3.3 mmol.h^{-1}.g^{-1} and was the first known facilitated diffusion system specific for fructose (Pina *et al.*, 2004). These three proteins, *Zr*Ffz1, *Zr*Ffz2 and *Zb*Ffz1, belong to a new family of sugar transport systems capable of mediating the uptake of hexoses via facilitated diffusion mechanism and their primary protein structure have more homology with drug/H$^+$ antiporters than with the other yeast sugar transporters members of the Major Facilitator Superfamily (Leandro *et al*., 2011; Leandro *et al*., 2013). *Zygosaccharomyces rouxii* also has a high-affinity, low capacity fructose/H$^+$ symporter called *Zr*Fsy1. Kinetic parameters of this transporter revealed a K_m of 0.45±0.07mM and a V_{max} of 0.57±0.02 mmol.h^{-1}.(gdw)$^{-1}$ (Leandro *et al*., 2013). The existence of this fructose/H$^+$ symporter was unexpected since *Z.rouxii* import sugars through low-affinity, high-capacity facilitators (*Zr*Ffz1 and *Zr*Ffz2) without spending energy on symport with protons. Thus, in environments with low sugar concentrations the yeasts invest energy in transport through active maintenance of the proton motive force, whereas when sugar concentration is high the concentration gradient across the plasma membrane is enough to maintain an active catabolism. In contrast with the two facilitators, *Zr*Fsy1 is phylogenetically related with the other sugar transporters that belong to the Major Facilitator Superfamily and is closely related to the other already characterized specific fructose/H$^+$ symporters (Leandro *et al*., 2013).

In the osmotolerant and fructophilic yeast *Candida magnoliae* JH110 two fructose-specific transporters, *Cm*Fsy1 and *Cm*Ffz1 were also identified and characterized, which demonstrate

high homology with known fructose transporters of other yeasts such as *Z.rouxii* (*Zr*Fsy1, *Zr*Ffz1, *Zr*Ffz2) and *Z.bailii* (*Zb*Ffz1), described above (Lee *et al*., 2013). Kinetic analysis showed that *Cm*Fsy1 is a high-affinity, low-capacity fructose-proton symporter with a K_m of 0.13±0.01mM and a V_{max} of 2.1±0.3 mmol.h^{-1}.(gdw)$^{-1}$, while *Cm*Ffz1 is a low-affinity, high-capacity fructose-specific facilitator with a K_m of 105±12mM and a V_{max} of 8.6±0.7 mmol.h^{-1}.(gdw)$^{-1}$. *Cm*Ffz1 along with *Zr*Ffz1, *Zr*Ffz2 and *Zb*Ffz1 form a new fructose transport family. As for *Z.rouxii,* when *C.magnoliae* JH110 is under low fructose concentration environments utilizes a proton motive force for fructose transport through the *Cm*Fsy1 transporter whereas, when fructose is present in high amounts the yeast transport this sugar through facilitated diffusion employing the *Cm*Ffz1 transporter (Lee *et al*., 2013).

1.3.3 Sugars phosphorylation by hexokinase enzyme activity

As already mentioned, one of the main steps that differs between glucose and fructose metabolism is the phosphorylation carried out by the enzyme hexokinase. Thus, another further likely target for fructose preference, apart from transport, is the hexose phosphorylation the first step of sugar metabolism. This is one of the key steps associated with the regulation of the fermentative metabolism in yeasts. Once the sugars (hexoses) such as glucose and fructose have been imported into the cell, hexokinase is responsible for phosphorylating them into their hexose-6-phosphate form and for this makes use of ATP as a phosphate group donor cofactor.

The yeast *S.cerevisiae* have three distinct isozymes involved in glucose phosphorylation, hexokinase I and II (Hxk I and II) and glucokinase. In a fermentation environment, hexokinase type II appears to play the main role during sugar phosphorylation, since it is the predominant enzyme during growth on glucose or fructose (Liccioli *et al*., 2011). Kinetic parameters values of *S.cerevisiae* Hxk I and II (Table 1.4) illustrate that affinity for fructose is the same in both enzymes but the affinity for glucose is slightly different between these two hexokinases. K_m values of Hxk I and II show that is 13 and 6 times higher for fructose, respectively. As K_m values are inversely proportional to affinity this indicate that Hxk I and II have a higher affinity for glucose. In terms of capacity, an interesting data is that Hxk I has a threefold higher V_{max} with fructose as a substrate than with glucose, while for Hxk II glucose and fructose V_{max} values are quite similar. Therefore, the higher affinity for glucose instead fructose displayed by *S.cerevisiae* hexokinases may play a critical role on the development of the glucophilic behaviour of this yeast (Berthels *et al*., 2008).

Table 1.4- Kinetic parameters, K_m and V_{max}, of *Saccharomyces cerevisiae* hexokinase I and II (based on Berthels *et al*., 2008).

Hexokinase	K_m (mM)		$K_{m\ Fruc}/K_{m\ Gluc}$	$V_{max\ Fruc}/V_{max\ Gluc}$
	Glucose	Fructose		
Hxk I	0.12	1.5	13	3
Hxk II	0.25	1.5	6	≈1

As already mentioned, enzymatic phosphorylation of sugars, glucose and fructose, has been proposed as one of the two possible triggers for the development of phenotypical hexose preference in yeasts. Although the enzyme responsible for this first step of sugar metabolism is the same, hexokinase, its activity might be modelled in terms of affinity and capacity depending on the substrate. Thus, the preference for phosphorylating a sugar rather than the other can be reflected on the final behaviour of the yeast, being glucophilic or fructophilic.

1.4 *Candida magnoliae*

1.4.1 Yeast with unusual properties

The osmotolerant yeast *Candida magnoliae* has very peculiar characteristics compared to other typical yeasts, facts that make it very interesting for industrial uses (Park *et al*., 2011; Yu *et al*., 2008). One of the particularities of this yeast is its fructophilic behaviour, i.e. in a mixture of sugars, it is able to consume fructose quickly compared to other sugars. This yeast is also able to grow under a wide range of pH values (at least between 2.5 and 8.0) in the presence of high concentrations of sugar (≥300g/L glucose and fructose) and can produce organic acids and sugar alcohols like erythritol, mannitol and glycerol (Kim *et al*., 2013; Yu *et al*., 2008). The nature, composition and concentration of media constituents directly influence the composition of sugar alcohols produced by this yeast (Savergave *et al*., 2011). This ability to withstand high solute content (hyper-osmotic environments) namely high sugar concentrations is called osmotolerance (Kim *et al*., 2013; Savergave *et al*., 2011; Yu *et al*., 2008). As mentioned above, *C.magnoliae* is able to produce sugar alcohols that are also found naturally in fruits and vegetables in small quantities. These compounds are a class of polyols in which the sugar's carbonyl (aldehyde or ketone) is reduced to the corresponding primary or secondary hydroxyl group therefore, they have characteristics similar to sugar and are used to improve the nutritional profile of food products (Akinterinwa *et al*., 2008).

Erythritol is a sugar alcohol that comprises four carbon atoms, has a pleasant taste, is non-cariogenic, since it cannot be fermented by dental caries-producing bacteria, and lack of insulin-stimulating properties, hence its use as a low-calorie sweetener (0.3 $kcal.g^{-1}$) and pharmaceutical excipient safe for diabetics (Park *et al*., 2011; Yu *et al*., 2006; Yu *et al*., 2008). This sugar alcohol is allowed to be used as a flavor enhancer, formulation aid, humectant, non-

nutritive sweetener, stabilizer, thickener, sequestrant and texturizer at maximum levels of 100% in sugar substitutes (Savergave *et al*., 2011). Animal studies showed that erythritol is almost entirely absorbed systemically, is not metabolized and is excreted unchanged in the urine. This sugar alcohol has about 70 to 80% of the sweetness of sucrose and occurs as a metabolite or storage compound on seaweeds and mushrooms and also composes a number of fruits like melons, grapes and pears. Erythritol also often occurs in fermented foods like cheese, wine, beer, soy sauce and miso (Park *et al*., 2011; Ryu *et al*., 2000; Yu *et al*., 2008). In biological production of erythritol by yeasts, Figure 1.4, sugars (glucose and fructose) are phosphorylated and routed though Pentose Phosphate Pathway for the production of erythrose-4-phosphate which is further dephosphorylated into erythrose by the erythrose-4-phosphate kinase enzyme. Then a NAD(P)H-dependent erythrose reductase catalyzes the hydrogenation of erythrose into erythritol (Moon *et al*., 2010; Park *et al*., 2011).

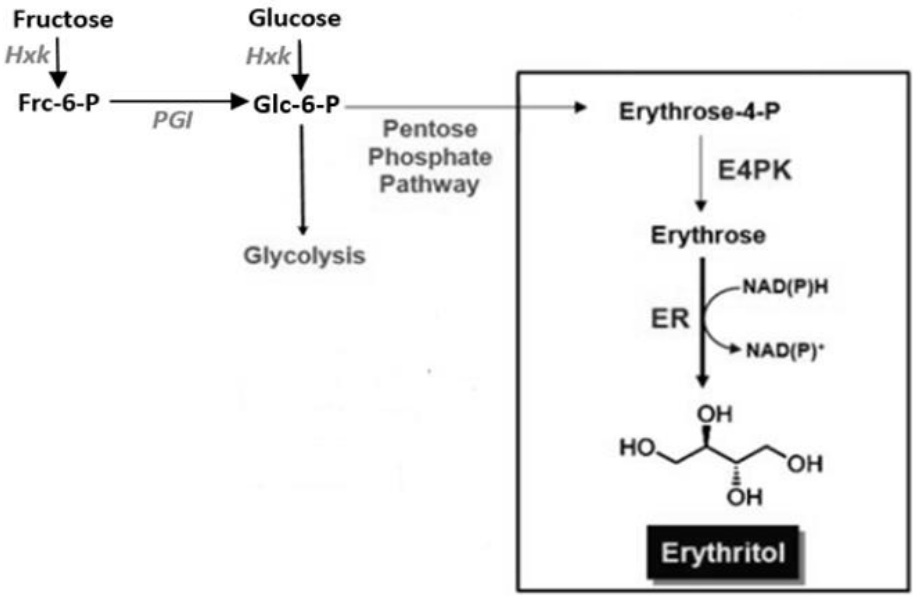


Figure 1.4- Pathway of erythritol biosynthesis in yeast. Abbreviations: *Hxk* (hexokinase), Frc-6-P (Fructose-6-phosphate), *PGI* (Phosphoglucose isomerase), Glc-6-P (Glucose-6-phosphate), E4PK (Erythrose-4-phosphate kinase), ER (Erythrose Reductase), NAD(P)H (Nicotinamide adenine dinucleotide phosphate reduced form) and $NAD(P)^+$ (Nicotinamide adenine dinucleotide phosphate oxidized form) (adapted from Moon *et al*., 2010).

In addition to erythritol, *Candida magnoliae* is also able to produce another sugar alcohol that is the most abundant in nature, mannitol, which is a fructose-derived six-carbon polyol (Song and Vieille, 2009). As well as erythritol, mannitol has gained at a commercial level immense importance as versatile and valuable ingredient for food and pharmaceutical industries (Saha and Racine, 2011). This compound is considered a low-calorie and low-cariogenic sweetener since it has about half the sweetness of sucrose, gives a pleasant taste, exhibits diuretic action and is not metabolized by the human body (Saha and Racine, 2011; Song and Vieille, 2009). Furthermore, its natural occurring form produced by bacteria, yeasts, fungi, algae, lichens and many plants

plays an important role in growth, carbon storage and fixation, proteins and cells protection against different stress conditions such as heat or osmotic changes due to its function as a compatible solute and free radical scavenger (Song and Vieille, 2009). Mannitol synthesis by microorganisms is performed by two main routes, as illustrated in Figure 1.5: the first one directly reduces fructose into mannitol by an NAD(P)H-dependent mannitol dehydrogenase, while in the second route the phosphorylation of fructose to fructose-6-phosphate by hexokinase occurs followed by the reduction to mannitol-1-phosphate by an NAD(P)H linked mannitol-1-phosphate dehydrogenase enzyme. Then the mannitol-1-phosphate is converted into inorganic phosphate and mannitol by the action of a mannitol-1-phosphatase. Mannitol metabolism is thought to be involved in growth regulation through a possible control of the cellular NADP/NADPH ratio since its oxidation produces NAD(P)H. A co-substrate such as glucose is needed for growth, to regenerate the reduced cofactor required in the reaction and to supply metabolic maintenance energy (Lee *et al*., 2003; Saha and Racine, 2011; Savergave *et al*., 2013; Song and Vieille, 2009).

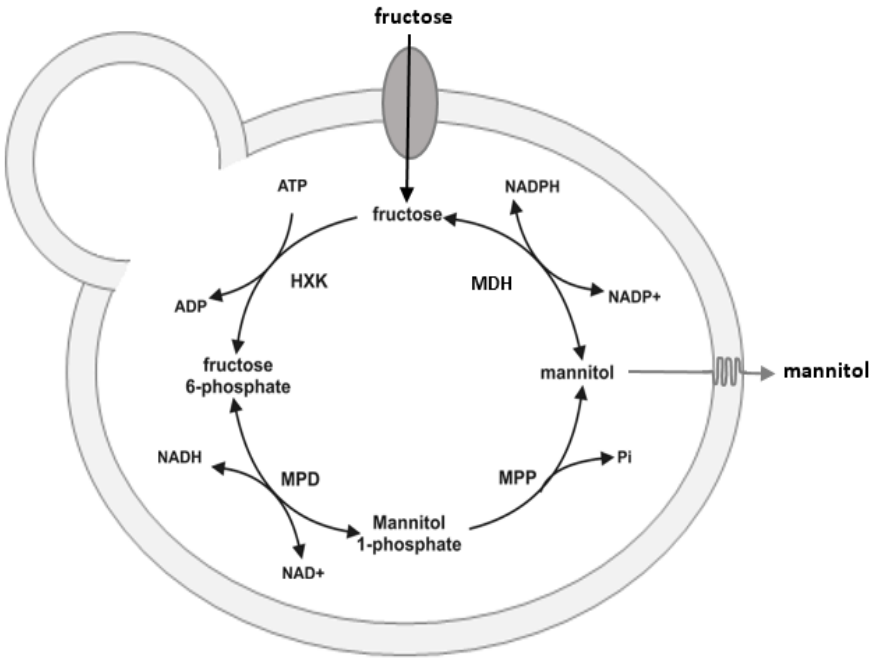


Figure 1.5- Pathway of mannitol biosynthesis in yeast. Abbreviations: HXK (hexokinase), ATP (Adenosine triphosphate), ADP (Adenosine diphosphate), NADH (Nicotinamide adenine dinucleotide reduced form), NAD^+ (Nicotinamide adenine dinucleotide oxidized form), MPD (mannitol 1-phosphate dehydrogenase), MPP (mannitol-1-phosphate phosphatase), Pi (inorganic phosphate), MDH (mannitol dehydrogenase), NADPH (Nicotinamide adenine dinucleotide phosphate reduced form) and $NADP^+$ (Nicotinamide adenine dinucleotide phosphate oxidized form) (based on Akinterinwa *et al*., 2008).

Candida magnoliae is also capable of producing glycerol, other sugar alcohol that plays an essential role in cell metabolism mainly in osmoregulation as a compatible solute and in redox balancing. Glycerol has many applications in food and other industries (Sahoo and Agarwal, 2002; Wang *et al*., 2001). The microbial glycerol biosynthesis pathway, shown in Figure 1.6, demonstrates that glycerol is a by-product of sugar fermentation to ethanol. During the production

of glycerol, the role of NADH consumed is to maintain the cytosolic redox balance particularly under anaerobic conditions, offsetting the production of NADH from cellular reactions. The rate and yield of glycerol production is affected by environmental factors as temperature, aeration, sugar concentration and osmotic stress (Sahoo and Agarwal, 2002; Wang *et al*., 2001).

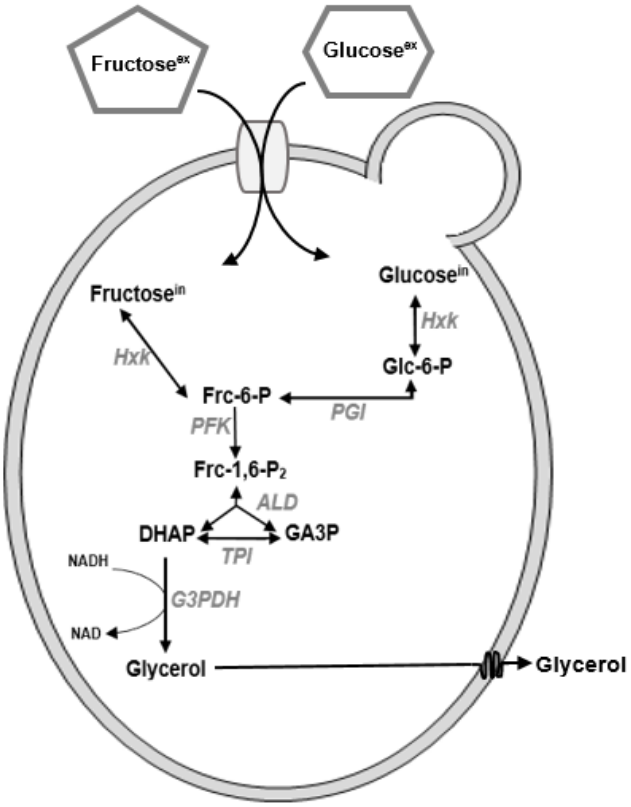


Figure 1.6- Pathway of glycerol biosynthesis in yeast. Abbreviations: *Hxk* (hexokinase), Glc-6-P (Glucose-6-phosphate), Frc-6-P (Fructose-6-phosphate), *PGI* (Phosphoglucose isomerase), *PFK* (Phosphofructokinase), Frc-1,6-P_2 (Fructose-1,6-biphosphate), *ALD* (Aldolase), DHAP (Dihydroxyacetone phosphate), GA3P (Glyceraldehyde 3-phosphate), *TPI* (Triosephosphate isomerase), NADH (Nicotinamide adenine dinucleotide reduced form), NAD (Nicotinamide adenine dinucleotide oxidized form) and *G3PDH* (Glyceraldehyde 3-phosphate dehydrogenase) (based on Wang *et al*., 2001).

1.4.2 Biotechnological application of *Candida magnoliae* to yield low-alcoholic and low-caloric fermented beverages from fructose-rich substrates

The yeast *Candida magnoliae* displays unique properties that may be exploited with the aim of applying them at a technological level. Taking advantage of the growing market for low-alcoholic beverages it is possible look at new approaches concerning this subject, aiming to improve process efficiency and the desired features of the final product. These new approaches can overcome the impasses caused by most of the methods used in the manufacture of such beverages. One of the most essential features of this yeast is its fructose consumption preference instead glucose, a completely opposite behaviour when compared with the majority of these microorganisms. Such preference is quite interesting mainly because this yeast is able to ferment

substrates in whose composition fructose prevails. Considering that, industrial fructose-rich substrates as fruit juices can be used as fermentation media, since it is constituted by fermentable sugars.

Fruits are a source of key nutrients, fibres and protective substances providing general well-being, satiety, maintenance of a balanced diet and highest energy intake. Besides this, fruit is associated to prevention of many diseases because it contains compounds (vitamins, minerals and antioxidants) known as nutraceuticals capable to have simultaneously nutritional and therapeutical activity (Bach-Faig *et al*., 2011; Kalt *et al*., 1999; Renuka *et al*., 2009). In fruits a high water content is also present which makes them very interesting to consume in the form of juice (Kalt *et al*., 1999; Mayer, 1997). Fruit and fruit products also have in their composition natural sugars such as glucose (monosaccharide), fructose (monosaccharide) and sucrose (disaccharide). However, the relative sugar composition of juice varies according to the fruit type and species, with maturity and as a result of environmental and climatic conditions of the growing season (Llamas *et al*., 2011; Sanz *et al*., 2004). In Table 1.5 the variation of fructose, glucose and sucrose content in different fruit juices is represented. Carbohydrates presence along with ascorbic acid, also present in a large variety of commercial fruit juices, can influence for example the pH, total acidity and sweetness, modifying chemical and sensory features of the product. Hence it is always necessary an adequate control to infer about authenticity and quality of the fruits used in food manufacturing (Llamas *et al*., 2011).

Table 1.5- Contents (g/100mL) of fructose, glucose and sucrose in some of the most consumed fresh fruit juices worldwide (from Sanz *et al*., 2004).

Sample	Fructose	Glucose	Sucrose	Glucose/fructose
Grapefruit	2.73 ± 0.01	2.66 ± 0.05	2.21 ± 0.03	0.97
Lemon	0.52 ± 0.04	0.50 ± 0.05	0.08 ± 0.01	0.96
Lime	1.18 ± 0.40	1.41 ± 0.3	0.26 ± 0.02	1.19
Mandarin	2.44 ± 0.07	2.22 ± 0.05	6.16 ± 0.46	0.91
Orange	1.90 ± 0.09	1.75 ± 0.10	4.71 ± 0.38	0.92
Grape	11.4 ± 0.06	9.72 ± 0.01	0.03 ± 0	0.85
Bilberry	4.91 ± 0.36	4.80 ± 0.30	0	0.98
Strawberry	2.08 ± 0.02	1.82 ± 0.01	1.73 ± 0.10	0.88
Guava	2.74 ± 0.26	0.95 ± 0.08	0.57 ± 0	0.35
Kiwifruit	5.33 ± 0.24	4.79 ± 0.23	0.66 ± 0.03	0.90
Apple	8.67 ± 0.30	2.62 ± 0.09	2.04 ± 0.10	0.30
Mango	2.47 ± 0.15	0.26 ± 0.01	4.50 ± 0.06	0.11
Peach	1.05 ± 0.08	0.69 ± 0.01	9.60 ± 0.40	0.66
Pear	11.2 ± 4.10	1.35 ± 0	0.26 ± 0.05	0.12
Pineapple	3.86 ± 0.19	3.51 ± 0.08	0.99 ± 0.07	0.91
Raspberry	2.51 ± 0.13	2.18 ± 0.14	0	0.87
Banana	3.54 ± 0.23	5.62 ± 0	13.9 ± 0	1.59
Redcurrant	4.46 ± 0.18	3.66 ± 0.14	0	0.82

From Table 1.5 it is possible to visualize that except for lime and banana, fructose content is always higher compared to glucose which is easily shown in glucose/fructose ratio being always lower than 1. Fruit juices which have a larger difference between fructose and glucose content

are guava, apple, mango, peach and pear. Besides the variation between glucose and fructose, sucrose also shows different concentration patterns depending on the fruit. Higher values for fructose compared to sucrose are displayed for most fruits excluding mandarin, orange, mango, peach and banana (Sanz *et al*., 2004). Considering these data, the majority of fruit juices consumed worldwide are excellent fermentable substrates but with fructose being the main sugar. Hence, fruit juices can be used as a substrate for fermentation maintaining its nutritional value, flavor and color even after the process, allowing the obtention of a low-caloric end product with significant quality.

Therefore, it might be practicable to use the behaviour of *Candida magnoliae* to ferment fructose, as well as the other sugars, to yield low-calory fermented beverages. In addition to that, is the ability of this yeast to direct the fermentation process to sugar alcohol formation reducing ethanol production. Also taking advantage of this feature there is a potential to apply *C.magnoliae* as a biological strategy to yield low-alcoholic fermented beverages.

