Potential of non-conventional yeasts for the production of added-value products from sugar beet pulp

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Preface

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), during the period February - October 2019, under the supervision of Professor Dr. Isabel Maria de Sá-Correia Leite de Almeida.

Declaration

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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"Eles não sabem, nem sonham,
que o sonho comanda a vida.
Que sempre que um homem sonha,
o mundo pula e avança"
António Gedeão, Pedra Filosofal, in "Movimento Perpétuo"

Abstract

The potential use of pectin-rich agro-industrial residues, namely sugar beet pulp (SBP), for production of biofuels and other bioproducts deserves attention. *Saccharomyces cerevisiae* ferments glucose and galactose but is unable to catabolize other pectin-sugar monomers, namely galacturonic acid, arabinose and xylose. Acetic acid, a potential growth inhibitor, is also present in pectin hydrolysates. Non-conventional yeasts (NCY) are emerging as alternatives for biotechnological applications, considering their metabolic diversity. In this work, five yeast species were isolated from SBP and macerated fruits and identified by molecular methods. Their performance and of other yeast strains to catabolize those carbon sources was assessed to be explored for SPB hydrolysates bioconversion. In aerobiosis, at 30ºC and pH 5.0, *Kluyveromyces marxianus* strains IST389 and CBS712 and *Meyerozyma guilliermondii* IST369 produced ethanol concentrations similar to *Saccharomyces cerevisiae* (0.8 %(v/v)) from glucose and galactose, but also metabolized arabinose and produced arabitol, *Kluyveromyces marxianus* being the greatest producer (6 g/L). All the studied species metabolized acetic acid, but none used galacturonic acid, despite *Rhodotorula mucilaginosa* ability to grow in this acid sugar. *Rhodotorula mucilaginosa* IST390 exhibited lower specific growth rates but produced high carotenoid yields (312 $\mu g/g_{dry\ biomass}$). The inhibitory effect of acetic acid in glucose or arabinose media was examined and only concentrations above 35 mM, the average concentration present in SBP hydrolysates, affected the yeasts' growth. The increase of temperature to 35ºC and pH decrease to 4.5 only affected *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa* growth. This study confirmed the potential of NCY for bioconversion of pectin-rich residues.

Keywords: *Kluyveromyces marxianus, Rhodotorula mucilaginosa, Meyerozyma guilliermondii*, pectin-rich agro-industrial residues, sugar beet pulp, bioproducts

Resumo

A utilização de resíduos agroindustriais ricos em pectina, nomeadamente polpa de beterraba (SBP), na produção de biocombustíveis e outros bioprodutos merece atenção. *Saccharomyces cerevisiae* fermenta glucose e galactose, mas não cataboliza outros monómeros de açúcar da pectina, como ácido galacturónico, arabinose e xilose. O ácido acético, um potencial inibidor do crescimento, está também presente nos hidrolisados de pectina. Considerando a diversidade metabólica, as leveduras não-convencionais (NCY) emergiram como alternativas em Biotecnologia. Neste trabalho, cinco espécies de leveduras foram isoladas de SBP e frutos e identificadas molecularmente. O seu desempenho e de outras estirpes no catabolismo daquelas fontes de carbono foi avaliado visando a bioconversão de hidrolisados de SBP. Em aerobiose (30ºC e pH 5,0), *Kluyveromyces marxianus* IST389 e CBS712 e *Meyerozyma guilliermondii* IST369 produziram, de glucose e galactose, concentrações de etanol semelhantes a *Saccharomyces cerevisiae* (0,8 %(v/v)) e metabolizaram arabinose produzindo arabitol, sendo *Kluyveromyces marxianus* a maior produtora (6 g/L). As espécies estudadas metabolizaram ácido acético, mas nenhuma utilizou ácido galacturónico, apesar de *Rhodotorula mucilaginosa* crescer neste açúcar ácido. *Rhodotorula mucilaginosa* IST390 exibiu menores taxas específicas de crescimento, mas produziu carotenoides com elevado rendimento (312 $\mu g/g_{biomassa}$). O efeito inibitório do ácido acético em meios com glucose ou arabinose foi estudado e apenas concentrações superiores a 35mM, a concentração média presente nos hidrolisados, afetaram o crescimento. O aumento da temperatura para 35ºC e diminuição do pH para 4,5 afetaram apenas o crescimento de *Meyerozyma guilliermondii* e *Rhodotorula mucilaginosa*. Este estudo confirmou o potencial das NCY na bioconversão de resíduos ricos em pectina.

Palavras-chave: *Kluyveromyces marxianus, Rhodotorula mucilaginosa, Meyerozyma guilliermondii*, resíduos agroindustriais ricos em pectina, polpa de beterraba, bioprodutos

List of Contents

List of figures

List of tables

List of abbreviations

ADH – Alcohol dehydrogenase

ALD – Aldehyde dehydrogenase

AR – Aldose reductase

ATP – Adenosine triphosphate

As – Arsenic

Cd – Cadmium

CO_2 – Carbon dioxide

Cu – Copper

DNA – Deoxyribonucleic acid

Fe – Iron

GalA – Galacturonic acid

GGPP – Geranylgeranyl pyrophosphate

GRAS – “Generally recognized as safe”

Hg – Mercury

HG – Homogalacturonan

HPLC – High-performance liquid chromatography

IST – Instituto Superior Técnico

ITS – Internal transcribed spacer

K. marxianus – Kluyveromyces marxianus

LAD – L-arabitol-4-dehydrogenase

LXR – L-xylulose reductase

M. guilliermondii – Meyerozyma guilliermondii

MDR – Multidrug Resistance

MFS - Major Facilitator Superfamily

MM – Minimal medium

MXR – Multixenobiotic Resistance

NCY – Non-conventional yeasts

NAD^+ – Nicotinamide adenine dinucleotide (oxidised form)

NADH – Nicotinamide adenine dinucleotide (reduced form)