The absence or presence of low-alcohol content is the main condition to produce low-alcoholic beverages however, such as alcoholic beverages these must meet certain requirements such as stability and organoleptic/sensory quality that depends on the complex balance between flavor, color, body and viscosity. Sugar alcohols produced by this yeast play an important role in achieving organoleptic characteristics required for the quality of the product. Given the reduced or absent ethanol production at the end of the fermentation process carried by this specific yeast there's no need for a dealcoholization step so there will be no loss of volatile compounds which are very important for flavor.

Thus, the implementation of this biological strategy might be an outstanding possibility since it does not require additional costs for equipment, makes use of accessible natural substrates like fruit juices which provide all compounds needed for fermentation, is based on a biological method equal to that used since thousands of years ago employing a microorganism and it can produce low-alcoholic and low-caloric fermented beverages with pleasant sensorial features.

2. Materials and Methods

2.1 Yeast strains

Two strains of *Candida magnoliae*, PYCC 2903 (CBS 166) isolated from the flower Magnolia sp. in Netherlands and PYCC 3191 (CBS 2677) isolated from concentrated orange juice with very high bisulphite content in South Africa, were used in this study. These strains belong to Portuguese Yeast Culture Collection (PYCC) that is associated to the Centre for Microbial Resources (CREM) and housed in the Department of Life Sciences (DCV) of "Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa" (FCT/UNL, Caparica).

2.2 Growth and fermentation conditions

2.2.1 Pre-inoculum preparation

Yeasts cells were routinely grown on YPD agar (1% Yeast Extract, 2% Peptone, 2% Glucose and 2% Agar) for 2 days at 25ºC.

Pre-inoculum was prepared in a 2.5L Erlenmeyer's flask containing 500 mL YPD medium inoculated to $O.D_{640nm}$≈0.1. Cultures were incubated at 25ºC, in an orbital (140 rpm) shaker until $O.D_{640nm}$ ≈ 15. Cells were harvested by centrifugation (3000 x g during 3min at room temperature), the supernatant was discarded and cells were washed (2x) with sterile bi-distilled water and resuspended in 1mL of sterile water.

2.2.2 Growth on YP medium with different sugar concentrations

Yeast were grown in 500 mL Erlenmeyer flasks containing 100 mL YP medium with different concentrations of glucose and fructose (duly indicated in results Part I) inoculated to $O.D_{640nm}$≈0.1. Cultures were incubated at 25ºC, either in an orbital shaker (185 rpm) or were kept, without agitation, to provide different oxygenation conditions. At specific time intervals, 1000 µL sample for posterior HPLC analysis were collected and 100 µL for serial dilutions in sterile water for optical density measurement.

2.2.3 Fermentation on YP medium with different ratios between sugars

Previously prepared pre-inoculum was added to Erlenmeyer flasks containing YP medium with 10% sugar but each flask with a different mixture of glucose: fructose (duly indicated in results Part I). To create different oxygen conditions, inoculum was added to 250 mL flasks with 50 mL of medium (aerobiose) or to 50 mL flasks with 50 mL of medium (oxygen limitation) and incubated, respectively in an orbital shaker (185 rpm) or kept without agitation. At specific time intervals, 1000 µL samples for posterior HPLC analysis and 100µL for serial dilutions in water for optical density measurement were collected.

2.2.4 Fruit juices used for fermentation

Juices fermentation assays were performed using four different fruit juices, graciously supplied by Sumol+Compal, which have different sugar concentrations and ratios as shown in Table 2.1.

Table 2.1- Sugar composition of fruit juices used for *Candida magnoliae* fermentation.

Juice	Sugar ($g.L^{-1}$)	Sugar (%)	Total sugar (%)
Orange	Sucr(52)+Gluc(22)+Fruc(24)	Sucr(5.2)+Gluc(2.2)+Fruc(2.4)	9.8
Apple	Sucr(11)+Gluc(26)+Fruc(63)	Sucr(1.1)+Gluc(2.6)+Fruc(6.3)	10
Pear	Sucr(10)+Gluc(13)+Fruc(59)	Sucr(1)+Gluc(1.3)+Fruc(5.9)	8.2
Peach	Sucr(73)+Gluc(17)+Fruc(13)	Sucr(7.3)+Gluc(1.7)+Fruc(1.3)	10.3

2.2.4.1 Juices preparation and initial control

Juices were diluted with sterile water until ºBrix value around 11.4. Degrees Brix (ºBrix) are used for total soluble solids (TSS) determination and were measured with an Abbe pocket refractometer Pal-1 ATAGO with a 0.05% graduations Brix scale at 20ºC. Apparatus calibration was made with sterile bi-distilled water.

For initial control, 1000µL samples were collected from juices substrates, centrifuged (13000 x g during 5min at room temperature), to remove the fruit pulp, and the supernatants were used for the ºBrix measurements. After that, supernatants were stored at -20ºC for later HPLC analysis. Before inoculation, initial juice pH was measured and titrated.

2.2.4.2 Juice inoculation and fermentation

Pre-inoculum was prepared using the same method described in 2.2.1 but in 500 mL Erlenmeyer flask. Fermentations were carried out in 500 mL Erlenmeyer's flasks containing 200 mL fruit juice.

In contrast to prepared synthetic culture medium, fruit juices have sucrose in their composition which is not easily consumed by *Candida magnoliae* leading to longer/incomplete fermentations. To solve this problem, juice was supplemented with 0.1µL/mL (0.24U/mL) of the enzyme invertase (Invertin MERCK® E1103), which catalyses the hydrolysis of sucrose into glucose and fructose. Cultures were incubated at 25ºC with gentle stirring, using stir bars to provide conditions for fermentation but also some homogenization of a very pulpy medium. At specific time intervals, 1000 µL samples were collected and centrifuged at 13000 x g for 5 minutes to remove the fruit pulp, and supernatants were used to measure ºBrix. Supernatants were then stored at -20ºC for later HPLC analysis.

At the beginning and end of fermentation, to control the number of viable cells, 100µL samples were collected for serial decimal dilutions with sterile water and 200µL of the 10^{-4}, 10^{-5} and 10^{-6} (beginning) and 10^{-6}, 10^{-7} and 10^{-8} (end) dilutions plated in YPD agar medium plates followed by incubation at 25ºC during 2 days.

2.2.4.3 Fermented juice pasteurization, bottling and sensorial evaluation

At the end of fermentation, the beverage was pasteurized by incubation in a thermostated water bath just for the time necessary to attained 76ºC. After pasteurization, ascorbic acid was added to the fermented juice before packaged in glass bottles and stored at 4ºC. For final control, fermented juice pH was measured and titrated. Subsequently, an organoleptic evaluation of the final product, in terms of texture, smell and taste was made.

2.3 Analytical methods: Quantification by HPLC

Sugars and alcohols were analysed by High-Performance Liquid Chromatography (HPLC). Supernatants stored at -20ºC were diluted 2, 5 or 10 times with bi-distilled water depending upon the sugar concentration. Diluted sample was then filtered through a 0.22 µm cellulose acetate membrane (CHROMAFIL PET-20/15MS) before injection (30 µL). HPLC operation mode is schematized in Figure 2.1.

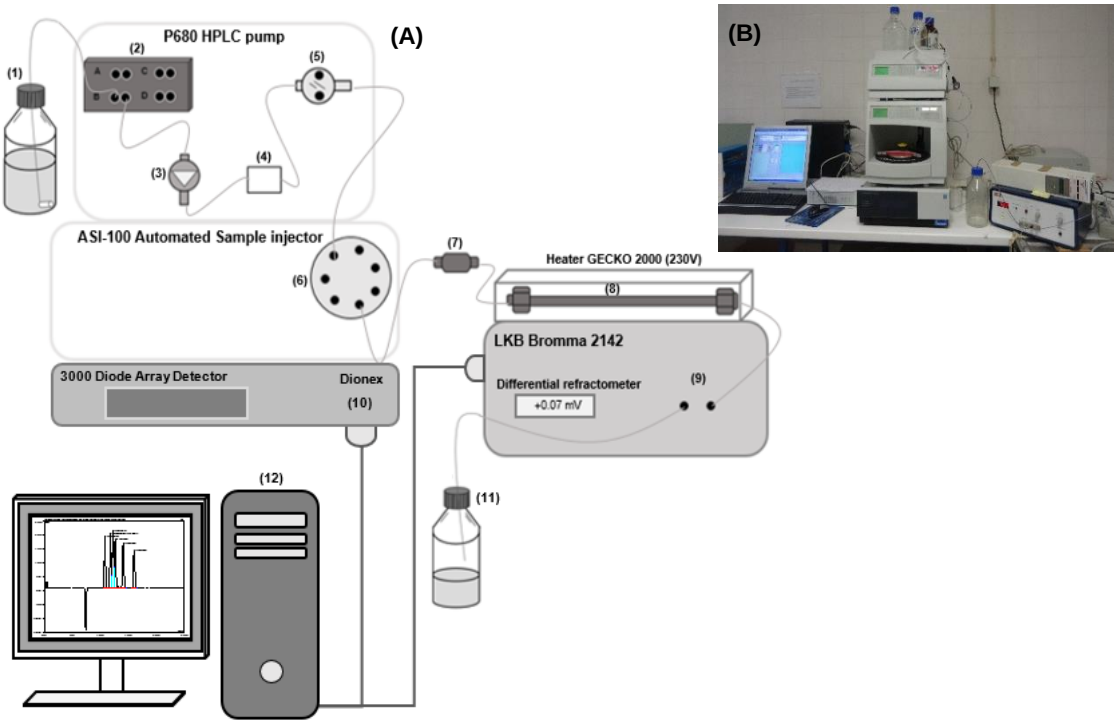


Figure 2.1- (A) Schematic diagram of a High-Performance Liquid Chromatography. (1) Eluent reservoir, (2) Eluent degasser, (3) Gradient valve, (4) Mixing vessel for delivery of the mobile phase, (5) High pressure pump, (6) Switching valve, (7) Pre-column (Bio-Rad), (8) Analytical column (Bio-Rad), (9) IR Detector, (10) UV Detector, (11) Waste and (12) Data acquisition. (B) Picture of HPLC equipment used.

Samples were quantified on a Dionex P680 instrument equipped with an automated sampler injector (Dionex, ASI-100) and a differential refractometer-LKB Bromma 2142 (9) Figure 2.1 using as mobile phase bi-distilled water at a flux rate of 0.6mL.min^{-1} at 75ºC. These compounds were separated on a HPLC Carbohydrate analysis Aminex HPX-87P column. Mobile

phase were prepared, filtered through a 0.22µm membrane filters and degassed under vacuum with a pump.

Peak identification was made by comparing the retention times of the sample peaks with those from pure standards. Calibration curves were made by sequentially diluting a multi-component standard containing sucrose, glucose, fructose, glycerol, mannitol, erythritol and ethanol between 10 and 25 g/L. Peak data was collected and processed by the CHROMELEON chromatography management system, version 6.8 (Dionex).

2.4 pH measurement and acidity titration

The pH was measured with a WTW pH-Electrode SenTix20 using a Radiometer PHM 82 Standard pH Meter as detector.

Titration was performed with 10 mL of juice diluted with 50 mL of bi-distilled water using a stir bar for correct homogenization. NaOH with a concentration of 0.1M was added until pH 8.1 (neutrality) and the volume of NaOH (mL) used for titrate acidity (TTA) was used in calculation (results Part III).

2.5 Hexokinase activity assays

2.5.1 Crude extracts preparation for enzymatic assays

Yeast cells, *Candida magnoliae* and *Saccharomyces cerevisiae*, were grown as explained in 2.2.2. Cultures were incubated at 25ºC with orbital agitation (185 rpm) until mid exponential phase (≈ after 24h of growth). Then cells were harvested by centrifugation (9000 rpm during 10 minutes at 4ºC), washed twice with TRIS buffer (50mM triethanolamine hydrochloride and 1µM PMSF pH 7.6), concentrated fourfold, and stored at -80ºC.

Immediately before assaying, cells were thawed, washed and resuspended in 400 µL of TRIS buffer. To disrupted cells, 400 µL of lysis buffer (0.1M triethanolamine hydrochloride, 2mM $MgCl_2$, 1mM DTT and 1µM PMSF) and 200 µL glass beads (230–320 nm) were subsequently added to the suspension followed by six alternating cycles of 1 minute vortexing alternated by 1 minute cooling on ice. Cell debris were removed by centrifugation (13000 rpm during 20 minutes at 4ºC) and supernatant was used for enzymatic assays.

2.5.2 Protein quantification assay

Total protein concentration in crude extracts was determined using **BCA protein assay kit (PIERCE)** with bovine serum albumin (BSA) as a standard according to manufacturer's instructions.

2.5.3 Enzymatic assays

For enzymatic assays, stock solutions of the reagents required for the reaction were prepared as illustrated in Table 2.2.

Table 2.2- Stock solutions prepared for hexokinase activity assays.

Stock solution	Concentration	Supplier
TRIS buffer	50mM	Sigma-Aldrich
ATP	50mM	Sigma-Aldrich
$NADP^+$	0.0125mmol/250µL	Sigma-Aldrich
$MgCl_2$	100mM	Merck
G-6-PDH	1U/50µL	Sigma-Aldrich
PGI	4.5U/µL	Sigma-Aldrich
Glucose	555mM	Sigma-Aldrich
Fructose	555mM	VWR-BDH prolabo

A UV-Vis Spectrophotometer (Thermo Scientific Evolution 300) set to Abs_{340nm} with controlled temperature at 25ºC was used to measure the Abs and the reaction was followed for 2 minutes. Data was collected and processed using the software Vision $_{pro}$ ™.

Firstly, the baseline of reaction with all the reagents except sugar, was performed to estimate enzyme residual activity in crude extract and then sugar was added for hexokinase activity measurement.

Slopes derived from sample and baseline straight equations were used for hexokinase activity determination, considering **Equation 2.1**.

Equation 2.1

$$\text{Hexokinase activity} = \frac{\Delta\text{Slopes}}{\varepsilon\ (\text{NADPH})}$$

in which ΔSlopes is the diference between sample and baseline slope (min^{-1}) and ε (NADPH) is NADPH molar extinction coefficient ($6.22*10^{-3}$mL.nmol^{-1}). From this equation is possible to calculate the hexokinase specific activity using **Equation 2.2**.

Equation 2.2

$$\text{Hexokinase specific activity} = \frac{\text{Hexokinase activity}}{[\text{Protein}] \times \frac{\text{Volume of extract}}{\text{Reaction volume}}}$$

where [Protein] is the concentration of total protein in crude extract (mg.mL^{-1}), Volume of extract is the volume used in enzymatic reaction (µL) and Reaction volume is the final volume where hexokinase was assayed (µL).

3. Results and Discussion

3.1 Part I

Fermentation profiles of two *Candida magnoliae* strains, including sugar consumption and fermentation products in different conditions, using synthetic culture medium

The yeast *Candida magnoliae* is crabtree-negative i.e. in the presence of oxygen promotes respiratory metabolism regardless of the sugar present in the medium, while in the absence of oxygen favours fermentative metabolism. Thus aiming to study and characterize the growth and fermentation profiles of two *Candida magnoliae* strains, PYCC 2903 and PYCC 3191, two conditions of oxygen supply were created in batch cultures: (1) plenty of oxygen (aerobiose), achieved by vigorous orbital shaking of the culture in a flask with a high flask-to-medium volume ratio to maximize the oxygen diffusion; and (2) oxygen limitation achieved by keeping the culture still in a flask with a low flask-to-medium volume ratio to avoid oxygen diffusion (see Table 3.1).

The growth and metabolism of *C.magnoliae* were tested with increasing sugar concentrations. For this purpose, only one of the sugars, 20g.L^{-1} (2%) of glucose or fructose, or a mixture of both, each at 10g.L^{-1} (1%), 50g.L^{-1} (5%), 100g.L^{-1} (10%) and 150g.L^{-1} (15%), were tested (see Table 3.1). In some conditions, the mixture of glucose and fructose reaches high sugar concentrations such as 200g.L^{-1}(20%) and 300g.L^{-1}(30%), which are well-tolerated conditions due to *C.magnoliae* ability to survive in hyper-osmotic environments.

Table 3.1- Conditions used to study and characterize the growth and fermentation profiles of two *Candida magnoliae* strains, PYCC 2903 and PYCC 3191.

	Growth and fermentation		**Fermentation simulating industrial fructose-rich substrates**	
Sugar	Glucose and Fructose • 15%G + 15%F • 10%G + 10%F • 5%G + 5%F • 1%G + 1%F Glucose or Fructose • 2%G • 2%F		Glucose and Fructose • 7%G + 3%F • 5%G + 5%F • 3%G + 7%F Glucose or Fructose • 10%G • 10%F	
Cell density inoculated	Low ($O.D_{640nm}$≈0.1)		High ($O.D_{640nm}$ ≈ 15)	
Conditions of oxygen supply	Aerobiose: orbital shaker (185 rpm)	Oxygen limitation: kept without agitation	Aerobiose: orbital shaker (185 rpm)	Oxygen limitation: kept without agitation
***Candida magnoliae* strains**	PYCC 2903 and PYCC 3191	PYCC 2903 and PYCC 3191	PYCC 2903	PYCC 2903 and PYCC 3191
Ratio of flask volume/medium	5:1	5:1	5:1	5:5
Section	3.1.2.1	3.1.2.2	3.1.3.1	3.1.3.2

3.1.1 Growth curves and specific growth rates

First of all, growth profiles and specific growth rates were determined for these two *C.magnoliae* strains. Growth curves (Appendix I and II) demonstrate that these two strains exhibit very similar growth profiles when subjected to the same conditions.

When cells were grown with plenty of oxygen higher optical densities ($O.D_{640nm}$= [28-50]) were achieved since they take advantage of high oxygenation to use respiratory metabolism, until the culture becomes so dense that probably growth is limited by oxygen (see Appendix I). On the other hand, when cells were grown under oxygen limitation, the cultures reached very low OD values ($O.D_{640nm}$= [0.6-1.6]) for the same incubation period since this condition favours the fermentative instead of the respiratory metabolism (see Appendix II). In this last case, regardless of sugar concentrations, optical densities for PYCC 2903 have achieved slightly higher values than for PYCC 3191.

The specific growth rate (μ) was calculated considering the linearization of exponential growth phase (**Equation 3.1**).

Equation 3.1

$$\mathrm{Ln(N)} = \mathrm{Ln(N_0)} + \mu \times \mathrm{t}$$

in which N is the final cell number, N_0 is the initial cell number, μ is the specific growth rate and t is the time of exponential growth phase.

Specific growth rates for *C.magnoliae* PYCC 2903 and PYCC 3191 are represented in Tables 3.2 and 3.3 and Figure 3.1.

Table 3.2- Specific growth rates for *Candida magnoliae* strains, PYCC 2903 and PYCC 3191, incubated aerobically at different sugar concentrations.

Growth conditions	PYCC 2903	PYCC 3191
	μ (h^{-1})	μ (h^{-1})
Glucose (15%) + Fructose (15%)	0.23	0.23
Glucose (10%) + Fructose (10%)*	0.25±0.00	0.24±0.00
Glucose (5%) + Fructose (5%)*	0.26±0.00	0.26±0.00
Glucose (1%) + Fructose (1%)	0.21	0.22
Glucose (2%)	0.21	0.22
Fructose (2%)	0.21	0.23

* Done in duplicate, values are mean with standard deviation (SD) (n=2).

Table 3.3- Specific growth rates for *C.magnoliae* strains, PYCC 2903 and PYCC 3191, incubated under oxygen limitation at different sugar concentrations.

Growth conditions	PYCC 2903	PYCC 3191
	μ (h^{-1})	μ (h^{-1})
Glucose (15%) + Fructose (15%)	0.09	0.06
Glucose (10%) + Fructose (10%)	0.09	0.07
Glucose (5%) + Fructose (5%)	0.10	0.09
Glucose (1%) + Fructose (1%)	0.07	0.06
Glucose (2%)	0.07	0.06
Fructose (2%)	0.06	0.05

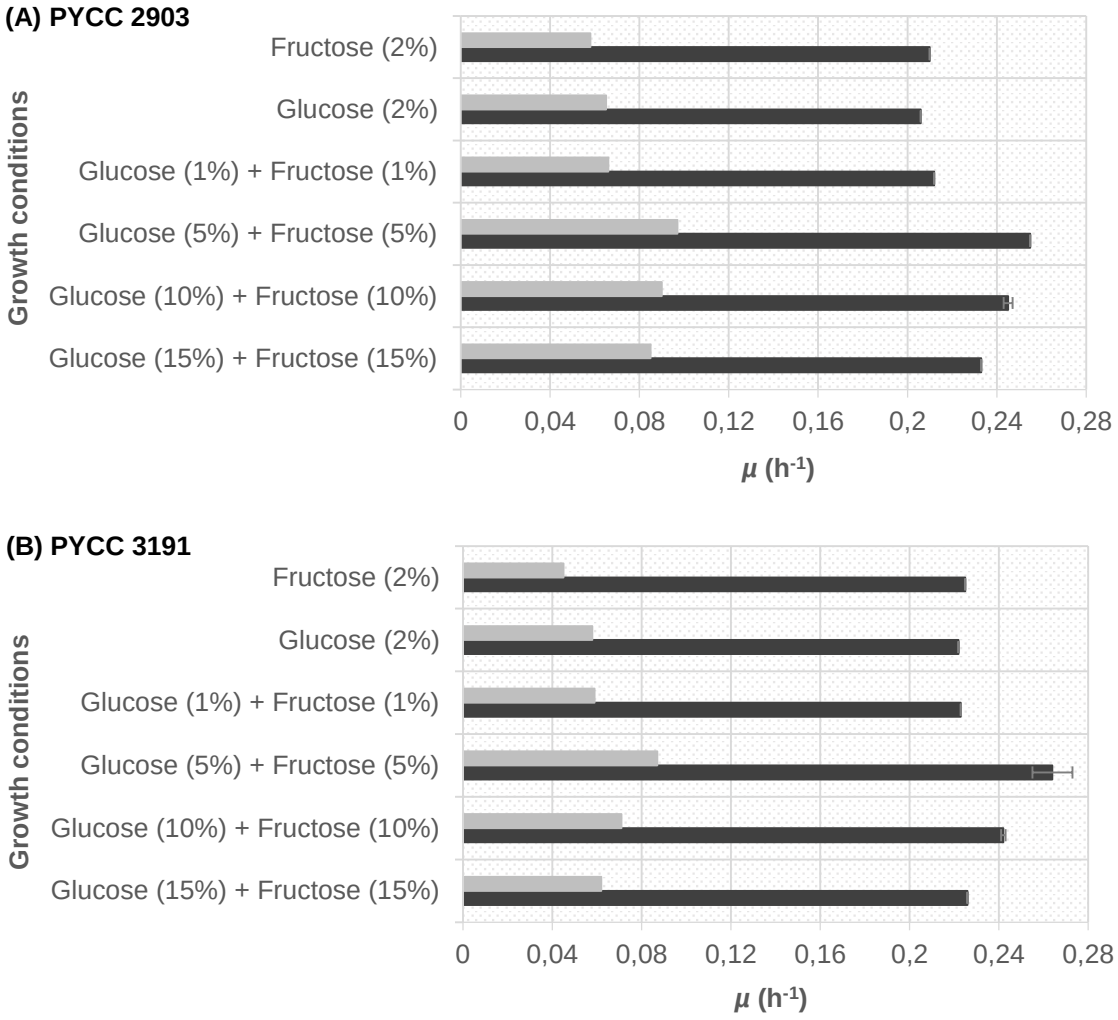


Figure 3.1- Comparison between specific growth rates of *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191, incubated aerobically (■) or under oxygen limitation (■) at different sugar concentrations. Bars for 10% and 5% sugars represent mean values with standard deviation (SD) (n=2).

The results of specific growth rates are similar for both strains and apparently with slightly higher values for higher (> 2%) sugar concentrations, with the greater values shown for 5% of each sugar in both cases. These results showed that increasing osmolarity (sugar concentration) neither affected specific growth rate nor the optical density reached, confirming the osmotolerant character of this yeast. Both strains show similar rates for glucose and fructose.

3.1.2 Glucose and fructose consumption. Fructophily analysis and fermentation products

Aiming to evaluate fructophily, glucose and fructose consumption rates were calculated using the slope of sugar consumption plots and correlated in terms of a ratio of consumption rates (**Equation 3.2**).

Equation 3.2

$$\text{Fructophily} = \frac{\text{Fructose consumption rate}}{\text{Glucose consumption rate}}$$

When the ratio between fructose and glucose consumption rates is higher than 1 (>1), the yeast exhibit a fructophilic behaviour consuming fructose faster than glucose, whereas if the ratio is lower than 1 (<1) exhibit a glucophilic behaviour consuming glucose faster than fructose.

In order to estimate more precisely the differences between products that may result from yeast fermentative metabolism, such as ethanol, glycerol, erythritol and mannitol, the fermentation products yield (g/g) was calculated using **Equation 3.3**,

Equation 3.3

$$Y_{\text{Fermentation products}} = \frac{[\text{Fermentation product}]}{\text{Total sugar consumed}}$$

where [Fermentation product] is the final concentration ($g.L^{-1}$) of ethanol, glycerol, erythritol or mannitol and Total sugar consumed is the difference between glucose and/or fructose concentration ($g.L^{-1}$) in the beginning and end of fermentation. To get a general idea of what happens with biomass production, a yield was determined using OD as a measured of total biomass, **Equation 3.4**,

Equation 3.4

$$Y_{\text{Biomass}} = \frac{O.D_{640\text{nm}}}{\text{Total sugar consumed}}$$

in which $O.D_{640nm}$ is the optical density of suspension in the end of fermentation and Sugar consumed is the difference between glucose and/or fructose concentration ($g.L^{-1}$) in the beginning and end of fermentation.

3.1.2.1 Aerobiose

Sugar consumption profiles for *C.magnoliae* PYCC 2903 and PYCC 3191 are illustrated in Figures 3.2 and 3.3.

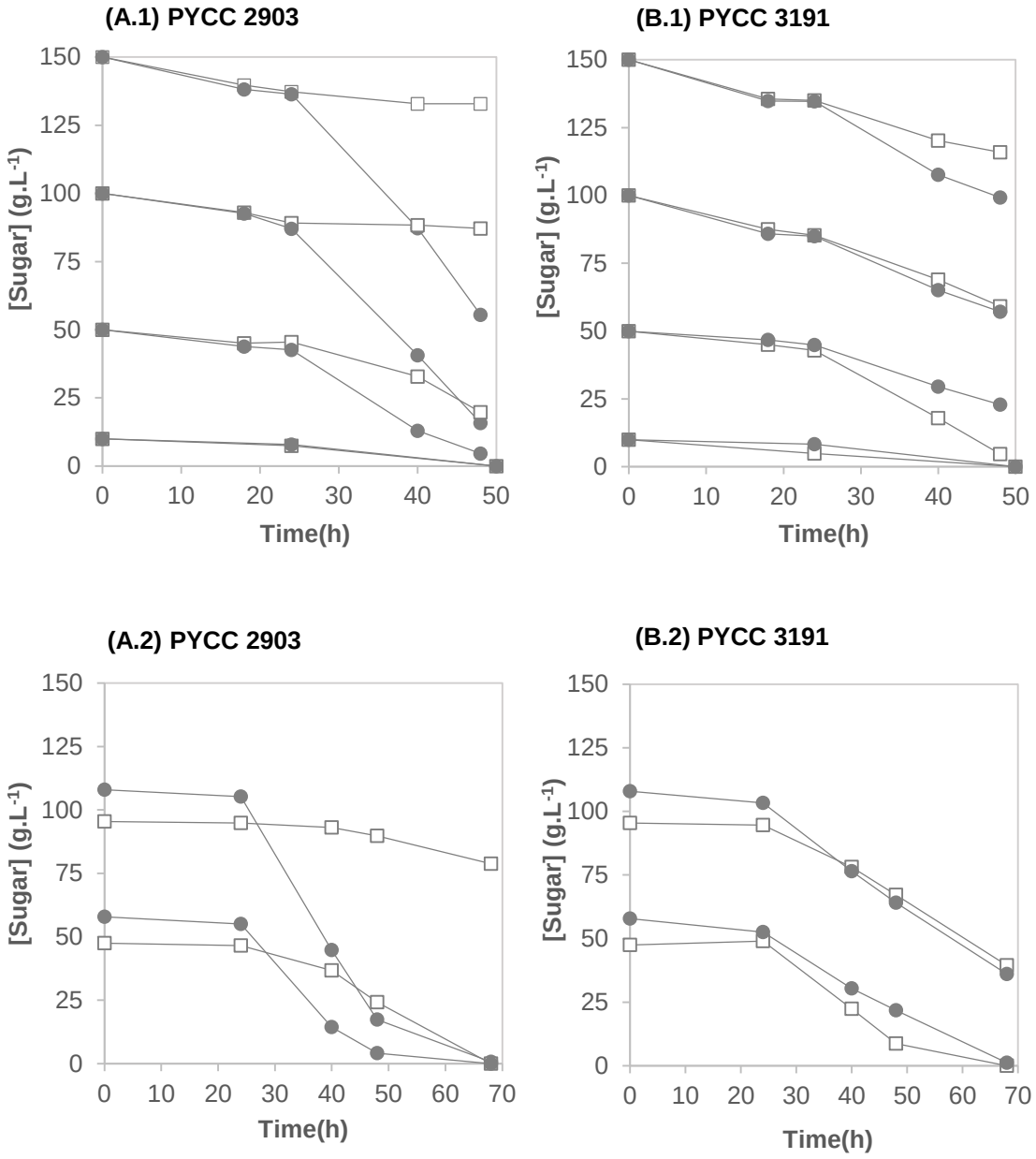


Figure 3.2- Glucose (□) and fructose (■) consumption for *Candida magnoliae* strains incubated aerobically at different sugar concentrations (15%, 10%, 5%, 1% of each sugar) (A.1) and (B.1). The lower graphs (A.2 and B.2) are duplicates for 10% and 5% sugar concentrations.

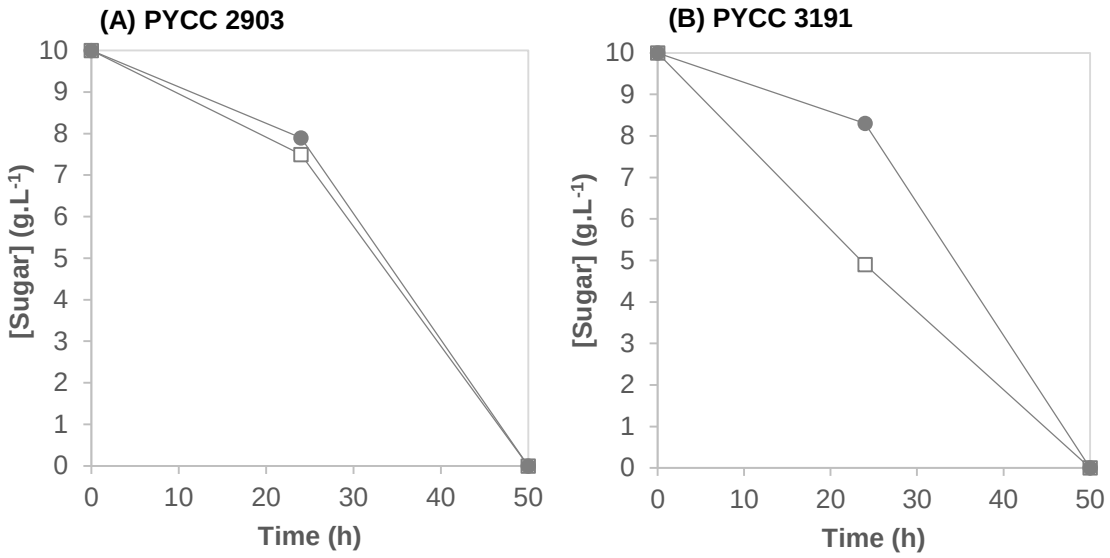


Figure 3.3- Glucose (□) and fructose (■) consumption for *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191, incubated aerobically at low sugar concentrations (1% glucose and fructose). Data re-plotted from Figure 3.2.

Experimental results for sugar concentrations with 5% and 10% of glucose and fructose are separated into two different graphs, Figure 3.2, because in assays A.2 and B.2 (duplicates of the assays 1) the fermentation time were extended so that the entire fructose initially added became depleted.

Graphs represented in Figures 3.2 and 3.3 show that sugar consumption occurs at a lower rate during the early stage (until ≈ 24h) and at a faster rate from 24h until the end, which may be due to different factors: Exponential increase of cell number in the presence of oxygen that leads to a higher cell density responsible for consuming sugar faster; Furthermore, when the yeast switches to fermentative metabolism, most probably because of oxygen limitation, sugar starts to be consumed at a higher rate (see Appendix I and Figure 3.5).

For higher sugar concentrations, both *C.magnoliae* strains show a preference for fructose over glucose, although this is more evident for PYCC 2903 than for PYCC 3191. Selective utilization of fructose by the first strain is observed in the presence of high sugar concentrations such as 15%, 10% and 5%, while for PYCC 3191 occurs only with 15% of both sugars. For these two strains in the presence of 1% of both sugars (re-plotted in Figure 3.3), the preferential consumption of fructose is not demonstrated but the trend towards fructose is still marked for PYCC 2903 whereas PYCC 3191, at 1% each sugar, shows the usual glucophilic behaviour present in the majority of yeast.

Graphs A.1 and B.1 show, for both strains that a fermentation time of 50 hours in conditions with high sugar content was insufficient for sugar depletion. So, assays 2, A.2 and B.2, were performed in order to confirm results obtained in assays 1, mainly for PYCC 3191 (B.1) in the presence of 10% and 5% of both sugars in which an inversion occurs in sugar consumption preference. The results from duplicate assays corroborate those obtained previously since PYCC 2903 consumes fructose faster than glucose in the presence of 10% and 5% of each sugar, while PYCC 3191 only shows some preference for fructose with 10% glucose and fructose. Thus, just as in the first assays, when PYCC 3191 cells grow in the presence of 5% of each sugar, glucose consumption is slightly higher than fructose.

Considering all these results with plenty of oxygen, *C.magnoliae* PYCC 2903 reveals a fructophilic behaviour more similar to that described in the literature for this yeast (Yu *et al*., 2006).

From sugar consumption profiles described above (Figure 3.2 and 3.3), it was possible to determine an average rate for glucose and fructose consumption and correlate them to provide a measurement of fructophily. Table 3.4 and Figure 3.4 show glucose and fructose consumption rates and fructophily values for *C.magnoliae* PYCC 2903 and PYCC 3191.

Table 3.4- Glucose and fructose consumption rates and fructophily analysis for *Candida magnoliae* PYCC 2903 and PYCC 3191 incubated aerobically at different sugar concentrations.

Strain	Growth conditions	Consumption rate (mmol/L.h)			Fructophily
		Glucose	Fructose	Total sugar	
PYCC 2903	Gluc (2%)	2.2	-	2.2	-
	Fruc (2%)	-	2.2	2.2	-
	Gluc (1%) + Fruc (1%)	1.1	1.1	2.2	1.0
	Gluc (5%) + Fruc (5%)	3.5/3.9	5.3/4.7	8.8/8.6	1.5/1.2
	Gluc (10%) + Fruc (10%)	1.5/1.4	9.7/8.8	11.2/10.2	6.6/6.5
	Gluc (15%) + Fruc (15%)	2.0	10.9	12.9	5.5
PYCC 3191	Gluc (2%)	2.2	-	2.2	-
	Fruc (2%)	-	2.2	2.2	-
	Gluc (1%) + Fruc (1%)	1.1	1.1	2.2	1.0
	Gluc (5%) + Fruc (5%)	5.2/3.9	3.1/4.6	8.3/8.5	0.6/1.2
	Gluc (10%) + Fruc (10%)	4.7/4.6	5.0/5.9	9.7/10.5	1.0/1.3
	Gluc (15%) + Fruc (15%)	3.9	5.9	9.8	1.5

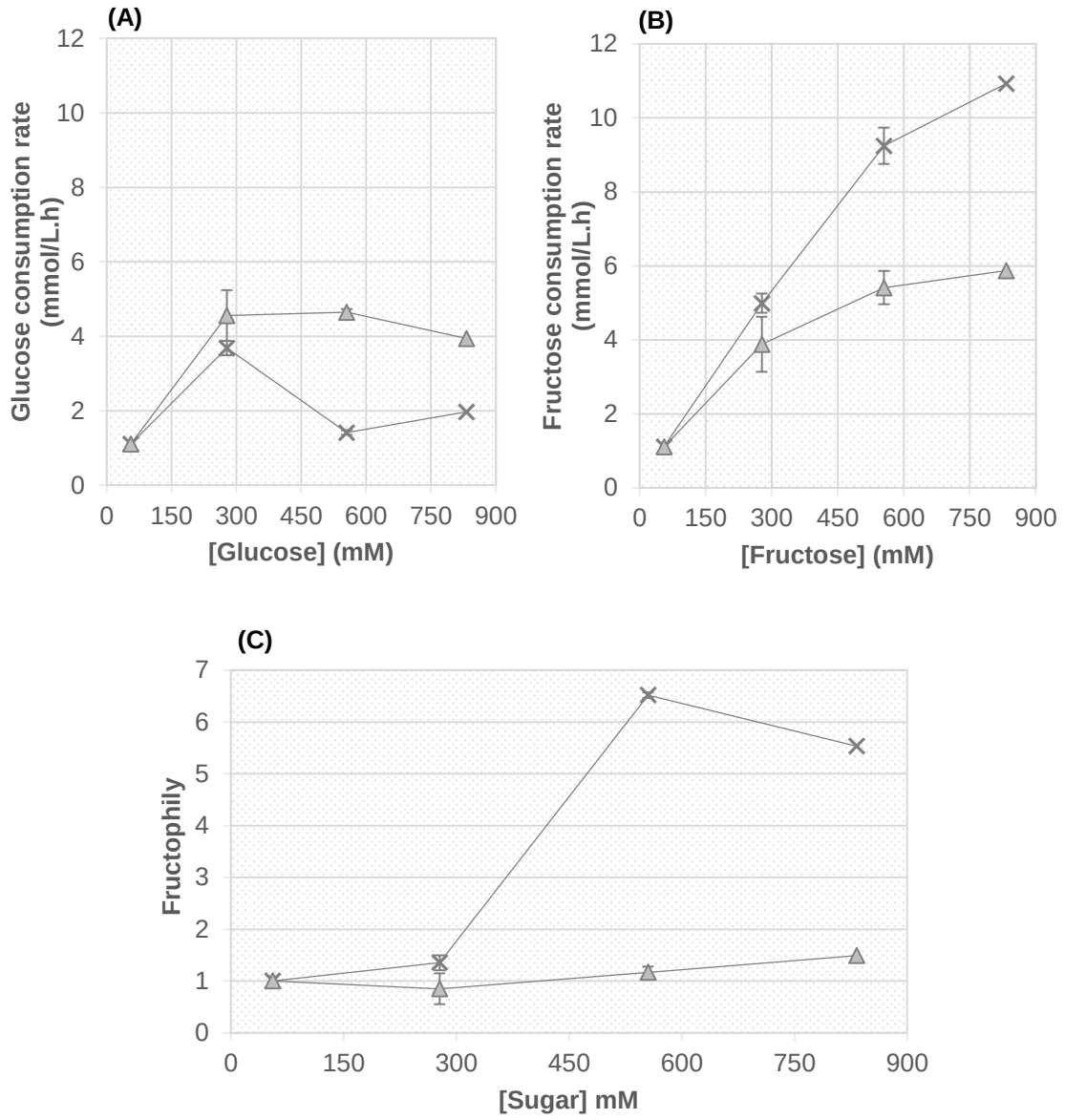


Figure 3.4- Glucose (A) and fructose (B) consumption rates and fructophily analysis (C) for *Candida magnoliae* strains, PYCC 2903 (✕) and PYCC 3191 (▲) incubated aerobically at increasing sugar concentrations. For 277.5 and 555.1mM (5 and 10%) of each sugar, bars represent mean values ±SD (n=2).

For PYCC 2903, fructose consumption rate is higher than glucose in the presence of high sugar content (15%, 10% and 5%), resulting in fructophily values much higher than 1 typical of a fructophilic behaviour. For these oxygenation conditions with 1% of each sugar, fructose and glucose are consumed at the same rate generating a fructophily value equal to 1. For PYCC 3191, fructose consumption rate is 2 times higher than glucose for 15% of each sugar, whereas for mixtures with 10% and 5% of each sugar fructose and glucose are consumed nearly at the same rate, resulting in fructophily values close but higher than 1 due to a slightly pronounced fructophilic behaviour. For 1% of each sugar, fructose and glucose are consumed at the same rate generating a fructophily value of 1, denoting neither a fructophilic nor a glucophilic behaviour.

C.magnoliae fructophily results demonstrate clear differences between these two strains mainly when cells were grown under high sugar concentrations (10 and 15%), reaching much higher values for PYCC 2903 strain.

Thus, this fructophily analysis based on the ratio between fructose and glucose consumption rates provides a more specific graphical visualization of the fructophilic behaviour of *C.magnoliae* strains, showing that this feature is more pronounced in PYCC 2903 than in PYCC 3191.

Interestingly, for both strains, the fructophily seems to arise from the specific increase in the fructose consumption rate for higher sugar concentrations.

A difference is visible between glucose and fructose curves: whereas glucose consumption rate (A) remains almost constant for sugar concentrations greater than or equal to 5% (277.5mM) of each sugar, fructose consumption rate (B) gradually increases with the increase of sugar content. For sugar concentrations of 5% (277.5mM) of each sugar or higher, glucose transporters appear to transport the maximum that they are capable of. Assuming a Michaelis-Menten behaviour for fructose consumption, it's possible to calculate a rough K_m value of approximately 250mM, which is close to the K_m of the Ffz1 fructose transporter described for *C.magnoliae* (K_m =105±12mM) (Lee *et al*., 2013). This result may indicate that *Cm*Ffz1 kinetic is directly associated with consumption rate, since the increase in fructose input through this transporter leads to an increased consumption of this sugar within the cell. The higher capacity of PYCC 2903 could contribute for the higher fructophilic character of these strain.

Another relevant aspect that needs confirmation is the decrease of glucose consumption at 10 e 15% sugars for strain 2903. This decrease may be related to a limit for the capacity of the yeast to consume sugar. In fact, in the Table 3.4, one can see that, in both strains, the average rate of total sugar consumption tends towards a maximum value.

After sugar consumption profile characterization and fructophily analysis, differences between ethanol and sugar alcohols ratios were evaluated aiming to study *C.magnoliae* strains fermentative metabolism in terms of fermentation products.

Fermentation products yield profiles and biomass yield for *C.magnoliae* PYCC 2903 (A) and PYCC 3191 (B) are shown in Figure 3.5.

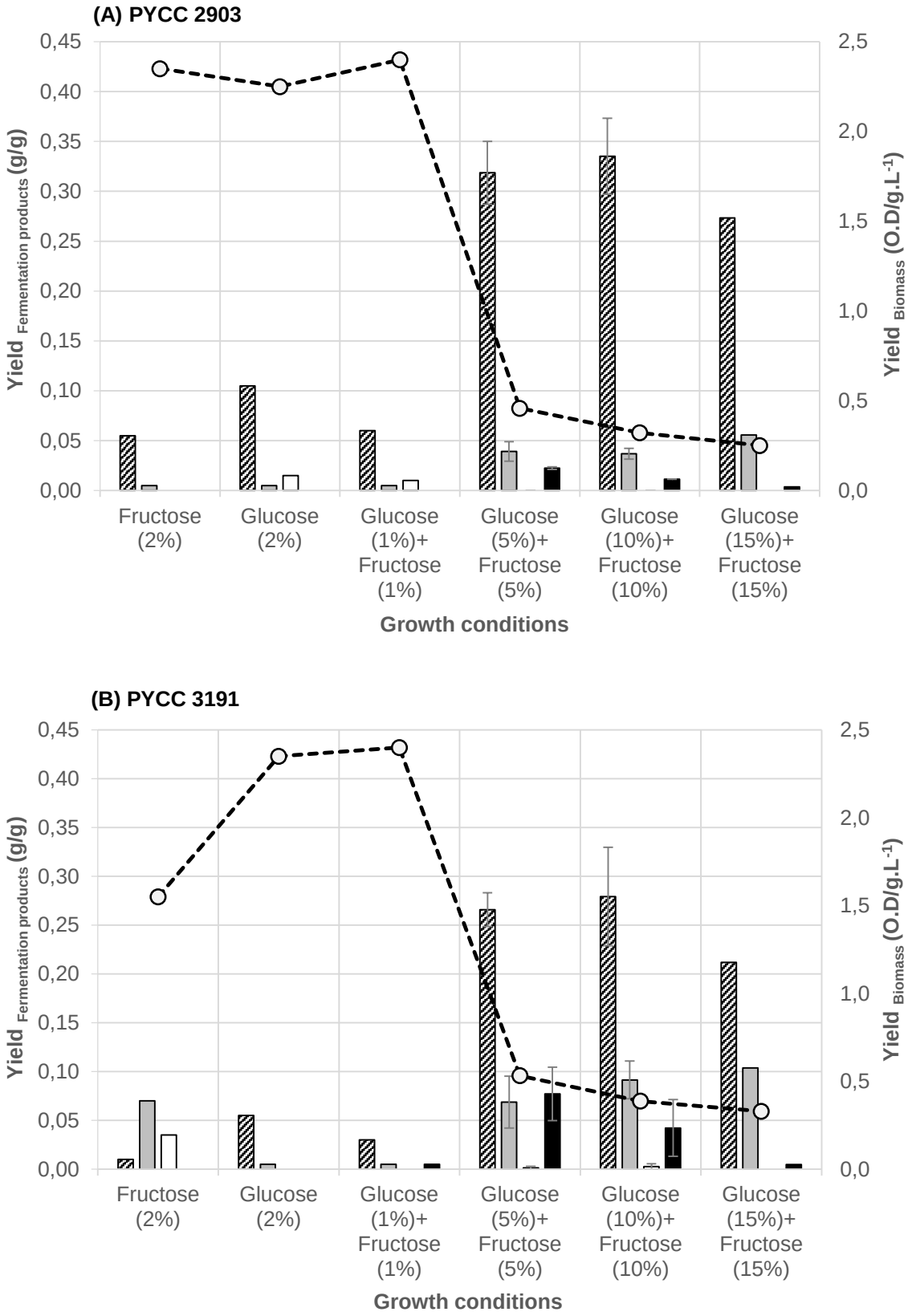


Figure 3.5- Ethanol (▨), glycerol (▩), erythritol (□) and mannitol (■) yield for *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191, incubated aerobically at different sugar concentrations. Bars for 10% and 5% sugars represent mean values with standard deviation (SD) (n=2).