NADPH – Nicotinamide adenine dinucleotide phosphate

OD – Optical density

Pb – Lead

PDC – Pyruvate decarboxylase

PDH – Pyruvate dehydrogenase

PG – Polygalacturonase

pKa – -log (Ka), being Ka the acid dissociation constant

PPP – Pentose phosphate pathway

R. glutinis – Rhodotorula glutinis

R. mucilaginosa – Rhodotorula mucilaginosa

rDNA – Ribossomal deoxyribonucleic acid

RF – Riboflavin

RG-I – Rhamnogalacturonan I

RG-II – Rhamnogalacturonan II

Rha – Rhamnose

rpm – Rotations per minute

S. cerevisiae – Saccharomyces cerevisiae

SBP – Sugar beet pulp

SCP – Single cell protein

Sp. – Specie

Spp. – Species

XDH – Xylitol dehydrogenase

XGA – Xylogalacturonan

XK – Xylulose kinase

YNB – Yeast nitrogen base

YPD – Yeast peptone dextrose

Z. bailii – Zygosaccharomyces bailii

Zn – Zinc

1. Motivation and thesis outline

Yeasts have been used by man for production of food and fermented beverages/products since pre-historical times [1]. The fermentation and catabolism of diverse carbon sources from organic residues by yeasts may allow their economically viable exploitation for bioethanol production and biorefinery processes and other bioproducts of interest in the Biotechnology industries. This versatile metabolic activity of yeasts is expected to become highly important in several relevant areas of economy, beyond food and beverages industries, such as those related with chemicals, detergents, textiles, cosmetic, pharmaceuticals or agro-industrial industries, and also in the environment [1–3].

Raw materials can be used as carbon, nitrogen and nutrients sources for yeast fermentation and growth. They are composed by a mixtures of sugars, amino acids and many other organic compounds [4]. Agro-industrial carbon sources can be divided into sugar materials (such as sugar beet, sugarcane or fruits and vegetables), starch feedstocks (like wheat, corn, rice or potatoes) and lignocellulosic substrates (wood, straw and grasses) [5]. Also, inulin, fats, oils, alcohols, hydrocarbons or different organic compounds can be suitable carbon sources [4]. Some crops, like corn and sugarcane, are still currently common feedstocks used to produce biofuels and other relevant bioproducts.

Sugar beet pulp is one of the raw materials with higher pectin content, alongside with citrus peels and apple pomace. They are generated in high amounts worldwide as waste products from the sugar industry or the industrial processing of fruits and vegetables (20 to >40%) [4–6]. The pectin fraction can be hydrolysed into its sugar monomers, namely D-galacturonic acid and neutral sugars xylose, arabinose, glucose and galactose, which are substrates that can be catabolized by yeasts [6]. On the other hand, the release of methanol and acetic acid from pectin during this hydrolysis process, can act as yeast stressors, leading to growth and fermentation inhibition [7,8]. Sugar beet pulp pectin presents high esterification with methoxy groups and the highest hydroxy groups acetylation levels among other pectin-rich fruits and vegetables [9]. This is one of the major anticipated problems associated with these residues.

Pectin-rich feedstocks also present problems with high variation due to geographical distribution and seasonality, but they are advantageously cheap and abundant [4,5]. Currently, most of the sugar beet pulp and citrus peels are dried and sold as cheap animal feed or put in landfills, but hold promise as inexpensive yeast feedstocks [6]. The utilization of organic waste residues, derived from crops and plant biomass, as substrates to produce added-value products allows the decrease of raw materials costs favouring environmentally friendly strategies, by saving and reutilizing resources. This may lead to the implementation of a circular bioeconomy, in which the metabolic versatility and the resistance to stresses shown by some non-conventional (non-*Saccharomyces*) yeasts point them as valuable cell factories of high potential for biorefineries [10,11]. Furthermore, these agro-industrial wastes can be used as environmentally friendly substrates for ethanol and interesting metabolites production, since they reduce greenhouse effect gases emissions compared to current bioprocesses, like corn ethanol production [12].

Saccharomyces cerevisiae is still the most important cell factory in the Biotechnological Industry. It is the chosen cell factory for food and beverage fermentation, as well as the major cell factory platform

for the production of bioethanol and other biofuels and for advanced biorefineries [13]. The metabolic engineering of *S. cerevisiae* is allowing the production of many compounds not naturally produced by this yeast species, and the use of pectin-rich agro-industrial sugars [6,13,14]. However, there are non-conventional yeast species that present industrial advantages in terms of metabolic pathways peculiarities, wider range of carbon sources of efficient assimilation, plus higher tolerance to some environmental stresses and cytotoxic compounds, that function as growth inhibitors [15,16]. Also, they are capable to produce the most diverse added-value products, beyond bioethanol, namely sugar alcohols (xylitol and arabitol) [17,18], lipids [19], enzymes [20–22], pigments (carotenoids) [23–25], among others. Different species and even strains differ in their interesting products synthesized, as well as in production rates and yields. Still, due to these relevant traits, some non-conventional yeasts are becoming so common, with their genome sequences and genetic tools available, that it is expected that will lose the current designation and become useful cell factories in the near future [2,15,16,26,27].

The present work was integrated in the EraNet Industrial Biotechnology project YEASTPEC - ERA-IB-16-013 - Engineering of the yeast *Saccharomyces cerevisiae* for bioconversion of pectin-containing agro-industrial side-streams. It aimed to study the conversion of sugar beet pulp (and other pectin-rich biomass) into added-value products, like bioethanol and bulk chemicals, using a *S. cerevisiae* strain engineered to resist multiple stresses and to utilize the different sugars present in the pectin-containing agro-industrial hydrolysates. The main goal of this work was to isolate and identify non-conventional yeast strains from pectin-rich sources and determine the potential of selected yeast species to utilize sugar beet pulp hydrolysates as a substrate for added-value products.

Study of different non-conventional yeasts isolated from fruits and other pectin-containing environments is expected to lead the identification of strains with potential for suitable bioconversion of pectin-rich wastes. With this purpose, samples of sugar beet pulp (SBP) and fruits were collected, and several yeast strains isolated were subsequently identified by sequencing specific regions of ribosomal DNA. Five biotechnologically interesting yeast strains were identified and selected for further analysis of their performance for growth in different sugars and in SBP hydrolysate. After a screening of 19 yeast strains to determine their growth ability in single pectin-sugar monomers and in sugar beet pulp hydrolysate, six strains were further studied considering their catabolic and fermentation capacity in sugar beet pulp hydrolysates, in both aerobic and microaerophilic conditions. Specifically, the isolated *Rhodotorula mucilaginosa* IST390 and *Kluyveromyces marxianus* IST389, other strains from the IST Collection, *Meyerozyma guilliermondii* IST369 and *Rhodotorula mucilaginosa* IST423, and reference strains. The carbon sources' consumption and products' production, throughout aerobic cultivation and in the end of microaerophilic fermentations, were monitored. Ethanol and arabitol were found to be produced by *M. guilliermondii* and *K. marxianus* strains in SBP hydrolysates. Since *R. mucilaginosa* is a notorious producer of pigments, the production ability in sugar beet pulp hydrolysates of the two strains examined was also assessed. The toxic effect of acetic acid, one of the most relevant inhibitors expected to be present in complex mixtures SBP hydrolysates, is a drawback in yeast performance that need to be understood, in order to characterize process bottlenecks and permit further improvement. So, the growth profiles of cultures

of glucose or arabinose supplemented with increasing concentrations of acetic acid were compared. Furthermore, the combined effects of temperature and pH, which may function as additional stress factors, in yeast growth profiles in SBP hydrolysates were assessed.

The results obtained in this study are important to highlight the potential of non-conventional yeasts as suitable cell factories or as sources of genes encoding metabolic pathways for *S. cerevisiae* engineering, in order to optimize the utilization of pectin-rich residues.

2. Introduction

2.1. Agro-industrial pectin-rich substrates for the production of bulk compounds

The majority of raw materials utilized as carbon sources are derived from sugars-, starch- or lignocellulose-containing feedstocks, as previously mentioned. Starch is composed by amylose and amylopectin, both formed by glucose monomers [4,16]. Lignocellulose has lignin (phenolic compound) cellulose, which is a glucose polymer, and hemicellulose, which is a heteropolymer containing short and highly branched sugar chains with hexoses (glucose, galactose and mannose) and pentoses (xylose and arabinose) [4,5]. Finally, sugar-containing substrates are mostly rich in glucose and fructose (sucrose). This last group is mainly composed by fruits and vegetables, which are rich in pectin.

2.1.1 Pectin composition

Pectin is a family of complex heteropolysaccharides and a structural component in plant cell walls [28]. It is composed by a linear chain of α-1,4 linked D-galacturonic acid (D-GalA) molecules, which corresponds to approximately 70% of its content and some have 2 to 4% of L-rhamnose (Rha) linked by β-1,2 and β-1,4 to it [28,29]. The side chains are composed by neutral sugars that include L-rhamnose, L-arabinose, D-xylose, D-galactose, L-fucose and D-glucose, among others [9]. Galacturonic acid (GalA) residues can be methyl-esterified at the C6 carboxyl group and/or O-acetylated at C-2 or C-3 and neutralized by ions, like sodium, calcium or ammonium [9,29].

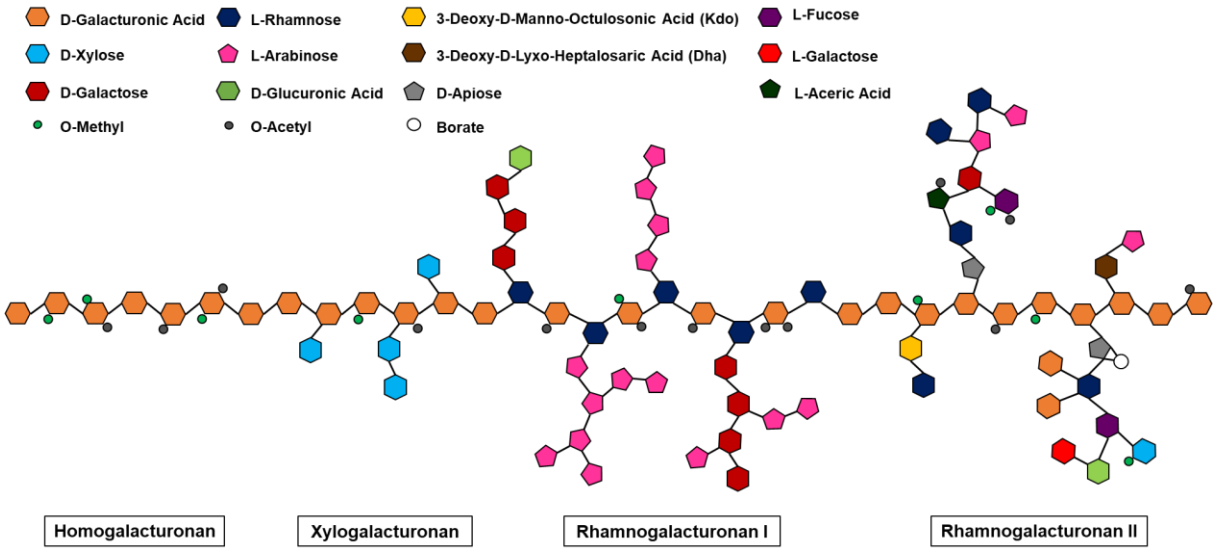

Figure 1 – Representation of the composition and structure of four pectic polysaccharides: homogalacturonan (HG), the simplest structure, substituted HG xylogalacturonan (XGA) and complex polysaccharides rhamnogalacturonan I and II (RG-I and RG-II).

A schematic representation of these types of pectin polymers is showed in Figure 1. There are three major pectin polymers: homogalacturonan (HG), which constitutes approximately 65% of pectin, being in higher amount; substituted galacturonan rhamnogalacturonan II (RG-II), composing only 10% of pectin; and rhamnogalacturonan I (RG-I), corresponding to 20 to 35 % of pectin [28,30]. There are

also different substituted homogalacturonans, xylogalacturonan (XGA), widely distributed in plants, and apiogalacturonan (AGA), only present in some duckweeds [30].

2.1.2. Pectin hydrolysis

The biochemical conversion of agro-industrial residues is subjected to several steps: pretreatment, hydrolysis/saccharification, fermentation (sugars bioconversion into products) and separation and recovery of the desired products (in case of bioethanol, a distillation process is used) [5,31].

Despite these wastes are abundant in pectin, they are lignocellulosic materials and still have a relative amount of lignin, cellulose and hemicellulose. The pretreatment step is necessary to improve cellulose and hemicellulose availability and help to degrade lignin, and consequently lignocellulose, since it is almost non-chemical and biodegradable [5,31]. The pretreatment may be physical (thermal, mechanical), physicochemical (steam explosion, ammonia fibre explosion, liquid hot water, supercritical CO_2), chemical (acids, bases, oxidation with oxygen or ozone) and biological (fungi) [5,31].