In Figure 3.5, it is possible to observe that higher fermentation product yields occur when cells grow under high sugar concentrations (15%, 10% and 5%). At these concentrations, cell density became very high thereby oxygen gets limited and the yeast switch to fermentative metabolism, raising the fermentation product yields. *C.magnoliae* PYCC 3191 (B) when compared with the other strain produces slightly less ethanol resulting in a higher sugar alcohol production. For this strain (B), ethanol yield varies between 0.21-0.28g/g and 0.01-0.06g/g for high and low sugar content, respectively. Glycerol yield is in a range between 0.01-0.1g/g, erythritol 0-0.04g/g and mannitol 0-0.08g/g. For PYCC 2903 (A), yield values for ethanol are among 0.27-0.34g/g and 0.06-0.11g/g for high and low sugar concentrations, respectively. Under high and low sugar content conditions glycerol yield is between 0.01-0.06g/g, erythritol 0-0.02g/g and mannitol 0-0.02g/g.

Interestingly, it is also observed that when glucose is absent from the culture medium (2% Fructose), ethanol production yield reaches its lowest value, 0.01 and 0.06g/g for PYCC 3191 (B) and PYCC 2903 (A), respectively, indicating that fructose might favour sugar alcohol formation. Fermentation product profiles illustrated in Figure 3.5 also show for both strains that mannitol production was only detected when a mixture of sugars, at high concentrations is present.

These fermentation product profiles (Figure 3.5) reveal that the type and yield of fermentation products varies between sugar concentrations and also differs from strains.

For both strains, in these oxygenation conditions, biomass yield curve (Figure 3.5) show higher values (1.55-2.40) for low sugar concentrations (1% each sugar and 2% glucose or fructose), while lower values (0.25-0.53) are for high sugar concentrations (5%, 10% and 15%). This indicates that the sugar consumed in the presence of low sugar content was used to produce biomass, hence the yield of fermentation products is low. For high sugar content the opposite occurs, sugar consumed was mostly to produce fermentation products instead of biomass.

3.1.2.2 Oxygen limitation

Sugar consumption profiles for *C.magnoliae* PYCC 2903 and PYCC 3191 are illustrated in Figures 3.6 and 3.7.

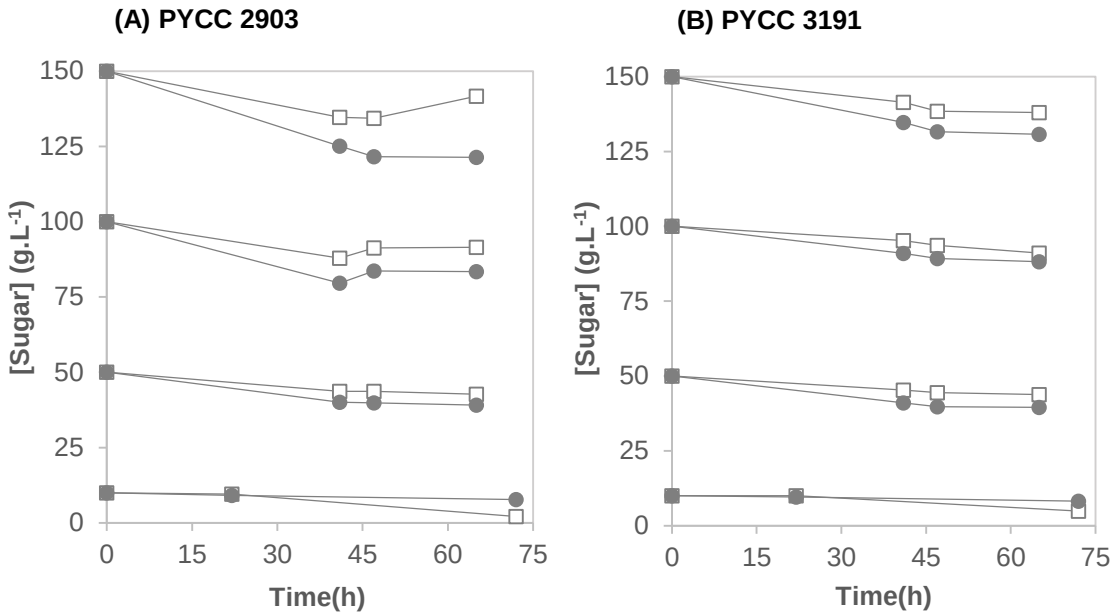


Figure 3.6- Glucose (□) and fructose (■) consumption for *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191, incubated with oxygen limitation at different sugar concentrations (15%, 10%, 5%, 1% of each sugar).

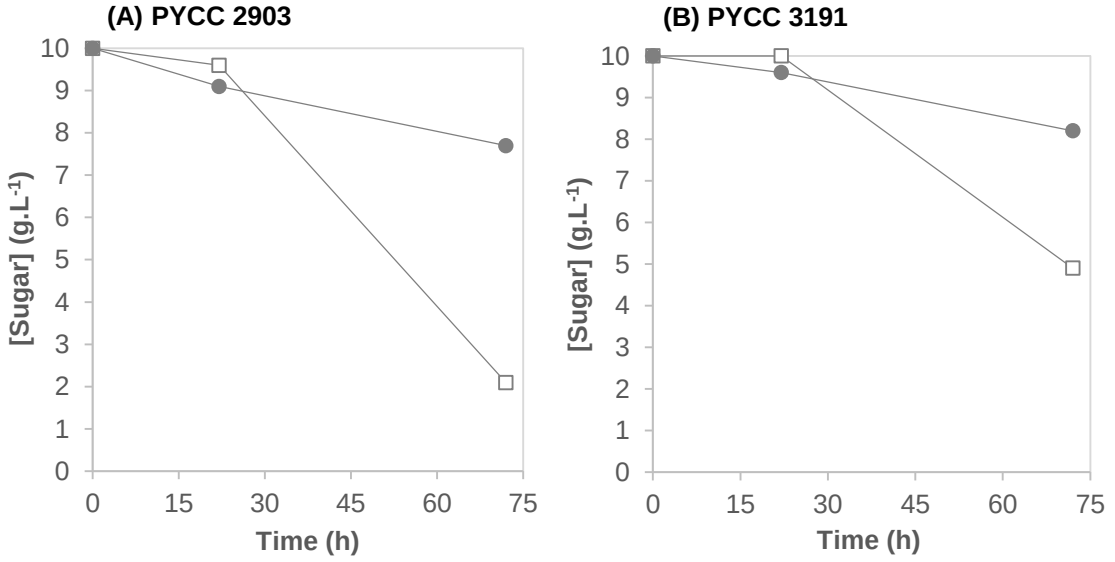


Figure 3.7- Glucose (□) and fructose (■) consumption for *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191, incubated with oxygen limitation at low sugar concentrations (1% glucose and fructose). Data re-plotted from Figure 3.6.

Graphs represented in Figures 3.6 and 3.7 demonstrate that sugar consumption was conducted with a very low rate as expected from the low cell density ($O.D_{640nm}$= [0.6-1.6]) attained under this condition (Appendix II) considering that oxygenation is significantly reduced, cells guide

the metabolism towards the fermentative pathway overlapping the respiratory metabolism that favors biomass production. Despite the low sugar consumption rate, both strains exhibit a fructophilic behaviour consuming fructose faster than glucose in the presence of high sugar content such as 15% of glucose and fructose. At low sugar content, of 1% each sugar, fructose is not preferentially consumed when compared with glucose.

From sugar consumption profiles described above (Figure 3.6 and 3.7), it was possible to determine glucose and fructose consumption rates and correlate them to provide a measurement of fructophily. Table 3.5 and Figure 3.8 show glucose and fructose consumption rates and fructophily values for *Candida magnoliae* PYCC 2903 and PYCC 3191.

Table 3.5- Glucose and fructose consumption rates and fructophily analysis for *Candida magnoliae* PYCC 2903 and PYCC 3191 incubated with oxygen limitation at different sugar concentrations.

Strain	Growth conditions	Consumption rate (mmol/L.h)			Fructophily
		Glucose	Fructose	Total sugar	
PYCC 2903	Gluc (2%)	1.1	-	1.1	-
	Fruc (2%)	-	0.9	0.9	-
	Gluc (1%) + Fruc (1%)	0.8	0.2	1.0	0.2
	Gluc (5%) + Fruc (5%)	0.2	0.2	0.4	0.9
	Gluc (10%) + Fruc (10%)	ND	ND	ND	ND
	Gluc (15%) + Fruc (15%)	ND	0.7	ND	ND
PYCC 3191	Gluc (2%)	0.7	-	0.7	-
	Fruc (2%)	-	0.7	0.7	-
	Gluc (1%) + Fruc (1%)	0.6	0.2	0.8	0.3
	Gluc (5%) + Fruc (5%)	0.3	0.3	0.6	0.9
	Gluc (10%) + Fruc (10%)	0.9	0.6	1.5	0.6
	Gluc (15%) + Fruc (15%)	0.6	0.8	1.4	1.2

ND-Not Determined

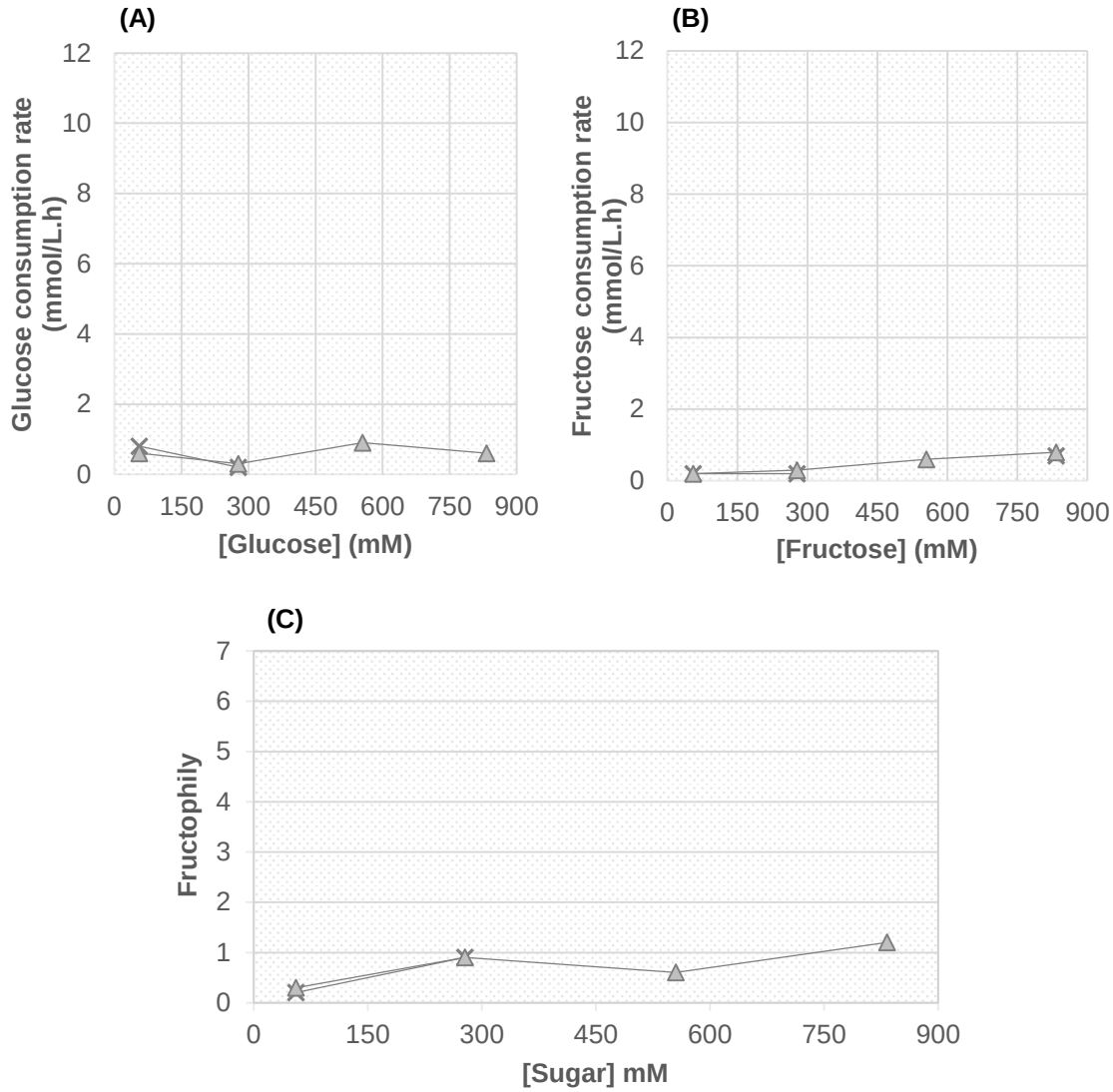


Figure 3.8- Glucose (A) and fructose (B) consumption rates and fructophily analysis (C) for *Candida magnoliae* PYCC 2903 (✕) and PYCC 3191 (▲) incubated with oxygen limitation at increasing sugar concentrations. For 277.5 and 555.1mM (5 and 10%) of each sugar, bars represent mean values ±SD (n=2).

Both strains exhibit a less pronounced fructophilic pattern to what was observed under aerobic conditions, once fructophily values are lower or close to 1, indicating that under these conditions glucose is consumed at the same rate or faster than fructose (Table 3.5 and Figure 3.8).

Fermentation products yield profiles and biomass yield for *C.magnoliae* PYCC 2903 (A) and PYCC 3191 (B) are shown in Figure 3.9.

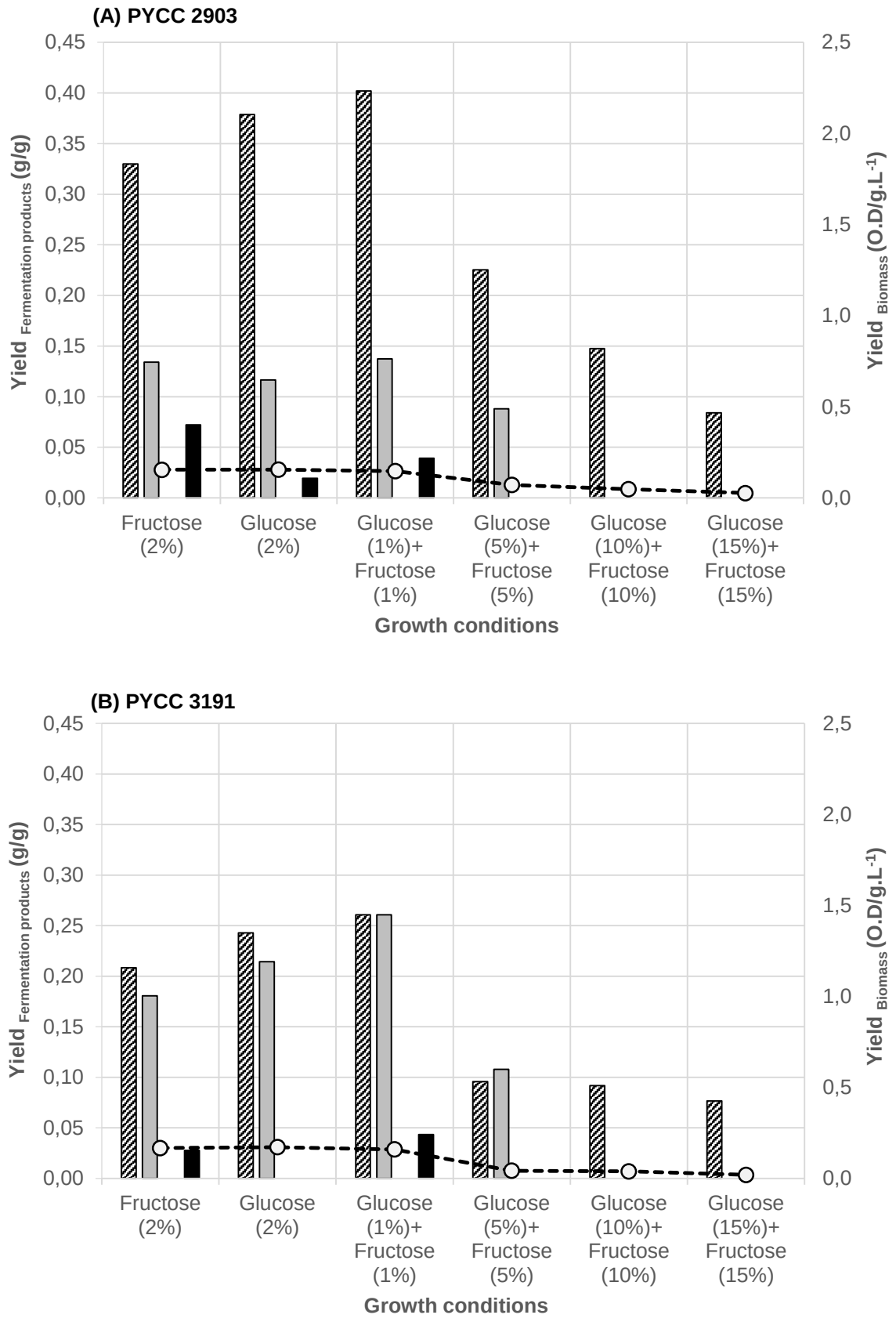


Figure 3.9- Ethanol (▨), glycerol (□), erythritol (□) and mannitol (■) yield for *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191, incubated with oxygen limitation at different sugar concentrations.

For lower sugar concentrations, and as expected under oxygen limitation, the fermentation metabolism predominated and ethanol and other fermentation products were produced. As was already observed in Figure 3.5, mannitol was produced by both strains confirming that this is a normal fermentation product for this species.

In this fermentation conditions, the higher sugar alcohols yield observed for PYCC 3191 in the previous section is even more evident. This strain has a more equilibrated distribution between glycerol and mannitol production and ethanol production. PYCC 2903 produces ethanol with a yield between 0.33-0.4g/g, glycerol 0.12-0.14g/g and mannitol 0.02-0.07g/g while for the other strain (B) ethanol yield is among 0.21-0.26g/g, glycerol 0.18-0.26g/g and mannitol 0-0.04g/g. Fermentation products profiles illustrated in Figure 3.9 also show for both strains, the lower yield of ethanol production is for 2% fructose considering the lower sugar concentrations (1% of each sugar and 2% of glucose or fructose).

These fermentation products profiles (Figure 3.9) reveal that the type and yield of fermentation products varies between sugar concentrations and also differs between strains.

For both strains, under these oxygen limitation conditions, biomass yield curve (Figure 3.9) show very low values (0.02-0.17) for low and high sugar concentrations. This suggests that regardless the sugar content, sugar was consumed for fermentation products formation rather than biomass production. However the results for the higher sugar concentrations are unclear and require further analysis.

3.1.3 Fermentations inoculated with high cell density

Aiming to evaluate the potential of *C.magnoliae* strains, PYCC 2903 and PYCC 3191, to yield low-alcoholic fermented beverages, a simulation of industrial fructose-rich substrates, such as fruit juices, was tested, using mixtures of sugars (glucose and fructose) in different and equal ratios or just one of the sugars maintaining the final concentration at $100g.L^{-1}$ (10%). For this study, cultures were inoculated with high cell density ($O.D_{640nm} \approx 15$) (see Table 3.1).

3.1.3.1 Aerobiose

Sugar consumption profiles for *C.magnoliae* PYCC 2903 simulating industrial fructose-rich substrates are demonstrated in Figure 3.10.

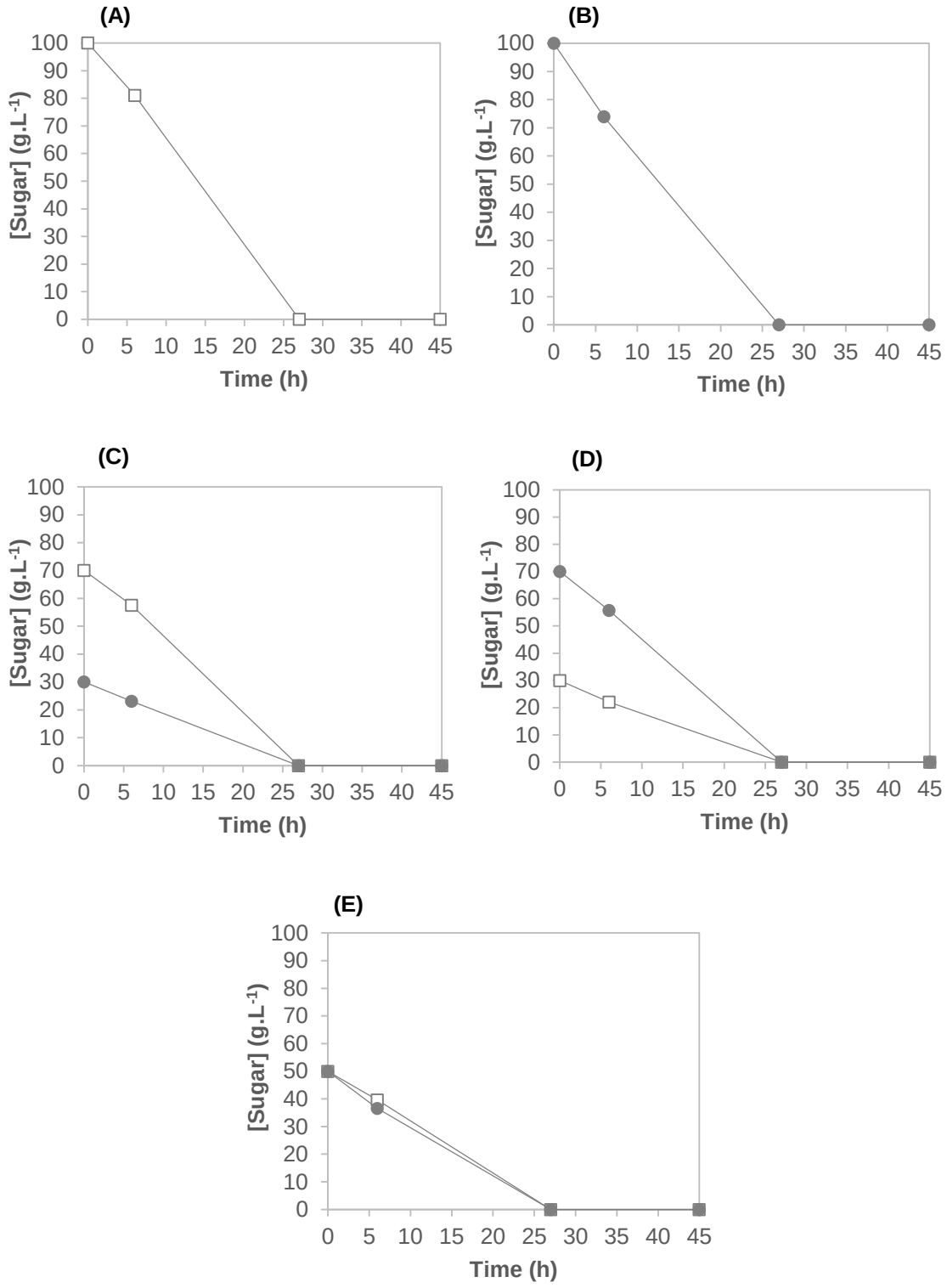


Figure 3.10- Glucose (□) and fructose (●) consumption for *Candida magnoliae* PYCC 2903 incubated aerobically at different and equal sugar ratios (A) 10% Glucose, (B) 10% Fructose, (C) 7% Glucose and 3% Fructose, (D) 3% Glucose and 7% Fructose and (E) 5% of each sugar.

Only one assay in aerobiose using PYCC 2903 was performed for comparison with profiles described in the previous chapter.

Although these fermentations have been inoculated with high cell density to create oxygen limitation conditions favourable to fermentative metabolism, cell number duplicated achieving an OD_{640nm} of approximately 65.