The saccharification process can occur by acid or enzymatic hydrolysis. Despite acid saccharifications is fast and easy, the enzymatic process presents advantages in terms of higher selectivity and yields of conversion into sugar monomers, less sugar degradation and inhibitors release and it is more environmentally friendly. The enzymes are produced by microorganisms, but the ones produced by filamentous fungi are the most used commercial preparations in biorefineries [5,31,32]. Cellulose is degraded into glucose by cellulases (endoglucanase, exoglucanase and β-glucosidase). Hemicellulose is converted into glucose, xylose, arabinose, galactose, mannose, rhamnose, glucuronic acid, acetic acid, ferulic acid by a mix of several enzymes, such as endoxylanase, exoxylanase, β-xylosidase, arabinosidase, α-arabinofuranosidase, α-glucoronisidase, acetyl xylan esterase and ferulic acid esterase [5,31,32]. Pectin is breached into D-galacturonic acid and other monomers, as the ones present in hemicellulose, e.g. glucose, xylose, arabinose, galactose and acetic acid, by addition of pectinolytic enzymes, like endopolygalacturonase, methyl esterase and arabinosidase [33]. The addition of pectinases and hemicellulases helps the degradation of all three types of polysaccharides (cellulose, hemicellulose and pectin) [31,33].

After hydrolysis, carbon sources are available to be converted into biomass and added-value products by microorganisms, having yeasts an important role. One important aspect to consider is the fact that the pretreatment and hydrolysis methods, as well as its conditions have a major influence in the efficiency and yield of the process, and in final composition of the hydrolysate. For examples, in enzymatic processes, the utilization of different enzyme cocktails, presenting different activities, leads to different final composition. Also, genetic and environmental factors generate residues with different initial composition. These problems limits the reproducibility of the process and the sugar availability, which affects products generation and their final yield [5,31–33].

2.1.3. Sugar beet pulp: pectin composition and hydrolysis

Sugar beet (*Beta vulgaris*) is a root crop that grows in temperate climates. It is planted in spring and collected in autumn or beginning of winter [4]. It is grown in 50 countries, being the main producers France, the United States of America and Germany, followed by Russia, Ukraine, Belgium, Poland, Netherlands, Turkey, China, Egypt and Iran [4,33].

Sugar beet pulp (SBP) is a residue from industrial sugar processing/extraction of sugar beet [34–36]. Approximately 400 million metric tons of SBP per year are being produced in the world [36]. It is mostly dried, and sometimes pelleted and massed, to be used as animal feed or discarded as waste [4,6]. Recently, sugar beet pulp reutilization has been exploited, since it can function as a substrate for ethanol, biofuels, acids, dietary fibres and food additives, single cell protein (SCP) and other biotechnological interesting chemicals and biomaterials, as well as heavy metal biosorbent (for wastewater treatment and bioremediation) [33,35–37].

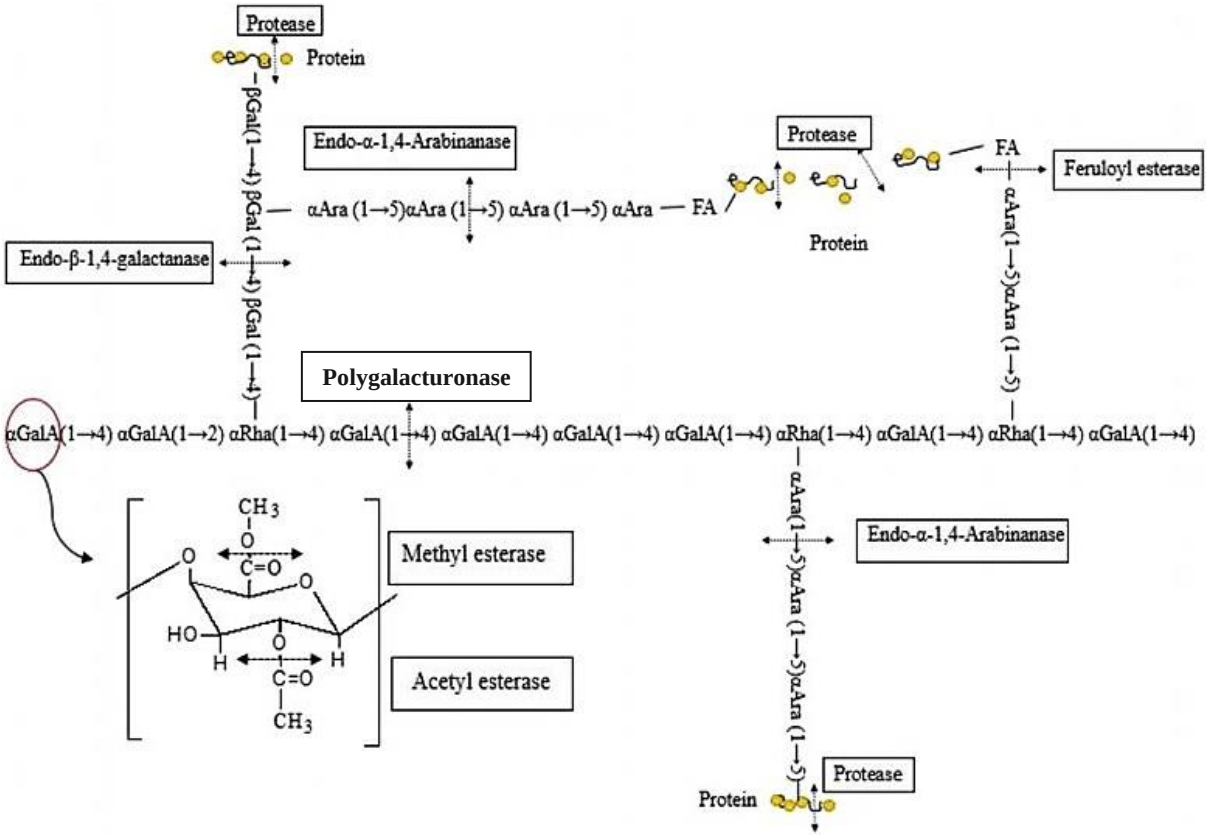

Figure 2 – Representation of SBP pectin and some pectinolytic enzymes. GalA: galacturonic acid; Rha: rhamnose; Gal: galactose; Ara: arabinose; FA: ferulic acid. Substitutions by some neutral sugars are missing. Taken from [35].

SBP is generally composed by 1/3 cellulose, 1/3 hemicellulose and 1/3 pectin, with low amounts of lignin, ash, fat, protein and residual sugars [33,36]. Still, SBP content in dry matter varies from 10 to 32% of pectin [29,33,34]. SBP pectin is composed by all three major types of pectin with already detailed characteristics, mostly by HG and RG-I with unbranched galactan and highly branched arabinan side chains, having high content of D-GalA, arabinose and glucose, with lower amounts of galactose and rhamnose [33]. RG-I and RG-II present some specific characteristics in terms of

existent neutral sugars and side chains, namely the substitution of GalA with a single glucuronic acid in RG-I, or the ferulic acid content [30,35]. A scheme of part of the SBP pectin composition and structure, as well as some hydrolytic pectinases that break it, is showed in Figure 2. Although the backbone and the most relevant side chains are present, some substitutions by various sugars are not represented. SBP pectin hydrolysis pretreatment is, most times, by steam explosion and enzymatic hydrolysis is nowadays the preferable saccharification method [33], considering the subsequent step of fermentation and bioconversion into bioethanol and other added-value products by microorganisms, and especially yeasts.

SBP pectin presents different properties from other common fruits' pectin, having low gelling capacity, and instead high emulsifying ability. This is due to its high acetylation levels (until more 5% than other pectins), high protein content (until more 10% than other pectins), high neutral sugars levels relatively to a low molecular weight and small size, and high number of side chains [33–35] . Also, it contains high number of ferulic acid residues (phenolic esters) esterified to the C-2 of arabinose and C-6 of galactose in the side chains [34,35]. These characteristics have potential in food, pharmaceutical and biomaterials applications [33].

2.2. Non-conventional yeasts as biotechnological cell factories

Yeasts have been used by humans for the production of fermented food and beverages predates 7000 BC. The majority of yeast research and industry is still based on *S. cerevisiae* due to historical reasons, like its utilization in the production of fermented food and beverages for centuries, and its tolerance, production and accumulation of high concentration of ethanol [38].

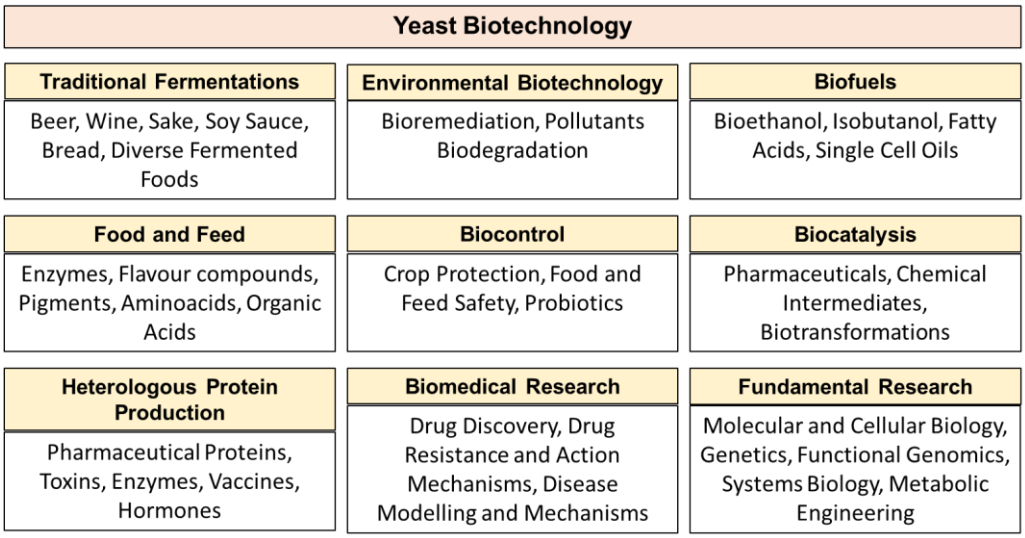

Figure 3 – Numerous biotechnological areas and applications of yeasts. Nowadays, besides their millennial use in fermented food and beverages, yeasts are relevant in several areas such as biofuels, pharmaceuticals and agriculture. Based on [1–3].

Yeasts are the greatest producers of biotechnologically relevant products, exceeding other microorganisms in abundance and profits [3]. As already mentioned, they have extended scientific and industrial interest in the most diverse areas, as food and beverages, agriculture, environment,

pharmaceuticals, biofuels, SCP, industrial enzymes, other bulk chemicals and metabolites production, [1,2]. A more detailed overview of current yeast applications is described in Figure 3.

In the past, non-conventional yeasts (NCY) were considered spoilage yeasts, being undesired or considered of reduced interest [15]. Recently, mostly in the last three decades, they have been acquiring importance, which led to their development and expansion. Although not all of the yeasts species are able to perform alcoholic fermentation efficiently, their assimilation and catabolism of different carbon sources permits to generate an high variety of added-value products, through different enzymatic pathways [3]. The variety of features and biotechnological roles [2] make them desirable cell factories.

2.2.1. Non-conventional yeasts ecology, general characteristics and advantages

NCY involve a huge biodiversity of yeast species, which are phylogenetically, morphologically and metabolically distinct. There are more than 1500 yeast species described, still is thought to be about 1% of existing yeasts in our planet [16,39]. Yeasts live in biotic communities with other organism in the most different environments, such as fresh or rotten plants (fruits, vegetables, flowers), trees, sea water, fresh water, soil, air, insects and other animals. The diverse ecological characteristics of their ecosystem have impact in their metabolism and morphology [39,40]. Most of known yeast species were isolated from fermented or spoiled food and beverages, clinical samples and natural environments, like soil and various plants [38].

NCY are able to produce several interesting compounds different from the ones synthesised by *S. cerevisiae* or produced in different desired conditions, which make them advantageous. They are able to tolerate various environmental stresses, like temperature, osmotic stress, pressure or inhibition by existent or produced cytotoxic compounds, which sometimes may be the desired product [15,16,41,42]. Numerous non-conventional yeasts can produce higher biomass, which is usually related with their inability to ferment, therefore directing their energy to growth. Nevertheless, they are valuable in terms of growth physiology, metabolic pathways (requirements and regulation) and production profile, which make them extremely important for production of a wide range of added-value products [41,42]. Some of the most biotechnological important non-*Saccharomyces* species are displayed in Table 1.

Although numerous NCY that have potential for the bioconversion of pectic sugars are considered “generally recognized as safe” (GRAS) label from FDA (Food and Drug Administration), there are several species that are well known animal pathogens. *Cryptococcus, Candida* and *Rhodotorula* genera possess several pathogenic species, such as *Cryptococcus neoformans* and *Cryptococcus gatii* [3] as well as *Rhodotorula mucilaginosa, Rhodotorula glutinis,* and *Rhodotorula minuta*, that have arose as opportunistic pathogens, with the ability to infect susceptible or immunocompromised patients [43]. However, they have high potential as producers of interesting compounds and they can also be used as sources of genetic information for engineering GRAS yeasts, like *S. cerevisiae*.