Sugar consumption profiles with 10% glucose (A) and 10% fructose (B) show that both sugars are consumed at the same rate. Graphs with 7% glucose and 3% fructose (C) and 3% glucose and 7% fructose (D) show that the sugar consumed faster is the one with the highest concentration, 7% glucose for (C) and 7% fructose for (D). Graph with equal sugar content (E) demonstrates that fructose consumption is slightly faster than glucose.

In these fermentations fructophily is only shown for fructose concentrations higher than 5%, which is slightly different compared with the results obtained in Figure 3.2. This might be due to pre-inoculum preparation on glucose. If the specific fructose transporters present in *C.magnoliae* (*Cm*Ffz1) are inducible, cells grown on glucose will transport fructose and glucose by the glucose transporters and the consumption ratio reflects the transport kinetic and sugar concentration and not fructophily. If this is the case, the fructophilic character of this species may be associated the presence of *Cm*Ffz1.

From sugar consumption profiles described above (Figure 3.10), it was possible to determine glucose and fructose consumption rates and correlate them to provide a measurement of fructophily. Table 3.6 and Figure 3.11 show glucose and fructose consumption rates and fructophily values for *Candida magnoliae* PYCC 2903 simulating industrial fructose-rich substrates.

Table 3.6- Glucose and fructose consumption rates and fructophily analysis for *Candida magnoliae* PYCC 2903 incubated aerobically at different and equal sugar ratios.

Growth conditions	**Consumption rate (mmol/L.h)**			**Fructophily**
	Glucose	**Fructose**	**Total sugar**	
Gluc (10%)	17.5	-	17.5	-
Gluc (7%) + Fruc (3%)	11.5	6.4	17.9	0.6
Gluc (5%) + Fruc (5%)	9.6	12.4	22.0	1.3
Gluc (3%) + Fruc (7%)	7.3	13.2	20.5	1.8
Fruc (10%)	-	24.1	24.1	-

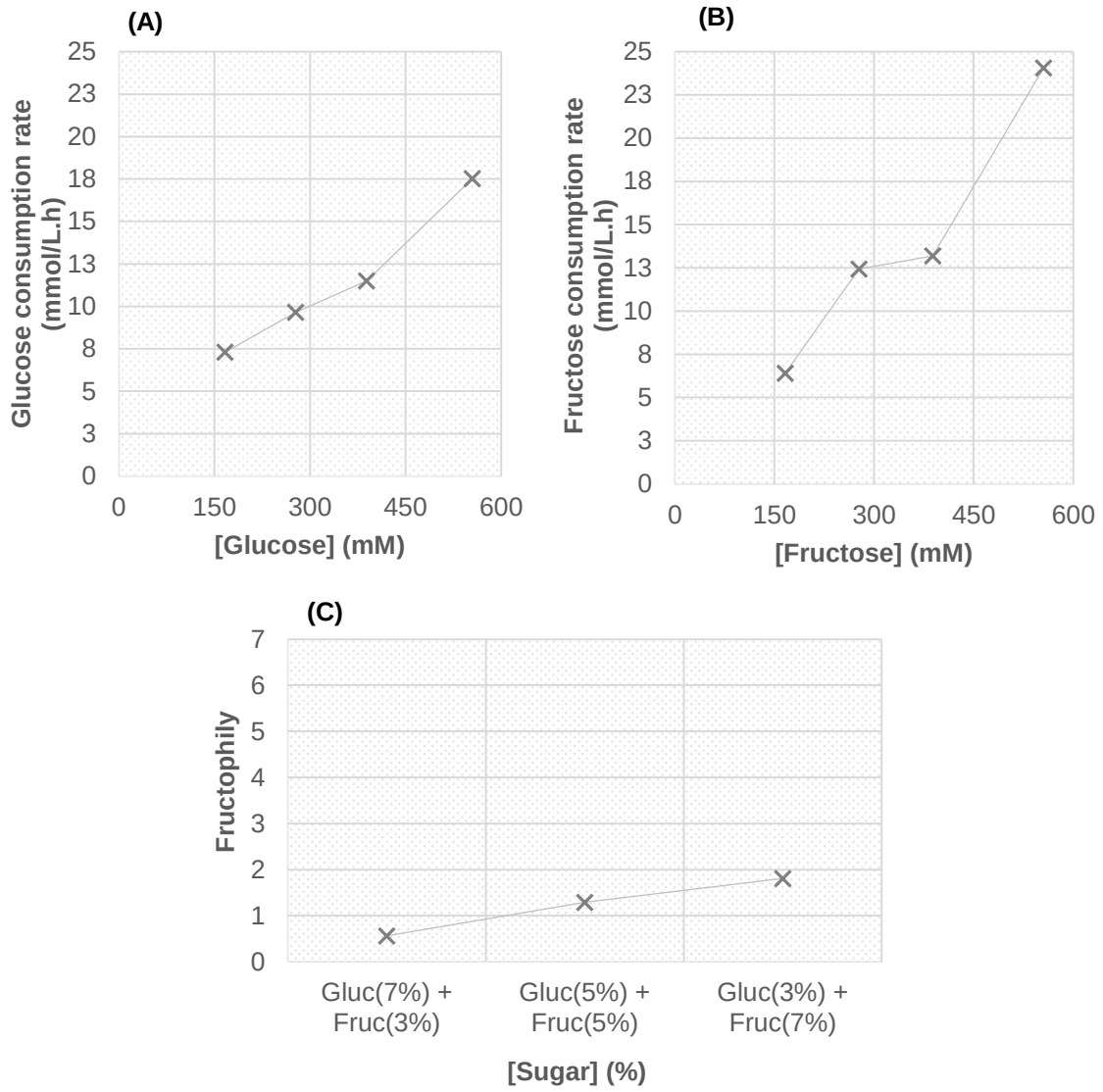


Figure 3.11- Glucose (A) and fructose (B) consumption rates and fructophily analysis (C) for *Candida magnoliae* PYCC 2903 (×) incubated aerobically at different and equal sugar ratios.

Results from Table 3.6 and Figure 3.11 demonstrate that fructose consumption rate is higher than glucose in conditions with 3% glucose and 7% fructose and 5% of each sugar, displaying a fructophily value higher than 1. In the presence of 7% glucose and 3% fructose, fructose consumption rate is lower than glucose and consequently fructophily value is lower than 1.

Fermentation products yield profiles for *Candida magnoliae* PYCC 2903 under conditions simulating industrial fructose-rich substrates are shown in Figure 3.12.

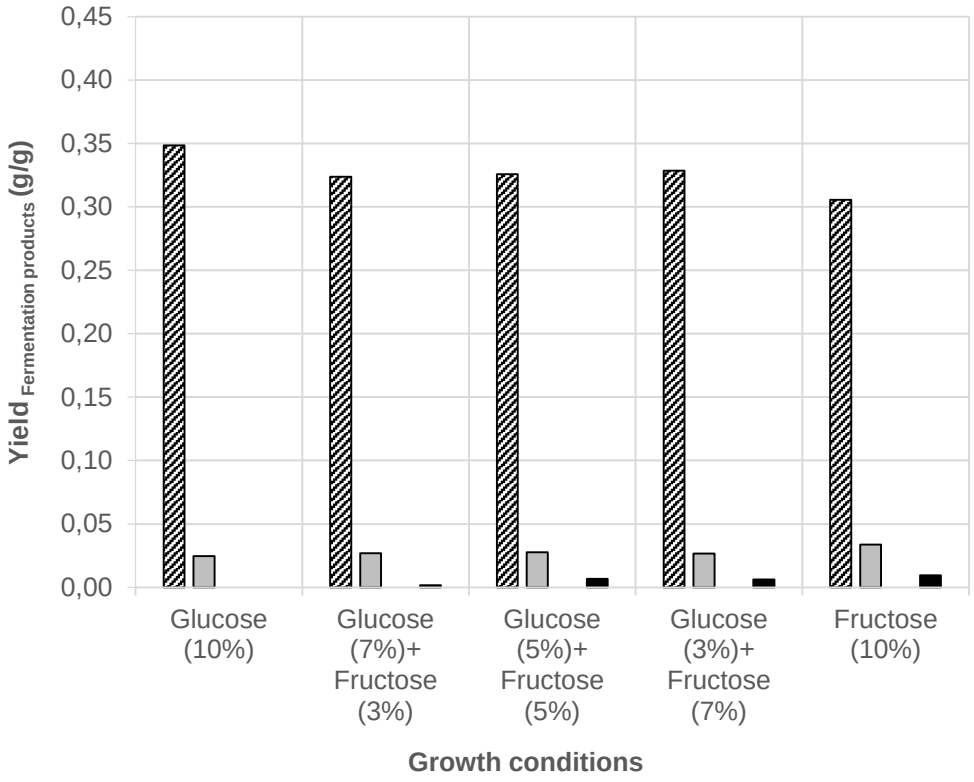
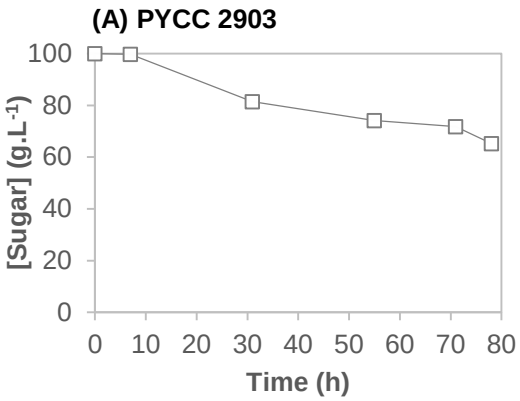


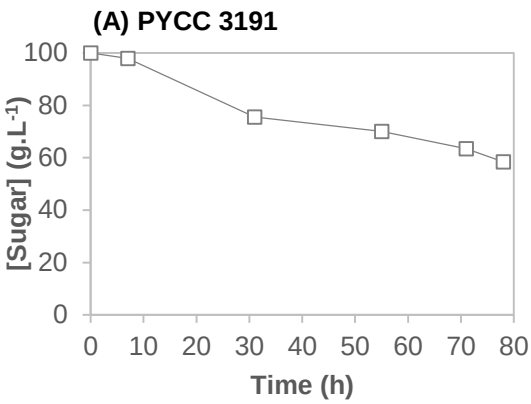
Figure 3.12- Ethanol (▨), glycerol (□), erythritol (□) and mannitol (■) yield for *Candida magnoliae* PYCC 2903 incubated aerobically at different and equal sugar ratios.

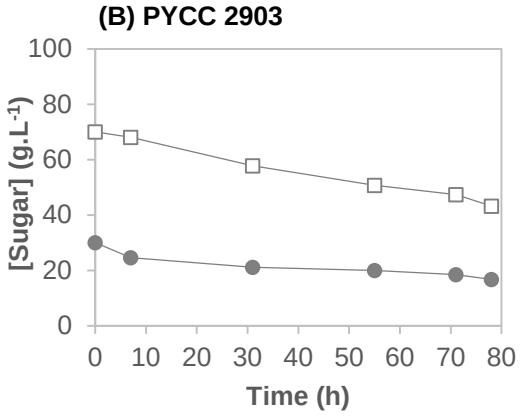
The fermentation product profiles corroborated the results from Figure 3.5, with the ratio between ethanol and sugar alcohols very high due to a high ethanol and low sugar alcohols yield production. Ethanol yield varies between 0.31 and 0.35g/g with the higher value shown when fructose is absent (10% glucose) and lowest value is observed for conditions when is only present fructose (10% fructose). On the other hand, glycerol yields are around 0.03g/g regardless of the conditions and mannitol varies from 0 to 0.01g/g with the highest value registered for conditions in which glucose is absent (10% fructose) and lowest value happen for conditions only with glucose (10% glucose).

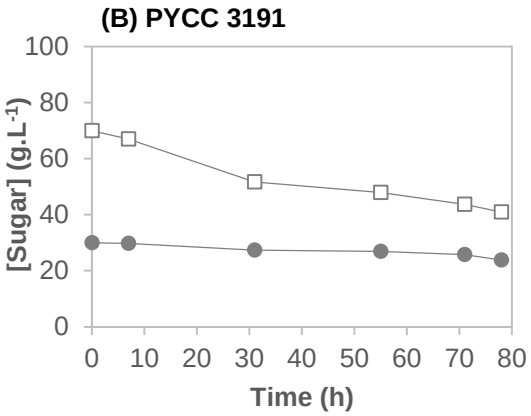
3.1.3.2 Oxygen limitation

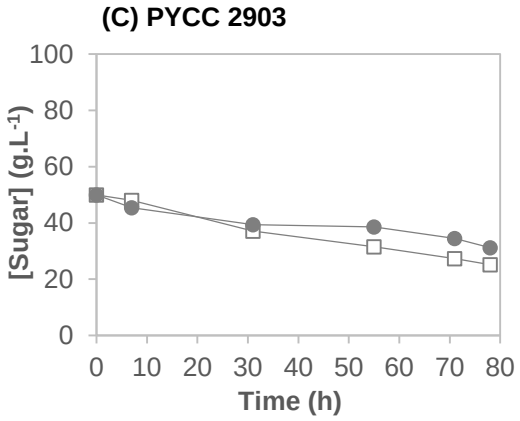
Sugar consumption profiles for *C.magnoliae* PYCC 2903 and PYCC 3191 simulating industrial fructose-rich substrates are demonstrated in Figure 3.13.

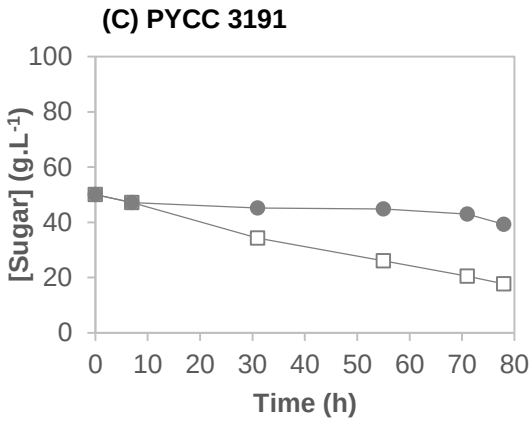

(A) PYCC 2903
[Sugar] (g.L-1)
Time (h)

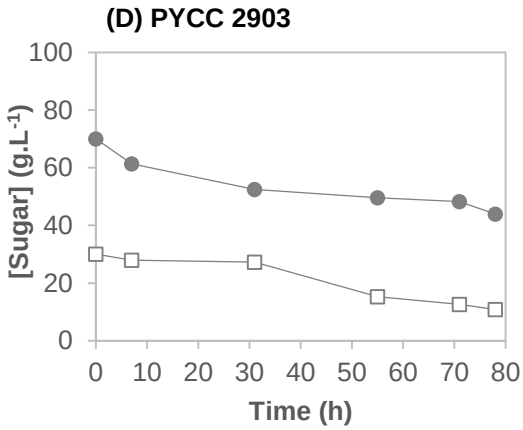

(A) PYCC 3191
[Sugar] (g.L-1)
Time (h)

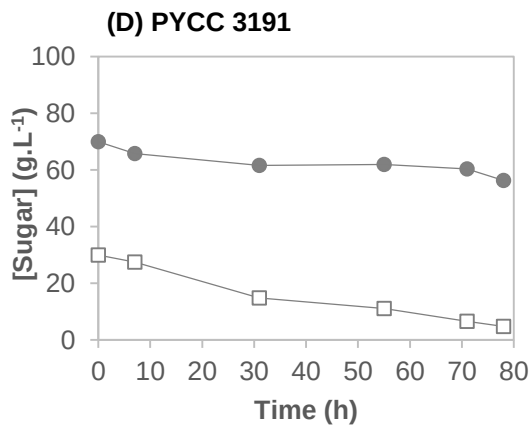

(B) PYCC 2903
[Sugar] (g.L-1)
Time (h)


(B) PYCC 3191
[Sugar] (g.L-1)
Time (h)


(C) PYCC 2903
[Sugar] (g.L-1)
Time (h)


(C) PYCC 3191
[Sugar] (g.L-1)
Time (h)


(D) PYCC 2903
[Sugar] (g.L-1)
Time (h)


(D) PYCC 3191
[Sugar] (g.L-1)
Time (h)

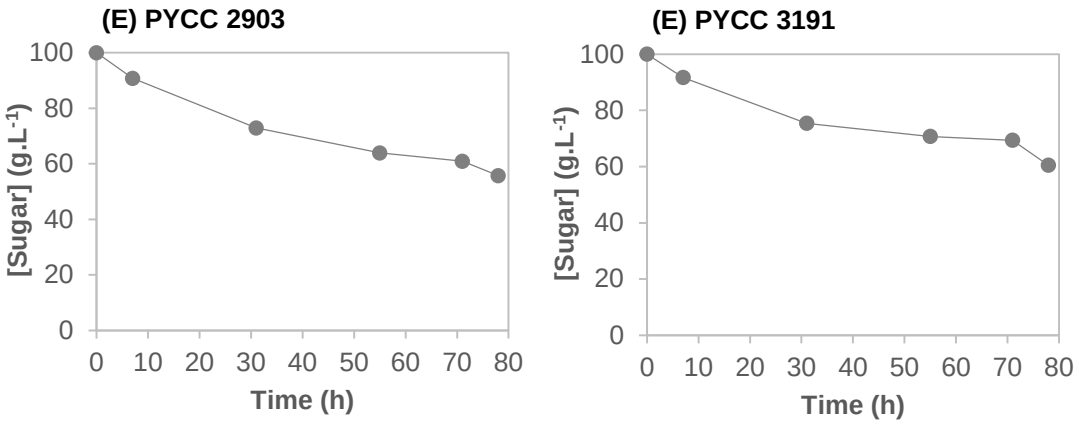


Figure 3.13- Glucose (□) and fructose (■) consumption for *Candida magnoliae* PYCC 2903 and PYCC 3191 incubated with oxygen limitation at different and equal sugar ratios (A) 10% Glucose, (B) 7% Glucose and 3% Fructose, (C) 5% of both sugars, (D) 3% Glucose and 7% Fructose and (E) 10% Fructose.

In graphs from Figure 3.13 occurs the same as in Figure 3.10 and the fact that fructophily it is not apparent might be related with the way that cells were pre-grown.

Table 3.7 and Figure 3.14 show glucose and fructose consumption rates and fructophily values, calculated from sugar consumption profiles represented in Figure 3.13, for *C.magnoliae* PYCC 2903 and PYCC 3191 simulating industrial fructose-rich substrates.

Table 3.7- Glucose and fructose consumption rates and fructophily analysis for *Candida magnoliae* PYCC 2903 and PYCC 3191 incubated with oxygen limitation at different and equal sugar ratios.

Strain	Growth conditions	Consumption rate (mmol/L.h)			Fructophily
		Glucose	Fructose	Total sugar	
PYCC 2903	Gluc (10%)	2.4	-	2.4	-
	Gluc (7%) + Fruc (3%)	1.9	0.8	2.7	0.4
	Gluc (5%) + Fruc (5%)	1.8	1.1	2.9	0.6
	Gluc (3%) + Fruc (7%)	1.4	1.5	2.9	1.1
	Fruc (10%)	-	2.9	2.9	-
PYCC 3191	Gluc (10%)	2.9	-	2.9	-
	Gluc (7%) + Fruc (3%)	2.0	0.4	2.4	0.2
	Gluc (5%) + Fruc (5%)	2.3	0.6	2.9	0.2
	Gluc (3%) + Fruc (7%)	1.8	0.7	2.5	0.4
	Fruc (10%)	-	2.4	2.4	-

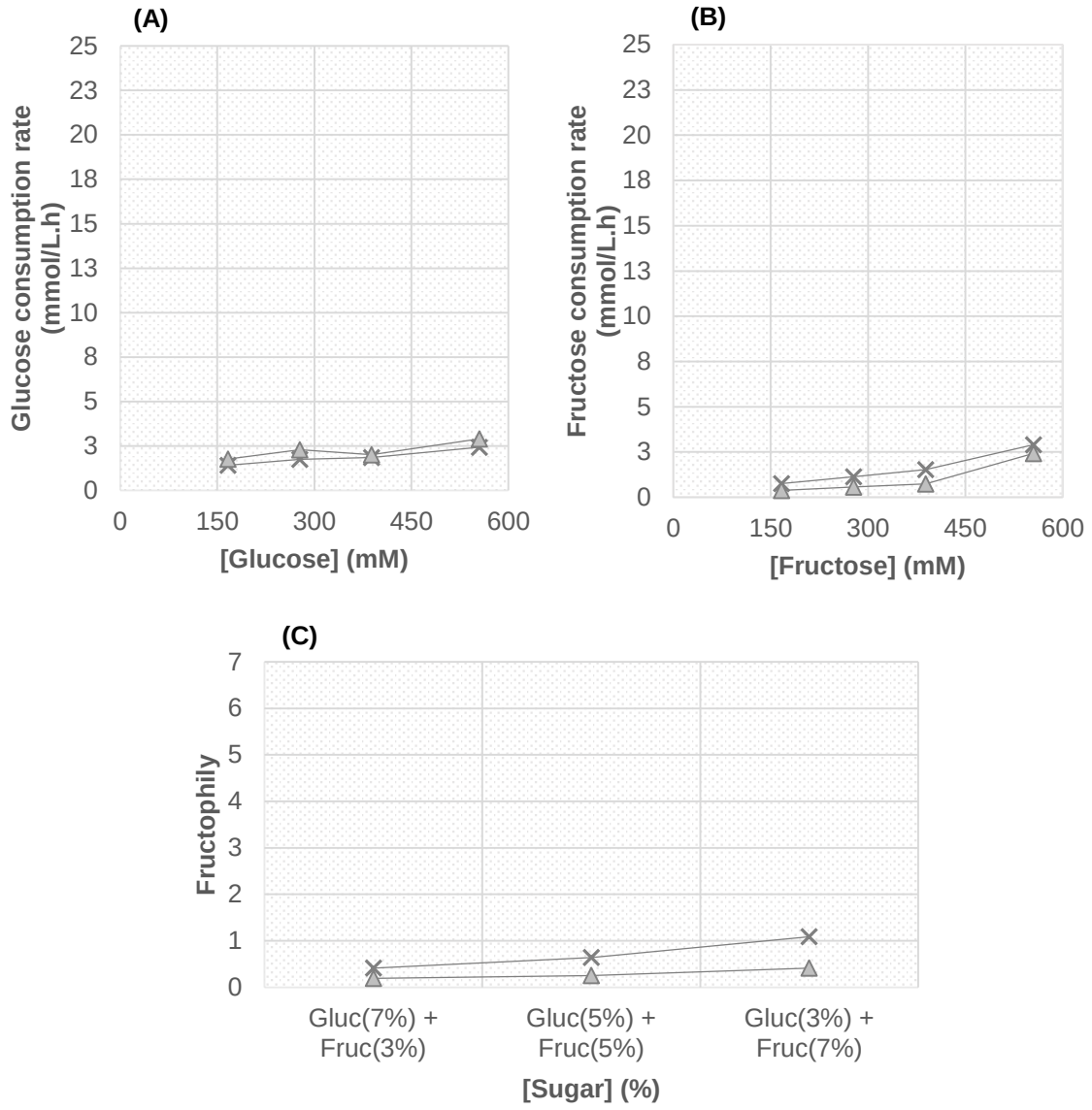


Figure 3.14- Glucose (A) and fructose (B) consumption rates and fructophily analysis (C) for *Candida magnoliae* PYCC 2903 (×) and PYCC 3191 (▲) incubated with oxygen limitation at different and equal sugar ratios.

Results from Table 3.7 and Figure 3.14 show for both strains fructophily values lower or close to 1, not revealing a fructophilic behaviour.

Although under these fermentation conditions, simulating industrial fructose-rich substrates, *C.magnoliae* fructophily is practically absent, higher values were displayed for PYCC 2903 than for 3191, which is in agreement with results obtained in **3.1.2.**

Fermentation products yield profiles for *Candida magnoliae* PYCC 2903 (A) and PYCC 3191 (B) under conditions simulating industrial fructose-rich substrates are shown in Figure 3.15.

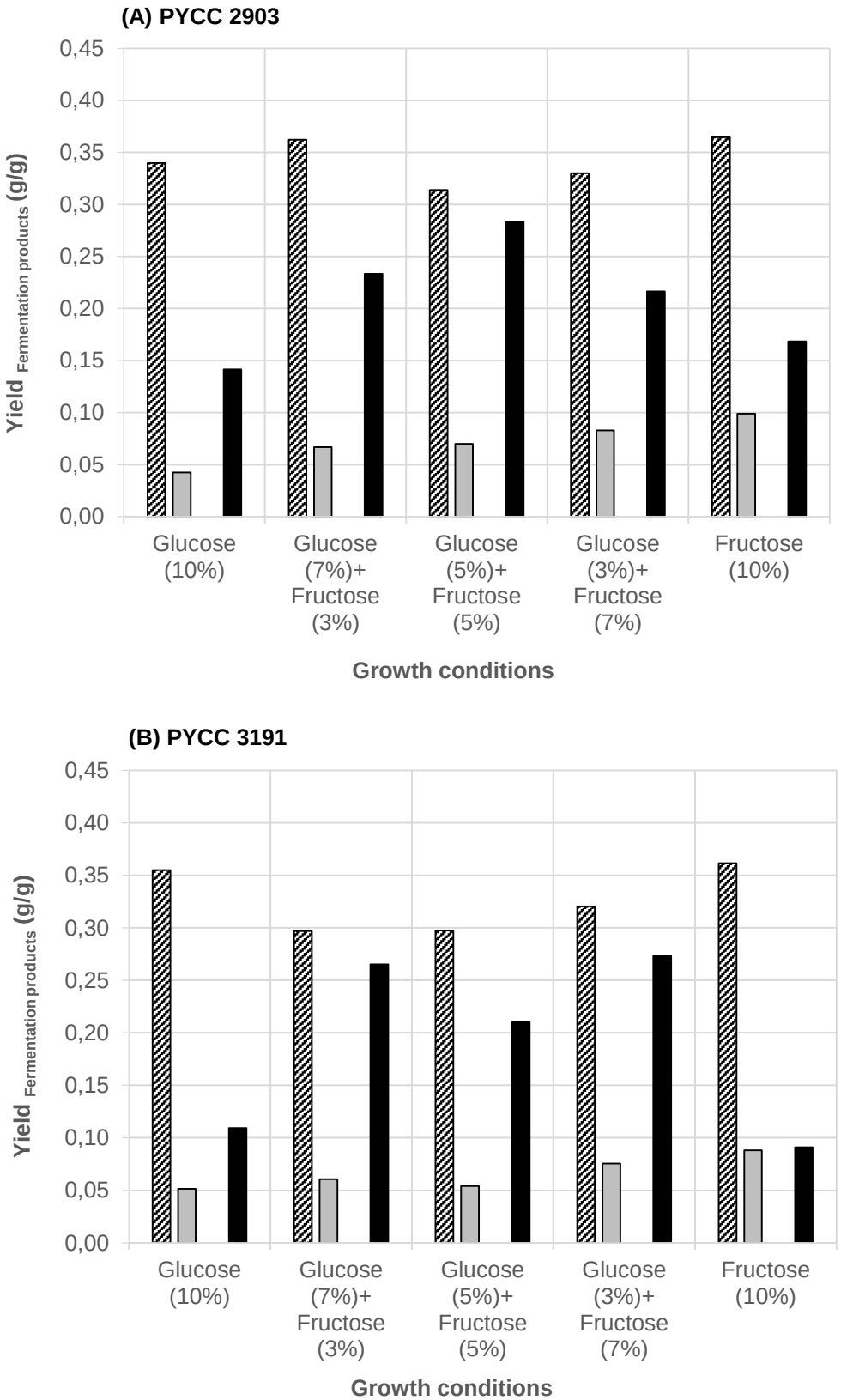


Figure 3.15- Ethanol (▨), glycerol (□), erythritol (□) and mannitol (■) yield for *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191, incubated with oxygen limitation at different and equal sugar ratios.

The conditions used in these experiments are likely to be those representing a better approach to anaerobiosis. In fact, the diffusion of oxygen is reduced due to the non-agitated cultures on flasks with a small headspace and the high cell density used as inoculum, which rapidly consumes some available oxygen. Graphs (A) and (B) in Figure 3.15 show that when oxygen is really limited, these strains use more sugar for the formation of glycerol and mannitol. These fermentation products profiles exhibit a different pattern when compared with Figure 3.12, representing a similar experiment but in agitation conditions on flask with high headspace. Apparently, the relative metabolic flux of glucose and fructose does not significantly contribute to modulate the fermentation products.

For both strains, ethanol production yield is between 0.3 and 0.37 g/g with the lowest value obtained in the presence of 5% of each sugar. Mannitol production yield is the one with a wider range, between 0.09 and 0.28 g/g, and the higher value differs from strains: for PYCC 2903 occurs for 5% glucose and fructose and for PYCC 3191 occurs with 3% glucose and 7% fructose. Glycerol production yield is also higher than in profile illustrated in Figure 3.12 with the values varying between 0.04 and 0.1 g/g and for both strains the higher value is displayed when culture medium simply have fructose (10% fructose). For these conditions, as well as in Figure 3.12, erythritol is not produced by both *C.magnoliae* strains, so the yield for this sugar alcohol formation is zero.

Results obtained for fermentation profiles of *Candida magnoliae* PYCC 2903 and PYCC 3191 slightly differs from that described in the literature mainly in terms of fermentation products (Lee *et al*., 2003; Ryu *et al*., 2000; Yu *et al*., 2006). These works described *C.magnoliae* as non-producing ethanol and to be a high erythritol producer, which is not observed for the strains used in the present work. The fermentation conditions such as temperature, shaking or stirring speed, sugar ratio and concentration and fermentation time for complete fructose depletion varies from those used in this work and all these variations might modulate the outcomes. *C.magnoliae* strains, wild type (KFCC 11023 and KCCM-10252) and a mutant (M2) with improve erythritol productivity, used in previous studies, were isolated in Korea from a fermentation sludge and honeycomb.

3.2 Part II

Enzymatic contribution for *Candida magnoliae* fructophilic behaviour: Study of hexokinase activity in terms of capacity (V_{max}) and sugar affinity (K_m)

Candida magnoliae fructophily might be due to the two main steps that differ in glucose and fructose metabolism: (1) Transport carried out by fructose transporters and/or (2) Sugar phosphorylation performed by hexokinases. In this project attention was given to phosphorylation step.

With the purpose of trying to explain this particular behaviour in this yeast, enzymatic assays were performed to evaluate the activity of this enzyme in terms of capacity (V_{max}) and affinity (K_m) for glucose and fructose.

During these enzymatic assays, hexokinase activity was indirectly measured considering the scheme in Figure 3.16. This enzyme integrates the glycolytic pathway and is responsible for phosphorylation of glucose to glucose-6-phosphate (Glc-6-P) and fructose to fructose-6-phosphate (Frc-6-P). However, the conversion of ATP cofactor into ADP resulting from the sugars phosphorylation cannot be measured directly. Therefore, it was necessary to make use of glucose-6-phosphate dehydrogenase as a coupling enzyme. This reaction consists in the formation of NADPH resulting from Glucose-6-phosphate dehydrogenase activity, which converts glucose-6-phosphate (Glc-6-P) into 6-phosphogluconate (6-PG). In the case of fructose a second coupled enzyme is necessary to convert Fructose-6-phosphate into Glucose-6-phodphate.

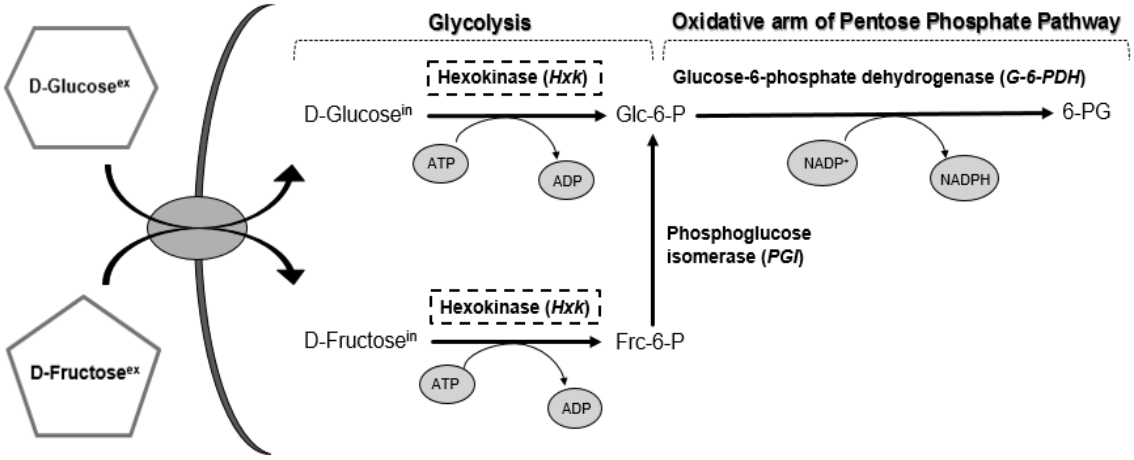


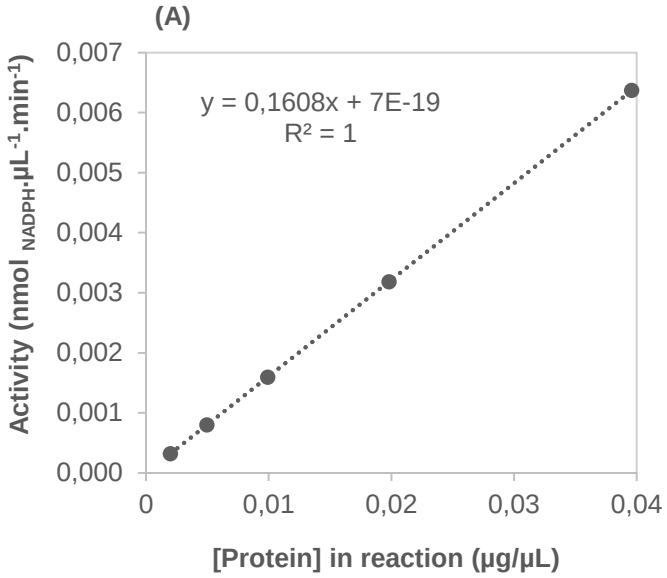
Figure 3.16- Illustrative scheme of hexokinase activity and subsequent steps used for this enzyme activity measurement. Abbreviations: ATP (Adenosine triphosphate), ADP (Adenosine diphosphate), Frc-6-P (Fructose-6-phosphate), Glc-6-P (Glucose-6-phosphate), $NADP^+$ (Nicotinamide adenine dinucleotide phosphate oxidized form), NADPH (Nicotinamide adenine dinucleotide phosphate reduced form) and 6-PG (6-phosphogluconate).

3.2.1 Hexokinase preliminary validation tests

Before hexokinase capacity and affinity assays, different preliminary validation tests were performed to confirm the rate-limitation conditions of the enzymatic assay, i.e. to confirm that the

coupled enzymes, *G-6-PDH* and *PGI*, are in excess. For that, increasing amounts of cell extract containing the hexokinase to be measured were used. Based on the known hexokinase kinetics of *Saccharomyces cerevisiae*, it was assumed that 50mM for glucose or fructose are substrate-saturated conditions.

Results obtained for *G-6-PDH* are shown in (A) Figure 3.17 as well as the conditions used for this assay (B).



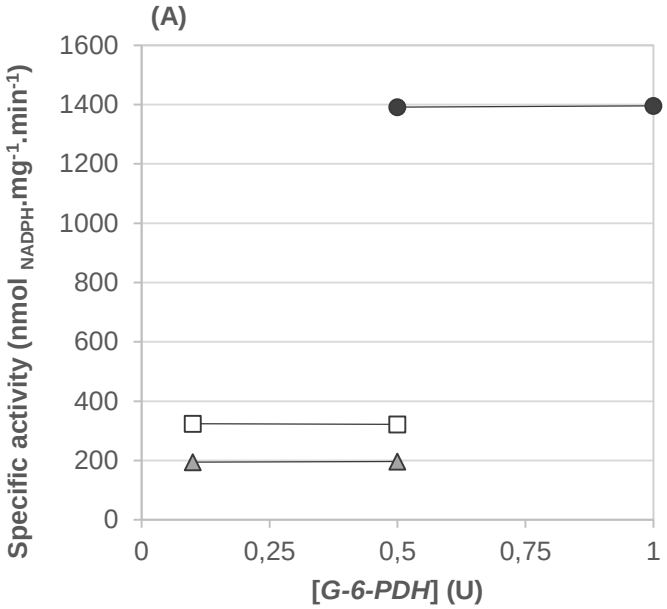
(B)

Enzymatic conditions	
[*G-6-PDH*] (U)	0.2

Figure 3.17- (A) Effect of hexokinase amount on the reaction rate, measured by the increase of NADPH over the first 60s after the addition of 50mM glucose. Cell extracts were prepared from *Candida magnoliae* PYCC 2903 grown in 10% of glucose and fructose.

For this assay, increasing amounts of cell extract: 0.002, 0.005, 0.010, 0.020 and 0.040µg total protein/µL were used. In Figure 3.17 (A), it is visible that hexokinase activity increased linearly with protein concentration suggesting that the reaction is limited by the enzyme to be measured.

In order to confirm these results, an additional assay for this enzyme was performed using extracts from three different yeast species, *S.cerevisiae*, *C.magnoliae* PYCC 2903 and PYCC 3191. Results obtained for this test (A) and conditions used (B) are demonstrated in Figure 3.18.



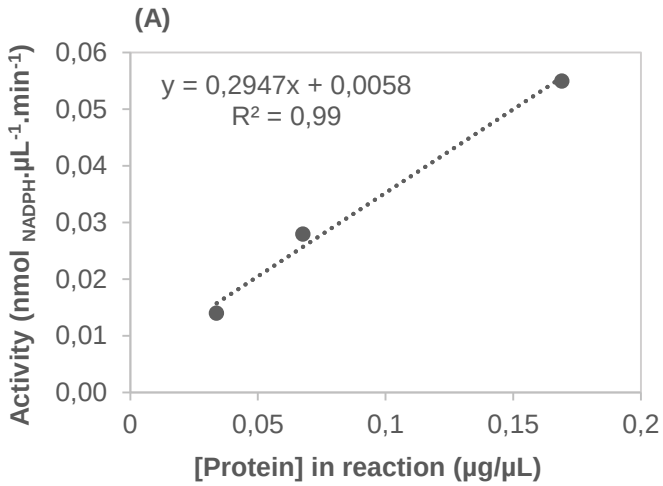
(B)

Enzymatic conditions	
S.cerevisiae [Extract] (mg/mL)	0.02
PYCC 2903 [Extract] (mg/mL)	0.02
PYCC 3191 [Extract] (mg/mL)	0.03

Figure 3.18- (A) Effect of *G-6-PDH* amount on the reaction rate, measured by the increase of NADPH over the first 60s after the addition of 50mM glucose. *S.cerevisiae* (●), *C.magnoliae* PYCC 2903 (▲) and PYCC 3191 (□). (B) Enzymatic conditions used for the assay with *S.cerevisiae*, *C.magnoliae* PYCC 2903 and PYCC 3191.

For this test, increasing *G-6-PDH* concentrations as 0.1 and 0.5U for *C.magnoliae* strains and 0.5 and 1U for *S.cerevisiae* were used. For both assays, hexokinase specific activity nearly does not varies within the same extract indicating that even the lower value of *G-6-PDH* enzyme (0.1U) can be used for enzymatic reaction once does not limit the reaction.

Results for Phosphoglucose Isomerase activity assay 1 are illustrated in (A) Figure 3.19. For this assay, the same *G-6-PDH* concentration, as specified in (B) was used.



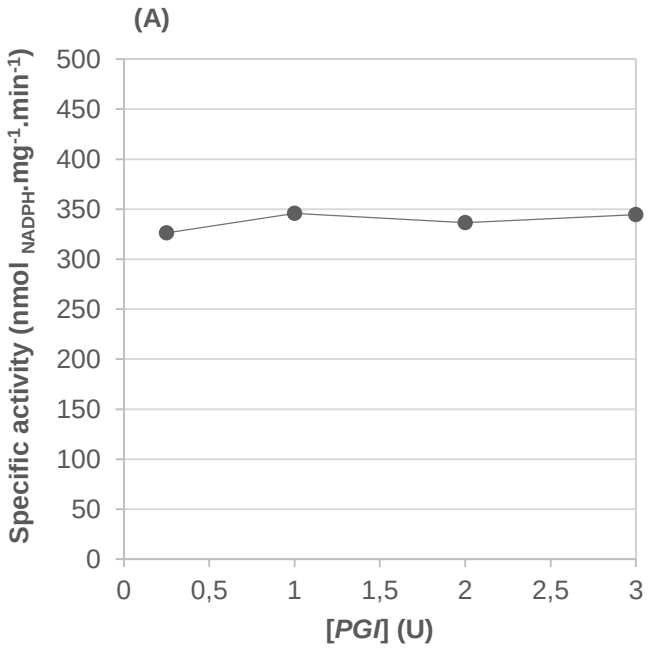
(B)

Enzymatic conditions	
[*G-6-PDH*] (U)	0.2
[*PGI*] (U)	1

Figure 3.19- (A) Effect of hexokinase amount on the reaction rate, measured by the increase of NADPH over the first 60s after the addition of 50mM fructose. Cell extracts were prepared from *Candida magnoliae* PYCC 2903 grown in 1% of glucose and fructose.

For this assay, increasing amounts of cell extract as 0.03, 0.07 and 0.17µg (total protein)/µL were used. In this test, hexokinase activity increased linearly with protein concentration indicating that 1U of *PGI* enzyme does not limit the reaction.

In order to confirm these results, an additional assay for this enzyme was performed using the same *G-6-PDH* concentration in the reaction, as demonstrated in (B) Figure 3.20, however changing *PGI* concentration. Results for this test are shown in (A) Figure 3.20.



(B)

Enzymatic conditions	
[Extract] (mg/mL)	0.1
[*G-6-PDH*] (U)	0.2

Figure 3.20- (A) Effect of *PGI* amount on the reaction rate, measured by the increase of NADPH over the first 60s after the addition of 50mM fructose. Cell extracts were prepared from *Candida magnoliae* PYCC 2903 grown in 1% of glucose and fructose.

In this assay, increasing concentrations of *PGI* enzyme as 0.25, 1, 2 and 3U were used. Results derived from this assay show that hexokinase specific activity is slightly lower (326 $nmol.mg^{-1}.min^{-1}$) when was added to reaction 0.25U of *PGI* enzyme. Despite the similarity of specific activity values, such results evidence that lower *PGI* concentration can slightly interferes in reaction and to make sure that this is not a limiting step, at least 1U of enzyme should be added to enzymatic reaction. This confirms the previous test for *PGI* wherein 1U of this enzyme is not a limiting condition for hexokinase activity measurement.

After performing these preliminary tests, the conditions used during enzymatic reaction were standardized and hexokinase activity measurement assays in terms of capacity (V_{max}) and affinity (K_m) for glucose and fructose were accomplished.

3.2.2 Enzyme capacity (V_{max}) and affinity (K_m)

Hexokinase capacity (V_{max}) results for *C.magnoliae* strains, PYCC 2903 and PYCC 3191, grown in different sugar concentrations (1%, 5% and 10% of each sugar) and using 20, 50 or 100mM of glucose or fructose during enzymatic reaction are illustrated in Table 3.8.

Table 3.8- *Candida magnoliae* PYCC 2903 and PYCC 3191 hexokinase capacity (V_{max}) results using 20, 50 or 100mM glucose or fructose for cells grown in 1%, 5% and 10% of each sugar.

Strain	Growth conditions	[Sugar] (mM)	Kinetic parameter V_{max} (nmol.mg^{-1}.min^{-1})	$V_{max\ Fruc}$ / $V_{max\ Gluc}$
PYCC 2903	Glucose (1%) + Fructose (1%)	Glucose (20)*	190±6	-
		Glucose (50) Fructose (50)	224 477	2.1
	Glucose (5%) + Fructose (5%)	Glucose (20)*	216±14	-
		Glucose (50) Fructose (50)	194 474	2.4
	Glucose (10%) + Fructose (10%)	Glucose (50) Fructose (50)	122 463	3.8
		Glucose (100) Fructose (100)	147 414	2.8
PYCC 3191	Glucose (1%) + Fructose (1%)	Glucose (20)*	204±1	-
	Glucose (5%) + Fructose (5%)	Glucose (20)*	252±0	-
	Glucose (10%) + Fructose (10%)	Glucose (20)*	241±5	-
		Glucose (100) Fructose (100)	304 527	1.7

*V_{max}'s using 20mM of glucose represent mean values with standard deviation (SD) (n=2).

For both strains and growth conditions, the fundamental confirmation obtained from these results regards the dissimilarity between glucose and fructose V_{max} values. Fructose V_{max} values are 2 or 3 times higher than glucose suggesting that hexokinase enzyme have more capacity for fructose. There are no relevant differences between cells grown on different sugar concentrations. Apparently, both glucose and fructose V_{max}'s are higher for PYCC 3191 than for PYCC 2903.

For enzymatic affinity (K_m) assays, increasing glucose (0.04, 0.08, 0.16, 0.4, 1.2 and 6mM) and fructose concentrations (0.5, 2, 5, 10 and 20mM) were used. Hexokinase activity profiles for glucose and fructose are illustrated in Figure 3.21 and Figure 3.22, respectively. These assays were carried out using *C.magnoliae* PYCC 2903 and PYCC 3191 extracts grown in 1% of each sugar.