Some of these yeasts are used for native or heterologous protein synthesis and metabolites production, namely for pharmaceutical purposes. They are advantageous for not being susceptible to viral infections, but especially to perform several post-translational modifications, many being similar to human modifications, which permits to avoid *S. cerevisiae* hyperglycosylation and mannose ligations, that are responsible for human immunological adverse reactions [41,42].

Table 1 – Several non-conventional yeasts species with most relevance in Biotechnology and Industry. When more than two species of the same genus are considered or the species itself is not identified, they are indicated by the name of the genus followed by spp. (species abbreviation). Adapted from [1].

Yeast species	
Kluyveromyces lactis	*Schwanniomyces occidentalis*
Kluyveromyces marxianus	*Debaryomyces hansenii*
Scheffersomyces (Pichia) stipitis	*Ogataea polymorpha*
Pichia spp.	*Komagataella (Pichia) pastoris*
Rhodotorula spp.	*Candida* spp.
Rhodosporidium spp.	*Blastobotrys adeninivorans*
Yarrowia lipolytica	*Trichosporon* spp.

2.3. Yeast inhibitory compounds present in pectin-rich biomass hydrolysates

The presence of toxic compounds in pectin-rich biomass hydrolysates is responsible for limitations in yeasts' metabolism and fermentation. These compounds, such as weak acids, furan derivatives, and phenolic compounds, are generated during pretreatment and hydrolysis of pectin-rich and lignocellulosic materials [32]. Although the toxicity of these compounds by themselves may be relatively low, when their combined effect is considered, higher toxicity values may be obtained, due to a synergistic effect [32,44]. In pectic substances, namely sugar beet pulp, acetic acid, methanol and ethanol, heavy metals and pesticides are some of the possible inhibitors present.

Table 2 – Degree of esterification and acetylation of several pectic materials. Sugar beet pulp shows the higher levels of acetylation and elevated esterification with methanol. Adapted from [9].

Pectin source	Acetylation (%)	Esterification (%)
Citrus fruits	3	64
Apple	4	71
Sugar beet	20	55

Pectin GalA carboxyl groups suffer esterification with methanol and some hydroxyl groups are partially acetylated, as already mentioned. The percentages of esterification and acetylation of several pectin-rich sources are displayed in Table 2. Sugar beet has elevated values of esterification (55%) and the highest percentage of acetylation (20%) [9]. Additionally, heavy metals presence in sugar beet, and other pectin-rich residues [45,46], results from plant uptake from the soil, due to intensive agriculture (with utilization of fertilizers) and other human activities, like industry, waste disposal and treatment or transports [38,45].

2.3.1. Acetic acid

Acetic acid is a weak acid that, despite its presence in pectin-rich residues, is also generated as a by-product of yeast alcoholic fermentation [44]. Acetic acid inhibits cell growth due to the ability of the non-dissociated form (pKa 4.7) to diffuse across plasma membrane and cause a flow of the weak acid into the cytosol. The subsequent dissociation/deprotonation of this acid in the yeast cells' cytosol (around neutrality) releases the acetate counter-ion and the accumulation of protons causing the acidification of the cytosol [7,47]. The effect of acetic acid is particularly drastic at medium pH below the pKa of the acid.

Although acetic acid is used in various low pH foods to prevent fungi spoilage, some non-conventional yeasts, known as spoilage yeasts, are able to thrive in these products, which is the case of the species Zygosaccharomyces *bailii.* It exhibits a remarkable tolerance to acetic acid, being able to grow at concentrations of acetic acid 3-fold higher (370-555 mM) than *S. cerevisiae* (80-150 mM), which already presents a significant resistance to acetic acid and to other weak acids at low pH [7,47]. Other yeasts species tolerant to acetic acid are *Pichia kudriavzevii* (133-167 mM) [48,49] and *Candida glycerinogenes* (100 mM) [50].

Acetic acid catabolization in several yeast species, like *S. cerevisiae, Candida utilis, Torulaspora delbruecki* and *Dekkera anomala*, is repressed by glucose [51,52], but in *Z. bailii* glucose and acetic acid are simultaneously catabolized [51,52]. However, acetic acid may also exert repression over ethanol respiration to be used as a carbon and energy sources [53]. Still, low acetic acid concentration (lower than 100 mM) have been showed to exert a positive effect in ethanol yields produced by *S. cerevisiae* at pH 5.5, whereas higher concentrations decrease ethanol production yields [32].

Depending on acetic concentration and the yeast strains, acetic acid can exert a positive effect in lipid accumulation. The yeast *Cryptococcus curvatus* has reportedly converted acetate into oils, with a final lipid accumulation up to 50% (w/w) [54]. *Rhodosporidium toruloides* was also able to grow in media with acetic acid as only carbon source, up to a concentration of 20 g/L and produced lipids [55]. This is consistent with the fact that most yeasts capable of growing in high acetic acid concentrations are oleaginous, since acetate can be assimilated and converted into acetyl-CoA, a lipid biosynthesis precursor [56,57].

Acetic acid formation and catabolism by yeasts is dependent of oxygen availability. The production depends on several enzymatic reactions, such as conversion of acetyl-CoA and acetyl adenylate by acetyl-CoA hydrolase; cleavage of citrate by the enzyme citrate lyase; transformation of acetyl-phosphate by acetyl kinase; oxidation of acetaldehyde by aldehyde dehydrogenase (ALD) (fermentation associated reaction); production from pyruvate, by pyruvate dehydrogenase (PDH), leading to acetyl-CoA, that is then converted into acetate through acetyl-CoA hydrolase [52], which is repressed in anaerobiosis. The formation of acetate may be important for cofactor regeneration (NADH and NADPH) and redox balance maintenance [52].

Still, when PDH is repressed, its bypass still allows the formation of acetyl-CoA from pyruvate used in the synthesis of fatty acids, through a sequential transformation of pyruvate to acetaldehyde by

pyruvate decarboxylase (PDC), acetate by aldehyde dehydrogenase (which is active in alcoholic fermentation), and acetyl-CoA by acetyl-CoA synthase (ACS). This suggests that acetic acid is mainly produced through the PDH bypass, despite other enzymatic reactions may be involved. When yeasts are subjected to aerobic conditions, they are producing fatty acids for growth, but then they may produce acetic acid after entry into semi-aerobic (fermentative) conditions as a mechanism for the regeneration of free CoA [52]. Both PDH and PDH bypass pathways are illustrated by a scheme in Figure 4.