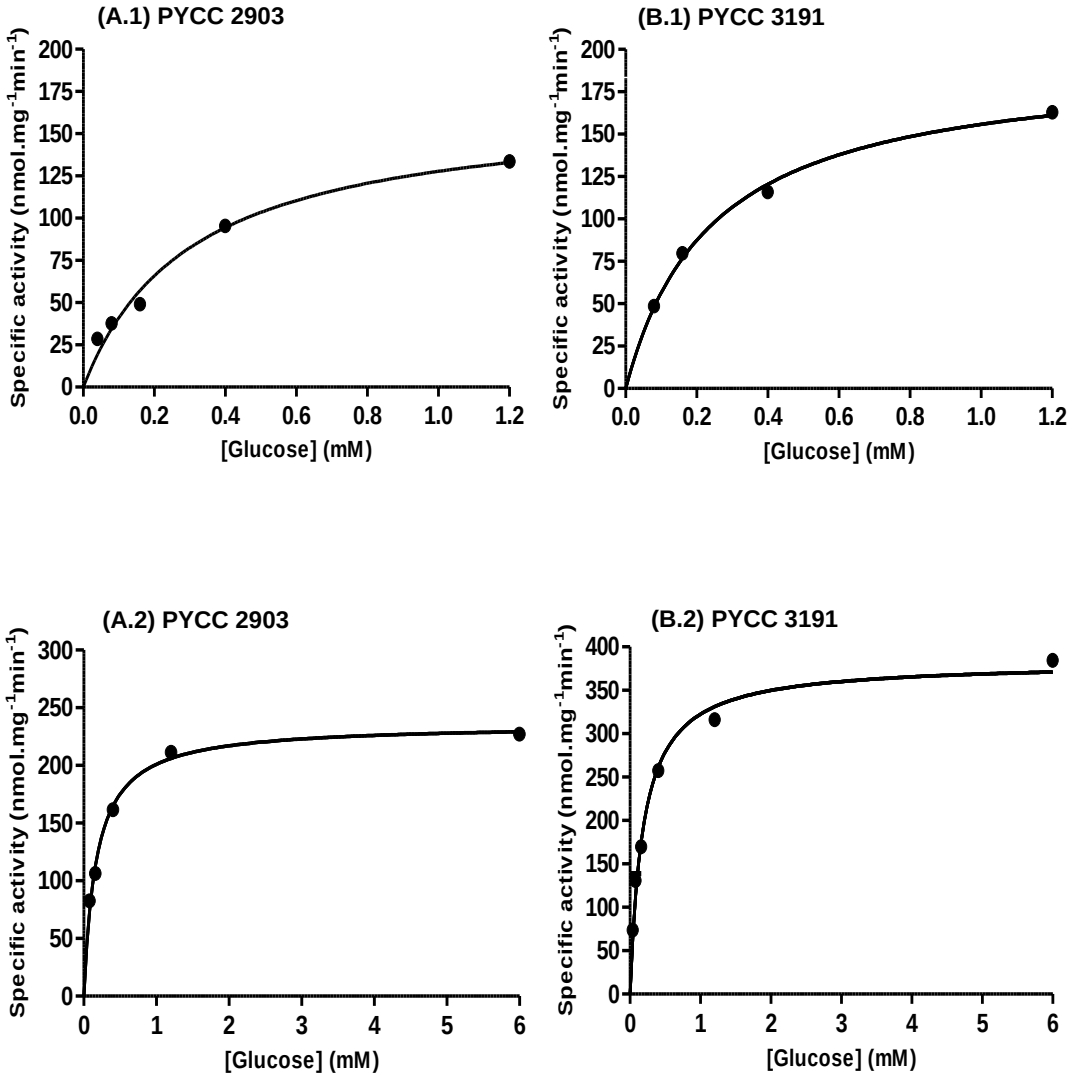


Figure 3.21- Michaelis-Menten plots of glucose phosphorylation by hexokinase of *Candida magnoliae* strains. (A.1) and (A.2) PYCC 2903 assay 1 and 2. (B.1) and (B.2) PYCC 3191 assay 1 and 2. Dots for (A.1), (B.1) and (B.2) represent mean values with standard deviation (SD) (n=2).

Results of hexokinase kinetic profiles for glucose, illustrated in Figure 3.21, are represented in two different graphs, (A.1), (A.2) and (B.1), (B.2), because extracts used for these assays were prepared in different occasions.

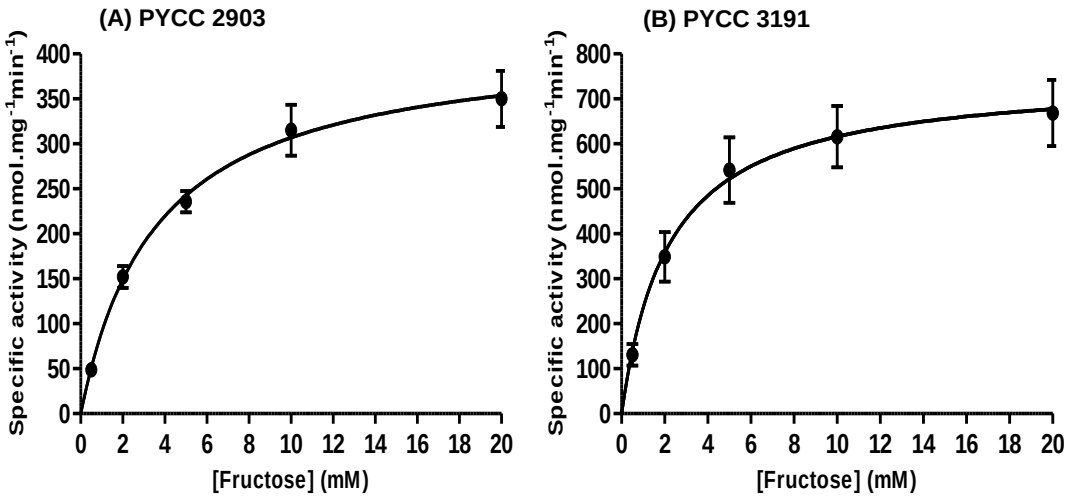


Figure 3.22- Michaelis-Menten plots of fructose phosphorylation by hexokinase of *Candida magnoliae* strains, (A) PYCC 2903 and (B) PYCC 3191. Dots represent mean values with standard deviation (SD) for (A) with n=3 and for (B) with n=4.

Table 3.9 resumes *C.magnoliae* strains hexokinase kinetic parameters values obtained from these assays (Figure 3.21 and Figure 3.22) and compares affinity and capacity of this enzyme between glucose and fructose.

Table 3.9- *Candida magnoliae* PYCC 2903 and PYCC 3191 hexokinase kinetic parameters, capacity (V_{max}) and affinity (K_m), and their ratio between glucose and fructose.

Strain	Assay	Kinetic parameters				K_m Fruc / K_m Gluc	V_{max} Fruc / V_{max} Gluc
		Glucose		Fructose			
		K_m (mM)	V_{max} (nmol.mg^{-1}.min^{-1})	K_m (mM)	V_{max} (nmol.mg^{-1}.min^{-1})		
PYCC 2903	A.1	0.3±0.04	167±9	3.6±0.5	417±17	12	2.5
	A.2	0.2±0.02	236±6			18	1.8
PYCC 3191	B.1	0.2±0.02	194±4	2.2±0.4	752±35	11	3.9
	B.2	0.2±0.02	383±8			11	2.0

These V_{max} results, Table 3.9, corroborate those previously, once hexokinase capacity is 2 or 3 times higher for fructose than glucose.

For both strains, glucose V_{max} from assay 1 and 2 are different however, these values are always lower than the V_{max} for fructose. On the other hand, glucose K_m values between assay 1 and 2 remain almost unchanged. For these two strains, glucose affinity values are quite similar,

around 0.2mM, but fructose varies, namely 3.6mM and 2.2mM for PYCC 2903 and PYCC 3191, respectively. Thus, *C.magnoliae* hexokinase K_m for fructose is between 11 and 18 times higher than for glucose. This condition implies that affinity for fructose is much lower than for glucose, so hexokinase affinity constant does not explain *Candida magnoliae* fructophilic behaviour.

C.magnoliae PYCC 2903 and PYCC 3191 hexokinase results suggests a similarity with hexokinase I of *Saccharomyces cerevisiae* which has a K_m for glucose of 0.12mM and for fructose of 1.5mM and a V_{max} three times higher for fructose than glucose (Berthels *et al*., 2008).

3.3 Part III

Evaluation the potential of *Candida magnoliae* yeast to yield low-alcoholic fermented beverages using real industrial fructose-rich substrates as fruit juices

The main objective of characterizing the fermentation profiles of these two *Candida magnoliae* strains under industrial fructose-rich substrates conditions was to evaluate the potential use of this yeast in industrial biotechnology, particularly in the low-alcoholic fermented beverages industry.

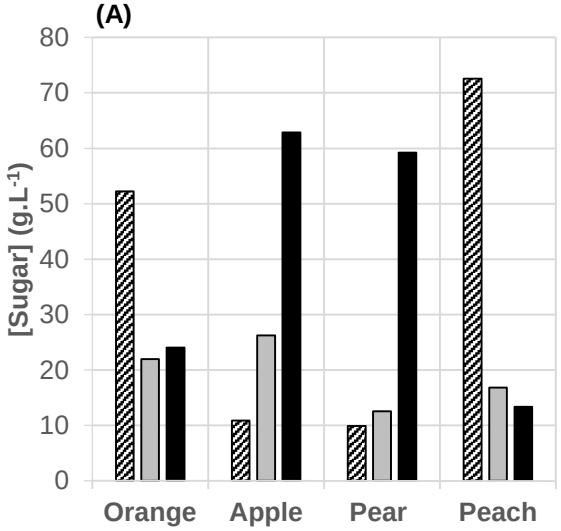
Candida magnoliae PYCC 2903 has a well-defined fructophilic profile in the majority of tested conditions, exhibiting preferential consumption of fructose compared to glucose when both sugars are present in the growth medium, and higher yield of sugar alcohols in fermentations simulating industrial fructose-rich substrates under oxygen limitation conditions.

3.3.1 Sugar composition profile of fruit substrates

Fermentations of fructose-rich substrates by *Candida magnoliae* PYCC 2903 were carried out on four fruit juices: orange, apple, pear and peach.

Orange, pear and peach juices were diluted in order to start the fermentation with a similar initial degree Brix (similar sugar concentration) around 11.4 and apple juice was used unaltered. Sugar composition (glucose, fructose and sucrose) of these four substrates was determined by HPLC and is illustrated in Figure 3.23.

(A)



(B)

	Glucose (%)	Fructose (%)	Fructose/ Glucose
Synthetic	7.0	3.0	0.4
culture	5.0	5.0	1.0
medium	3.0	7.0	2.3
Orange	2.2	2.4	1.1
Apple	2.6	6.3	2.4
Pear	1.3	5.9	4.5
Peach	1.7	1.3	0.8

Figure 3.23- (A) Sugar composition profile of orange, apple, pear and peach juices used in fermentation assays. Sucrose (▨), glucose (□) and fructose (■). (B) Comparison of fructose/glucose ratio between fermentations simulating industrial fructose-rich substrates of Part I and fruit juices.

As shown above, (A) Figure 3.23, juices used in fermentation assays have different amounts of sucrose, glucose and fructose in their composition. Peach and orange are those with the highest amounts of sucrose, 72.6 and 52.2g.L^{-1}, respectively. Apple (62.9g.L^{-1}) and pear

(59.2g.L^{-1}) have the highest amounts of fructose. Glucose concentration varies between 12.5 and 26.2g.L^{-1} with the lowest value for pear and highest value for apple.

Similar fructose/glucose ratios, (B) Figure 3.23, between synthetic medium conditions, previously tested in Part I, and fruit juices are shown for 5% of each sugar with orange and peach and for 3% glucose and 7% fructose with apple. In synthetic medium conditions identical to those created in pear where fructose concentration is almost five times higher than glucose were not tested.

3.3.2 Fruit juice fermentations

Juice fermentations were conducted under the same conditions as those in 3.1.3 Parte I, this using gentle magnetic stirring. *C.magnoliae* PYCC 2903 inoculum was grown on YPD medium and inoculated at $O.D_{640nm} \approx 15$.

Sugar consumption during fermentation was followed by measuring total soluble solids (TSS), which is commonly expressed as ºBrix (Terry *et al*., 2005). The results are shown in Figure 3.24 where degree Brix values for different fruit juices and growth (logarithm CFU/mL) were plotted against fermentation time, in hours.

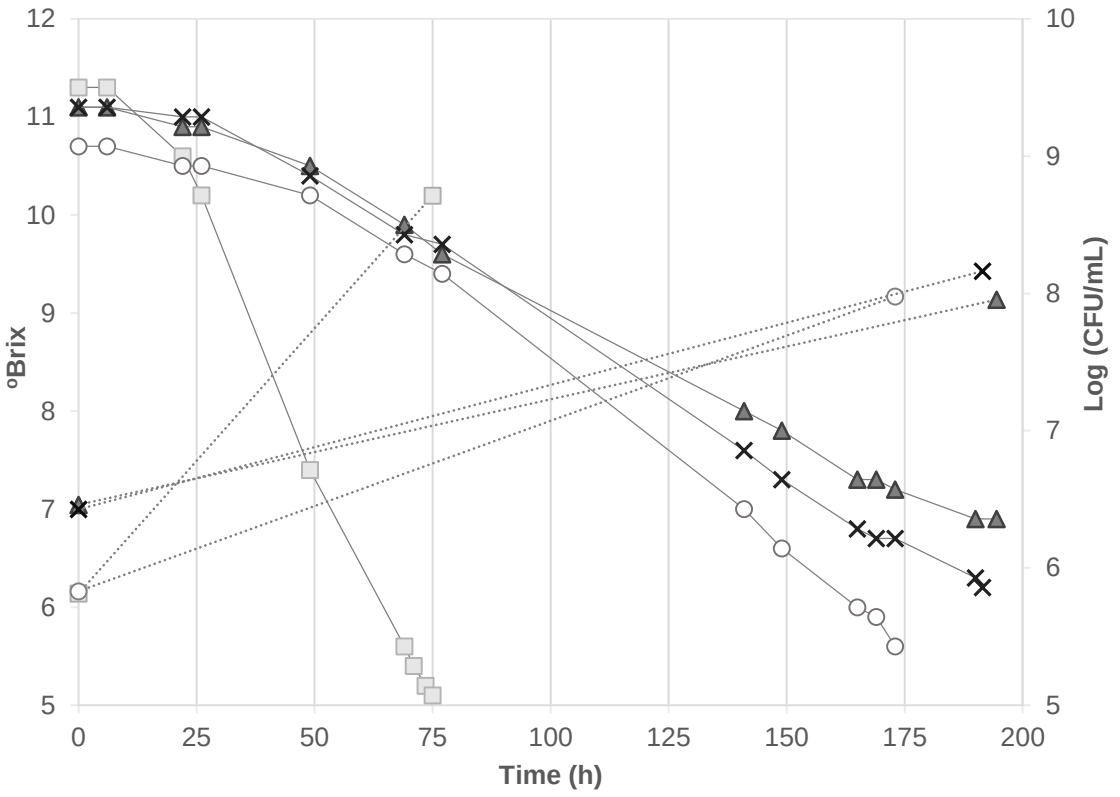


Figure 3.24- Juices fermentation, orange (□), apple (○), pear (▲) and peach (×), conducted by *C.magnoliae* PYCC 2903 at 25ºC and Log (CFU/mL) in these fructose-rich substrates.

Orange juice started with 11.3 ºBrix, pear and peach with 11.1 ºBrix and apple with 10.7 ºBrix. In orange juice fermentation sugars were consumed fastest. A ºBrix value of 5.1 was reached in just in 75 hours. Fermentation medium texture might explain such difference when compared with the other fruit juice fermentations. Orange juice being less viscous, it is easier to achieve, through gentle stirring, an equal distribution of the nutrients, allowing yeast access to the entire medium.

Pear, peach and apple juices exhibit a similar sugar reduction profile with a fermentation length between 170 and 195 hours, which are two and three times higher than orange. In the end of these fermentations, ºBrix values were 5.6, 6.9 and 6.2 for apple, pear and peach, respectively, which are higher values than for orange juice, probably due to the hampered access to sugars imposed by the high viscosity.

For both fruit juices, cell number immediately after inoculation is between 5.8 and 6.5 Log (CFU/mL) and at the end of fermentation process is between 8.0 and 8.7 Log (CFU/mL). CFU/mL results indicate that higher growth occurred when inoculation was performed in orange juice (8.7) which is not surprising since due to its lower viscosity, there is an equal distribution of nutrients and a higher oxygenation of the medium, and cells can take advantage of this sugar and oxygen availability to grow.

Table 3.10 displays total sugar reduction after the fermentations. Sugar concentrations ($g.L^{-1}$) were determined by HPLC and total sugar reduction was calculated using **Equation 3.5**.

Equation 3.5

$$\text{Total sugar reduction } (\%) = (1 - \frac{\text{Sugar consumed}}{\text{Total [Sugar]}_{\text{initial}}}) \times 100$$

where “Sugar consumed” is the difference between the sum of glucose, fructose or sucrose concentrations at the beginning and end of fermentation and Total $[Sugar]_{initial}$ is the sum of glucose, fructose and sucrose concentration at the beginning of fermentation.

Table 3.10- Fermented juices sugar reduction (%).

Juice	Total $[Sugar]_{initial}$ ($g.L^{-1}$)	Total $[Sugar]_{final}$ ($g.L^{-1}$)	Total sugar reduction (%)
Orange	98.2	5.3	94.6
Apple	100	22.1	77.9
Pear	81.7	7.8	90.5
Peach	102.7	20.9	79.6

Fermentations with higher sugar reduction were orange and pear with 94.6% and 90.5%, respectively. The other two juices, apple and peach, shows a lower total sugar reduction with values less than 80%.

3.3.3 Fermentation products

Fruit juices fermentation samples were analysed to evaluate fermentation products profiles, in order to determine the ratio between ethanol and sugar alcohols production. Figure 3.25 shows a comparison between ethanol and sugar alcohols yields.

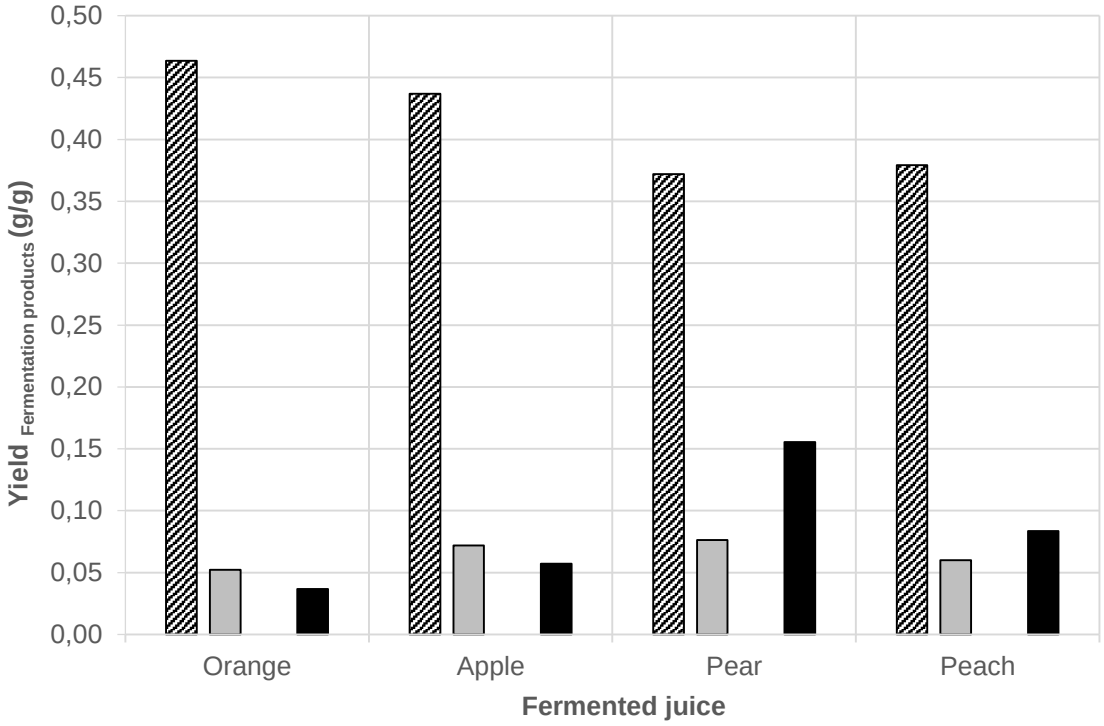


Figure 3.25- Ethanol (▨), glycerol (□), erythritol (□) and mannitol (■) yield from different fermented fructose-rich substrates such as orange, apple, pear and peach juice.

In the end of fruit juices fermentation, ethanol has a production yield between 0.37 and 0.46g/g with lower values displayed for pear (0.37g/g) and peach (0.38g/g). This fact coincides with higher yields of mannitol (0.08 and 0.16 g/g) production for peach and pear, respectively. The poorest results, in terms of fermentation products profile, were shown for fermentation in orange since it was the one that produced higher ethanol and lower sugar alcohols amounts. On the other hand, the best results were from pear and peach fermentation where the ratio between ethanol and sugar alcohols production yield was lower, evidencing a decrease in ethanol and an increase in glycerol and mannitol formation, when compared with fermented orange juice results.

3.3.4 Juices pH and titratable acidity

Besides analytes content (sugars and alcohols), fruit acids concentration can also affect flavour directly. Aiming to evaluate the acidity, the pH and total acidity, of fruit substrates and fermented juices was measured and titratable acidity (TTA) in citric acid was calculated using **Equation 3.6**.

Equation 3.6

$$\mathrm{TTA} = \mathrm{V} \times 0.064$$

in which V is the volume of NaOH (mL) used for acid titration and 0.064 was the conversion factor for citric acid.

Figure 3.26 compares pH with TTA (g citrate/100mL) values considering each juice fermentation time, in hours.

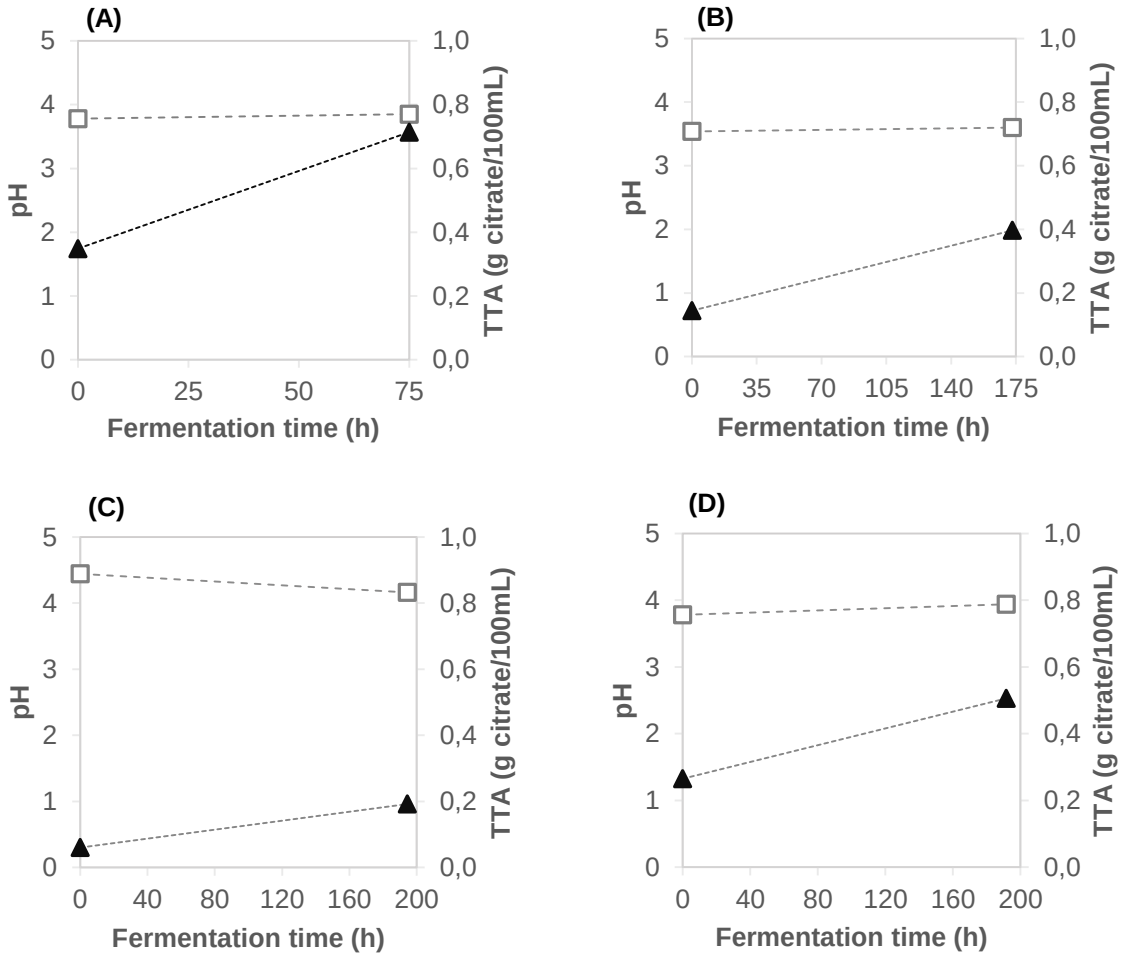


Figure 3.26- Comparison between pH (-□-) and TTA (g citrate/100mL) (-▲-) in different fruit juices. (A) Orange, (B) Apple, (C) Pear and (D) Peach.