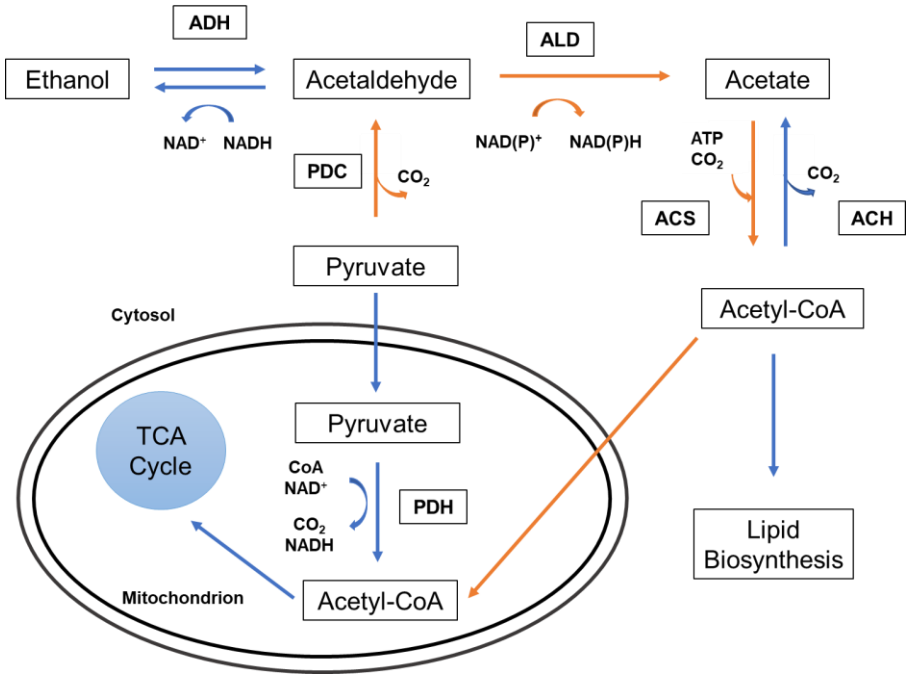

Figure 4 – Representation of the yeast acetic acid metabolic pathways. PDH pathway is indicated by blue arrows, while PDH bypass is showed in orange arrows. PDH: pyruvate dehydrogenase; PDC: pyruvate decarboxylase; ALD: aldehyde dehydrogenase; ACS: acetyl-CoA synthetase; ADH: alcohol dehydrogenase. Adapted from [52].

S. cerevisiae mechanisms of uptake and adaptive responses to acetic acid toxicity are well known, being schematically represented in Figure 5. In pH < pKa and glucose repression conditions, not only acetic acid diffuses across the membrane, but it is also thought to also pass through an aquaglyceroporin, Fps1. When there is not repression or pH > pKa, acetate enters the cells through the acetate carrier Ady2, and it is unable to diffuse, so its accumulation leads to increase of the turgor pressure and oxidative stress [47].

To respond to stress induced by acetic acid, there is the necessity to recover the intracellular pH, which is achieved by the activation of both a plasma membrane H^+-ATPase, encoded by the PMA1 gene, and a vacuolar H^+-ATPase. The remodeling of plasma membrane to reduce acetic acid diffusion into the cell is achieved by activation of diverse genes involved in the synthesis of cell wall polysaccharides, structure and remodeling , as well as synthesis of sphingolipids and sterol [7,44,47]. There is also the activation of Major Facilitator Superfamily (MFS) transporters, involved in Multidrug/Multixenobiotic Resistance (MDR/MXR), Tpo2, Tpo3 and Aqr1, to export it to the exterior of the cells. The genes that encode them are activated by the transcription factor Haa1 [44,47].

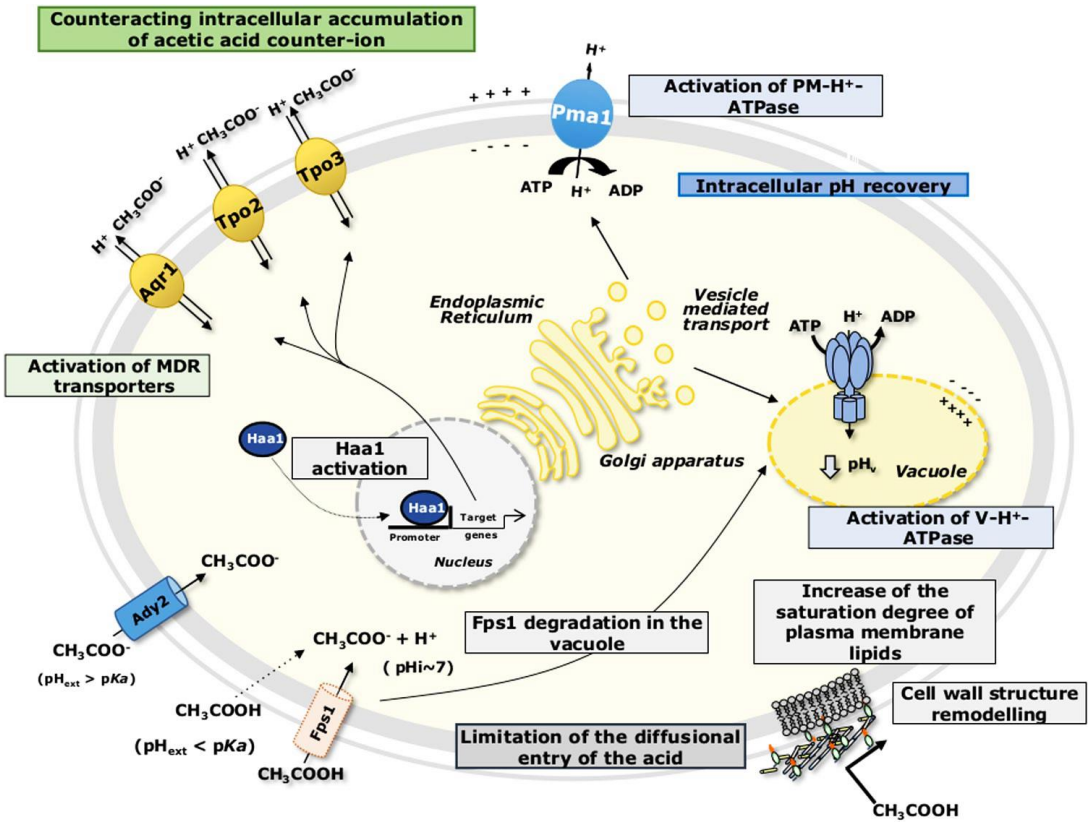

Figure 5 – Representation of *S. cerevisiae* adaptive mechanisms and responses to acetic acid toxicity. pHext: external pH, pHi: intracellular pH. Taken from [47].