Graphs from Figure 3.26 show small variations in pH values between fruit substrates and fermented juices with the values within a range of 3.54 and 4.44. Lower values were observed for apple (3.54 and 3.60) and higher for pear (4.44 and 4.16).

As illustrated above (Figure 3.26), TTA values are more variable between fruit substrates and fermented juices and also among juices. Higher (0.35 and 0.71 g citrate/100mL) and lower (0.06 and 0.19 g citrate/100mL) total acidity was showed for orange and pear, respectively.

3.3.5 Organoleptic evaluation: Texture, smell and taste

The fermented fruit juices were sensorially evaluated in terms of texture, smell and taste. For that, a sensorial evaluation was accomplished by two different non professional appraisers. Results of fermented juices organoleptic appreciation are described in Table 3.11.

Table 3.11- Fermented fruit juices organoleptic evaluation in terms of texture, smell and taste with an overall appreciation.

Appraiser	Juice	Texture	Smell	Taste	Overall evaluation
1	Orange	Soft and liquid	Oxidized orange	Dry flavour	+/-
	Apple	Very pulpy	Apple nectar	Floury apple; No yeasty flavour	+
	Pear	Pulpy	Pear nectar	Fruity flavour; No yeasty flavour	++
	Peach	Very pulpy	Peach nectar	Freshly cut fruit; No yeasty flavour	++
2	Orange	Soft and liquid	Oxidized orange	Very dry flavour	+/-
	Apple	Very pulpy	Apple nectar	Floury apple; No yeasty flavour	+
	Pear	Very pulpy	Freshly cut pear	Fruity flavour; No yeasty flavour	+++
	Peach	Very pulpy	Peach nectar	Freshly cut fruit; No yeasty flavour	++

Sensorial evaluation of fruit substrates fermented by *Candida magnoliae* PYCC 2903, Table 3.11, show, in general, a positive result. Fermented pear and peach were those with high classification (+++ or ++) showing minimal changes to original fruit substrates. These juices exhibit pleasant taste features without a yeasty flavour and smells like nectar or freshly cut fruit. Fermented apple achieved a classification lower than the previous ones (+) even so demonstrating enjoyable attributes such nectar smell and no yeasty flavour. As in initial substrate, apple, pear and peach juices have a pulpy texture. Fermented juice with less pleasurable sensorial characteristics was orange with a classification of (+/-). Despite orange fermentation having been the fastest consuming sugars, the final product smells like oxidized fruit and its taste is dry.

4. Conclusions

In this work, the assessment of fermentation profiles of two *Candida magnoliae* strains, PYCC 2903 and PYCC 3191, brought to light differences in sugar consumption preference and fermentation product yields. In general, PYCC 2903 strain showed the best results with well-defined fructophilic profile in the majority of the conditions tested and with the higher yield of sugar alcohols in fermentations simulating industrial fructose-rich substrates under oxygen limitation conditions. However, fermentation profiles of those *C.magnoliae* strains demonstrated some variations when compared with literature results for this yeast, mainly in terms of fermentation products. These works described *C.magnoliae* as non-producing ethanol and to be a high erythritol producer which is opposite to the results obtained from this study. Such differences might be due to differences in the yeast strains and fermentation conditions used.

The attempt to uncover the basis of the fructophilic behaviour in this yeast, through evaluation of hexokinase enzyme activity in terms of capacity (V_{max}) and affinity (K_m) for glucose and fructose was not successful, since the kinetic profile does not explain the preferential consumption of fructose by *C.magnoliae*. For both strains, fructose V_{max} and K_m values are higher than glucose suggesting that hexokinase has more capacity to transport fructose but lower affinity for this sugar than for glucose.

The use of *C.magnoliae* PYCC 2903 in fermentation of fructose-rich substrates aiming to evaluate the potential of this yeast in low-alcoholic fermented beverages industry revealed satisfying results. Although pear and peach fermentations have been the slowest, they exhibited the best results. These fermentations showed the lowest ratio between ethanol and sugar alcohol production yields, as a result of a decrease in ethanol and an increase in glycerol and mannitol formation. Moreover, sensorial evaluation of pear and peach fermented juices were those with the highest classification exhibiting pleasant taste and smell.

5. References

Akinterinwa, O., Khankal, R. and Cirino, P.C. 2008.
Metabolic engineering for bioproduction of sugar alcohols.
Current Opinion in Biotechnology. 19; 461-467.

Bach-Faig, A., Berry, E.M., Lairon, D., Reguant, J., Trichopoulou, A., Dernini, S., Medina, F.X., Battino, M., Belahsen, R., Miranda, G. and Serra-Majem, L. 2011.
Mediterranean diet pyramid today. Science and cultural updates.
Public Health Nutrition. 14; 2274-2284.

Bai, F.W., Anderson, W.A. and Moo-Young, M. 2008.
Ethanol fermentation technologies from sugar and starch feedstocks.
Biotechnology Advances. 26; 89-105.

Bennett, J.W. 1998.
Mycotechnology: the role of fungi in biotechnology.
Journal of Biotechnology. 66; 101-107.

Berthels, N.J., Otero, R.R.C., Bauer, F.F., Pretorius, I.S. and Thevelein, J.M. 2008.
Correlation between glucose/fructose discrepancy and hexokinase kinetic properties in different *Saccharomyces cerevisiae* wine yeast strains.
Applied Microbiology and Biotechnology. 77; 1083-1091.

Blandino, A., Al-Aseeri, M.E., Pandiella, S.S., Cantero, D. and Webb, C. 2003.
Cereal-based fermented foods and beverages.
Food Research International. 36; 527-543.

Brányik, T., Silva, D.P., Baszczynski, M., Lehnert, R. and Almeida e Silva, J.B. 2012.
A review of methods of low alcohol and alcohol-free beer production.
Journal of Food Engineering. 108; 493-506.

Caplice, E. and Fitzgerald, G.F. 1999.
Food fermentations: role of microorganisms in food production and preservation.
International Journal of Food Microbiology. 50; 131-149.

Diezemann, A. and Boles, E. 2003.
Functional characterization of the Frt1 sugar transporter and of fructose uptake in *Kluyveromyces lactis*.
Current Genetics. 43; 281-288.

Doehlemann, G., Molitor, F. and Hahn, M. 2005.
Molecular and functional characterization of a fructose specific transporter from the gray mold fungus *Botrytis cinerea*.
Fungal Genetics and Biology. 42; 601-610.

Francesco, G.D., Freeman, G., Lee, E., Marconi, O. and Perretti, G. 2014.
Effects of Operating Conditions during Low-Alcohol Beer Production by Osmotic Distillation.
Journal of Agricultural and Food Chemistry. 62; 3279-3286.

Galeote, V., Novo, M., Salema-Oom, M., Brion, C., Valério, E., Gonçalves, P. and Dequin, S. 2010.
FSY1, a horizontally transferred gene in the *Saccharomyces cerevisiae* EC1118 wine yeast strain, encodes a high-affinity fructose/H+ symporter.
Microbiology. 156; 3754-3761.

Ghorai, S., Banik, S.P., Verma, D., Chowdhury, S., Mukherjee, S. and Khowola, S. 2009.
Fungal biotechnology in food and feed processing.
Food Research International. 42; 577-587.

Gonçalves, P., Sousa, H.R. and Spencer-Martins, I. 2000.
FSY1, a novel gene encoding a specific fructose/H+ symporter in the type strain of *Saccharomyces carlsbergensis*.
Journal of Bacteriology. 182; 5628-5630.

Kalt, W., Forney, C.F., Martin, A. and Prior, R.L. 1999.
Antioxidant Capacity, Vitamin C, Phenolics, and Anthocyanins after Fresh Storage of Small Fruits.
Journal of Agricultural and Food Chemistry. 47; 4638-4644.

Kim, H.J., Lee, H-R., Kim, C.S., Jin, Y-S. and Seo, J-H. 2013.
Investigation of protein expression profiles of erythritol-producing *Candida magnoliae* in response to glucose perturbation.
Enzyme and Microbial Technology. 53; 174-180.

Leandro, M.J., Sychrová, H., Prista, C. and Loureiro-Dias, M.C. 2011.
The osmotolerant fructophilic yeast *Zygosaccharomyces rouxii* employs two plasma-membrane fructose uptake systems belonging to a new family of yeast sugar transporters.
Microbiology. 157; 601-608.

Leandro, M.J., Sychrova, H., Prista, C. and Loureiro-Dias, M.C. 2013.
*Zr*Fsy1, a High-Affinity Fructose/H^+ Symporter from Fructophilic Yeast *Zygosaccharomyces rouxii*.
Public Library of Science. 8; 1-10.

Lee, D-H., Kim, S-J. and Seo, J-H. 2013.
Molecular cloning and characterization of two novel fructose.specific transporters from the osmotolerant and fructophilic yeast *Candida magnoliae* JH110.
Applied Microbiology and Biotechnology. 98; 3569-3578.

Lee, J-K., Song, J-Y. and Kim, S-Y. 2003.
Controlling Substrate Concentration in Fed-Batch *Candida magnoliae* Culture Increases Mannitol Production.
Biotechnology Progress. 19; 768-775.

Liccioli, T., Chambers, P.J. and Jiranek, V. 2011.
A novel methodology independent of fermentation rate for assessment of the fructophilic character of wine yeast strains.
Journal of Industrial Microbiology and Biotechnology. 38; 833-843.

Lin, Y. and Tanaka, S. 2006.
Ethanol fermentation from biomass resources: current state and prospects.
Applied Microbiology and Biotechnology. 69; 627-642.

Llamas, N.E., Nezio, M.S.D. and Band, B.S.F. 2011.
Flow-injection spectrophotometric method with on-line photodegradation for determination of ascorbic acid and total sugars in fruit juices.
Journal of Food Composition and Analysis. 24; 127-130.

Mayer, A-M. 1997.
Historical changes in the mineral content of fruits and vegetables.
British Food Journal. 99; 207-211.

Meier, S., Karlsson, M., Jensen, P.R., Lerche, M.H. and Duus, J. 2011.
Metabolic pathway visualization in living yeast by DNP-NMR.
Molecular BioSystems. 7; 2834-2836.

Moon, H-J., Jeya, M., Kim, I-W. and Lee, J-K. 2010.
Biotechnological production of erythritol and its applications.
Applied Microbiology and Biotechnology. 86; 1017-1025.

Park, E-H., Lee, H-Y., Ryu, Y-W., Seo, J-H. and Kim, M-D. 2011.
Role of Osmotic and Salt Stress in the Expression of Erythrose Reductase in *Candida magnoliae*.
Journal of Microbiology and Biotechnology. 21; 1064-1068.

Pickering, G.J. 2000.
Low- and Reduced-alcohol Wine: A Review.
Journal of Wine Research. 11; 129-144.

Pina, C., Gonçalves, P., Prista, C. and Loureiro-Dias, M.C. 2004.
Ffz1, a new transporter specific for fructose from *Zygosaccharomyces bailii*.
Microbiology. 150; 2429-2433.

Renuka, B., Kulkarni, S.G., Vijayanand, P. and Prapulla, S.G. 2009.
Fructooligosaccharide fortification of selected fruit juice beverages: Effect on the quality characteristics.
Food Science and Technology. 42; 1031-1033.

Room, R., Babor, T. and Rehm, J. 2005.
Alcohol and public health.
Lancet. 365; 519-530.

Ross, R.P., Morgan, S. and Hill, C. 2002.
Preservation and fermentation: past, present and future.
International Journal of Food Microbiology. 79; 3-16.

Ryu, Y-W., Park, C.Y., Park, J.B., Kim, S-Y. and Seo, J-H. 2000.
Optimization of erythritol production by *Candida magnoliae* in fed-batch culture.
Journal of industrial Microbiology & Biotechnology. 25; 100-103.

Saha, B.C. and Racine, F.M. 2011.
Biotechnological production of mannitol and its applications.
Applied Microbiology and Biotechnology. 89; 879-891.

Sahoo, D.K. and Agarwal, G.P. 2002.
Effect of Oxygen Transfer on Glycerol Biosynthesis by an Osmophilic Yeast *Candida magnoliae* I_2B.
Biotechnology and Bioengineering. 78; 545-554.

Sanz, M.L., Villamiel, M. and Martínez-Castro, I. 2004.
Inositols and carbohydrates in different fresh fruit juices.
Food Chemistry. 87; 325-328.

Savergave, L.S., Gadre, R.V., Vaidya, B.K. and Jogdand, V.V. 2013.
Two-stage fermentation process for enhanced mannitol production using *Candida magnoliae* mutant R9.
Bioprocess and Biosystems Engineering. 36; 193-203.

Savergave, L.S., Gadre, R.V., Vaidya, B.K. and Narayanan, K. 2011.
Strain improvement and statistical media optimization for enhanced erythritol production with minimal by-products from *Candida magnoliae* mutant R23.
Biochemical Engineering Journal. 55; 92-100.

Scott, R. and Sullivan, W.C. 2008.
Ecology of Fermented Foods.
Human Ecology Review. 15; 25-31.

Song, S.H. and Vieille, C. 2009.
Recent advances in the biological production of mannitol.
Applied Microbiology and Biotechnology. 84; 55-62.

Sousa-Dias, S., Gonçalves, T., Leyva, J.S., Peinado, J.M. and Loureiro-Dias, M.C. 1996.
Kinetics and regulation of fructose and glucose transport systems are responsible for fructophily in *Zygosaccharomyces bailii*.
Microbiology. 142; 1733-1738.

Steinkraus, K.H. 2002.
Fermentation in World Food Processing.
Comprehensive Reviews in Food Science and Food Safety. 1; 23-32.

Terry, L.A., White, S.F. and Tigwell, L.J. 2005.
The Application of Biosensors to Fresh Produce and the Wider Food Industry.
Journal of Agricultural and Food Chemistry. 53; 1309-1316.

Wang, Z-X., Zhuge, J., Fang, H. and Prior, B.A. 2001.
Glycerol production by microbial fermentation: A review.
Biotechnology Advances. 19; 201-223.

Yu, J-H., Lee, D-H., Oh, Y-J., Han, K-C., Ryu, Y-W. and Seo, J-H. 2006.
Selective Utilization of Fructose to Glucose by *Candida magnoliae*, an Erythritol Producer.
Applied Biochemistry and Biotechnology. 131; 870-879.

Yu, J-H., Lee, D-H., Park, Y-C., Lee, M-G., Kim, D-O., Ryu, Y-W. and Seo, J-H. 2008.
Proteomic Analysis of Fructophilic Properties of Osmotolerant *Candida magnoliae*.
Journal of Microbiology and Biotechnology. 18; 248-254.

Appendices

Appendix I. Growth curves for *Candida magnoliae* strains incubated aerobically at different sugar concentrations

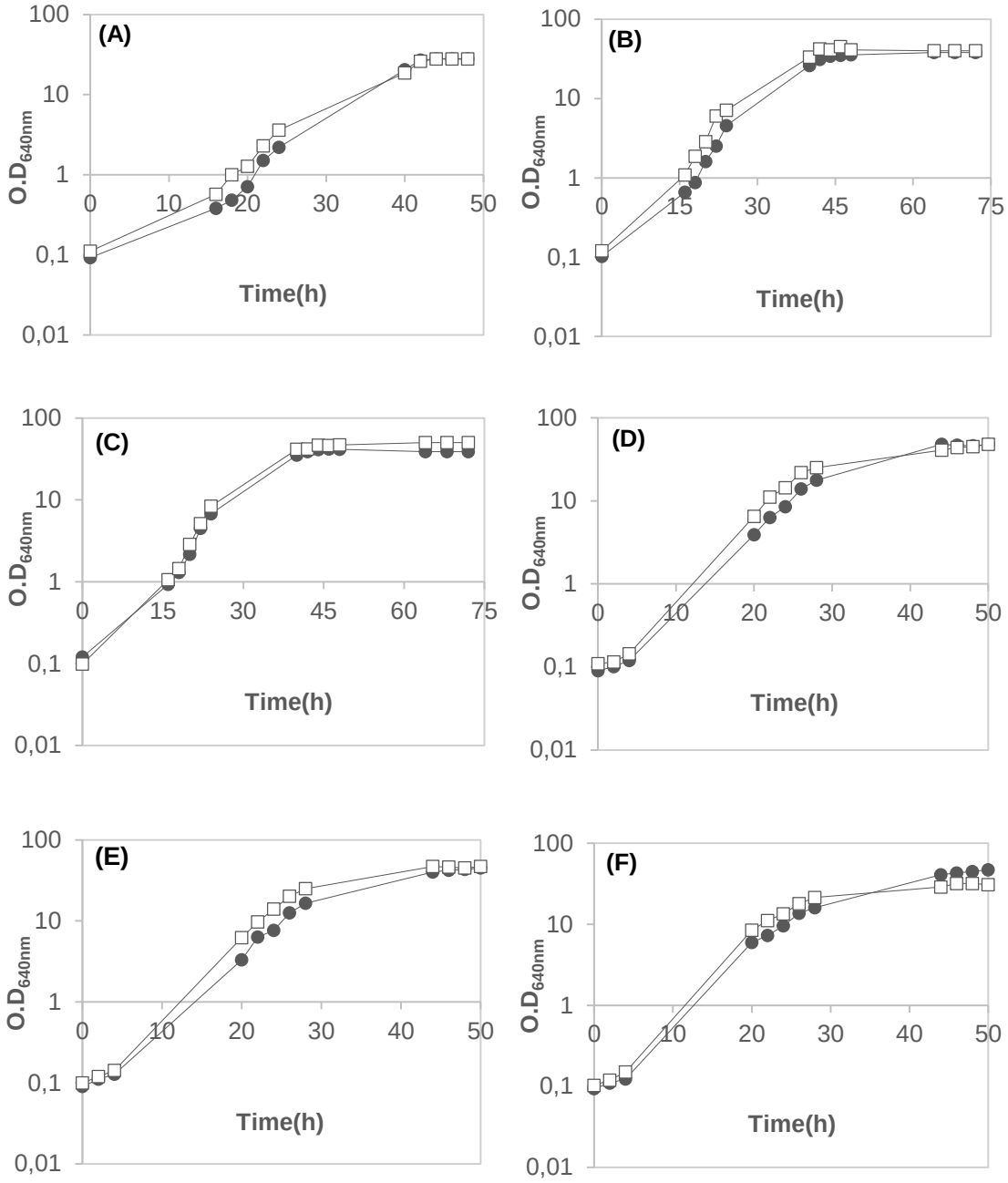


Figure A.1- Growth curves for *Candida magnoliae* PYCC 2903 (●) and PYCC 3191 (□) incubated aerobically at different sugar concentrations. (A) 15% Glucose and fructose, (B) 10% Glucose and fructose, (C) 5% Glucose and fructose, (D) 1% Glucose and fructose, (E) 2% Glucose and (F) 2% Fructose.

Appendix II. Growth curves for *Candida magnoliae* strains incubated with oxygen limitation at different sugar concentrations

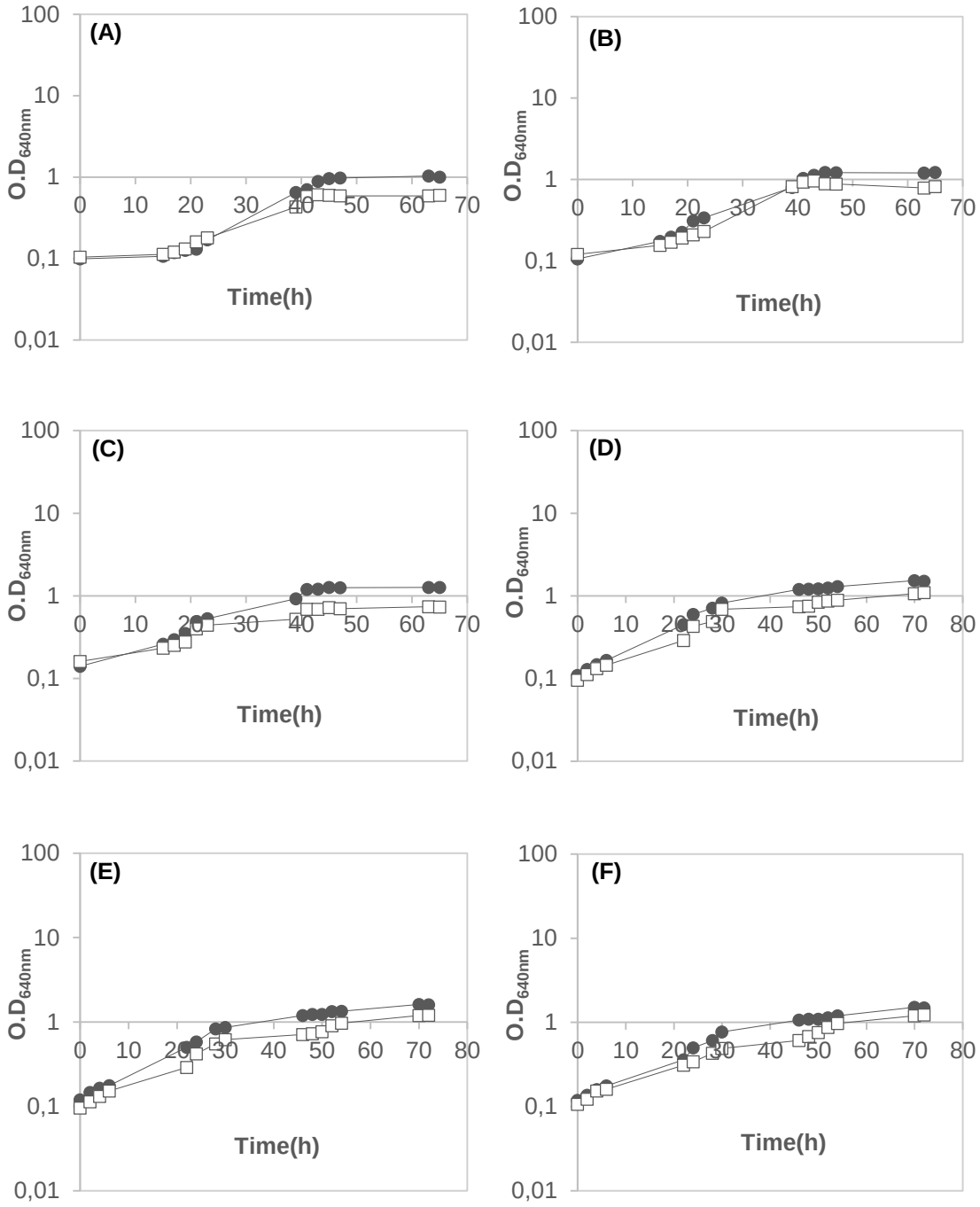


Figure A.2- Growth curves for *Candida magnoliae* PYCC 2903 (●) and PYCC 3191 (□) incubated with oxygen limitation at different sugar concentrations. (A) 15% Glucose and fructose, (B) 10% Glucose and fructose, (C) 5% Glucose and fructose, (D) 1% Glucose and fructose, (E) 2% Glucose and (F) 2% Fructose.