The activation of about 80% of the genes responsible to acetic acid responses is due to Haa1. It binds to the motif Haa1-responsive element (HRE), which is present in 55% of the genes upregulated in response to acetic acid, being considered direct targets: Tpo2 and Tpo3 (transporters involved in response to several weak acids), Ypg1 (cell-wall glycoprotein present in nutrient deficiency conditions) and Yro2 (Hsp30 protein homologue involved in acetic acid tolerance and potassium depletion) [7,58]. The indirect targets do not contain the HRE motif, which is the case of transcription factors genes *MSN4* (involved in general yeast stress response), *FKH2* (oxidative stress response), *NRG1* (several yeast stress responses), *STP4* and *COM2*, highlighting the existence of a great and complex network. Two of the most significant genes activated by Haa1 that confer the highest protection to acetic acid are of *SAP30* (encodes a Rpd3L histone deacetylase complex subunit) and *HRK1* (encodes a protein kinase belonging to a family related to post-translational regulation of plasma membrane transporters) [7,47,58].

2.3.2. Methanol and ethanol

In general, the toxicity of alcohols that have cellular membranes as targets increases with their lipophilicity that can be assessed by the logarithm of the partition coefficient octanol/water (Pow). The log Pow of methanol is -0.74, below ethanol log Pow (-0.30), being less toxic [59].

Methanol is also produced during fermentation [60]. Its toxicity mechanisms are not completely unveiled due to the lack of published studies or the fact that methanol can be converted in

formaldehyde, a much more toxic compound [8]. For *S. cerevisiae,* 1.23 M of methanol and 1.8 mM of formaldehyde, are concentrations that inhibit growth without causing cell death [60]. Methanol promotes ALDs expression, while ADHs are only slightly induced. This is probably related with the promotion of a fast formaldehyde removal, which is an efficient mechanism to reduce intracellular concentrations of a very toxic intermediate [60]. The pathway of methanol detoxification is shown in Figure 6.

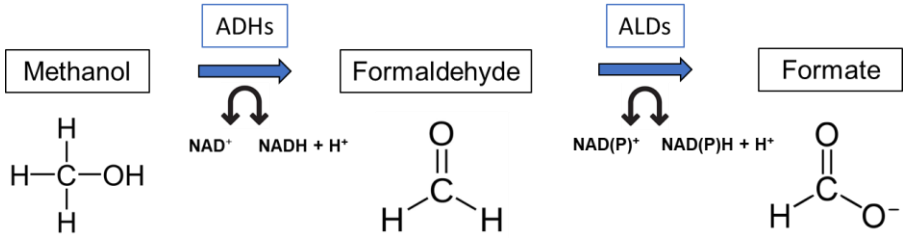

Figure 6 – Putative methanol detoxification mechanism in yeast. The first step is methanol conversion into formaldehyde, catalysed by alcohol dehydrogenases (ADHs), while the second step is the conversion of formaldehyde into formate by aldehyde dehydrogenases (ALDs) Adapted from [60].

Despite *S. cerevisiae*'s inability to grow on methanol, several non-conventional yeasts can efficiently use it as the sole carbon and energy source. This methylotrophic yeasts have been exploited for biotechnological applications, such as, the production of single-cell protein (SCP), heterologous recombinant proteins and chemical compounds from methanol, an inexpensive source of carbon [2,27,61]. These yeast stains belong to a number of yeast genera including *Candida, Pichia* and some genera that were recently separated from Pichia, namely *Ogataea, Kuraishia* and *Komagataella* [61,62]. Some of the most known methylotrophic yeast species are *Candida boidinii*, *Ogataea (Pichia) methanolica*, *Komagataella (formerly Pichia) pastoris, Ogataea minuta* and *Ogataea (formerly Hansenula) polymorpha,* as well as *Candida parapsilosis, Candida (formerly Torulopsis) glabrata* and *Ogataea (formerly Pichia) thermomethanolica* [2,61,62].

The ethanol toxicity mechanisms and *S. cerevisiae* adaptive responses have been thoroughly studied due to its relevance in alcoholic fermentation [44,63]. Ethanol tolerance mechanisms in yeast rely on multidrug resistance mechanisms, specifically in a combination of the transcription factors expression Msn2/4, (controls general stress response), Yap1 (oxidative stress) and Hsf1 (heat shock responses) [44,63]. Additionally, aquaglyceroporin Fps1p is related with the decrease of ethanol intracellular accumulation, which may be due to the promotion of ethanol efflux, when abruptly exposed or as result of plasma membrane lipid composition alteration. Also, production of ethanol is increased in high-density fermentation when it is overexpressed [44,63].

S. cerevisiae is usually known as the most ethanol tolerant species. Several strains have been described as being able to grow in ethanol concentrations in the range 12 and 14% (v/v), in particular the industrial strain Ethanol Red [64,65]. Strains of *Hanseniaspora valbyensis, Lachancea thermotolerans, Pichia kudriavzevii, Saccharomycodes ludwigii, Torulaspora delbrueckii, Wickerhamomyces anomalus (Pichia anomala)*, and *Zygosaccharomyces rouxii* were reported to tolerate growth at 13% (v/v) of ethanol, while *Brettanomyces anomalus, Brettanomyces (Dekkera) bruxellensis* and *Brettanomyces naardenensis* are able to grow in 12% (v/v) ethanol [38]. *Dekkera*

bruxellensis strains isolated from wine exhibit growth in ethanol concentrations between 10 and 16% (v/v) [66]. This species is also known for accumulating acetic acid, being more resistant to it than *S. cerevisiae,* and also for producing, in anaerobiosis, similar amounts of ethanol as *S. cerevisiae* [15,67]. *Pichia kudriavzevii* can grow at concentrations up to 20% (v/v) ethanol at 40ºC, [48] and it is reported to produce more ethanol than *S. cerevisiae* (approximately more 20% of ethanol at pH 4.0 and 35% or 200% of ethanol at temperatures 40ºC or 45ºC, respectively) [49].

2.3.3. Heavy metals and agricultural pesticides

Heavy metals are essential micronutrients for yeasts when in small amounts. But when they reach toxic concentrations, they induce the generation of reactive oxygen species (ROS), consequent oxidative stress and affection of proteins, lipids and nucleic acids functionality [38]. In the case of agricultural pesticides, mechanisms of toxicity and tolerance in yeast are more poorly studied, although the global effects of the herbicide 2,4-D and the agricultural fungicide mancozeb, among many others, have been examined [68].

The major heavy elements present in pectin-rich residues, namely in sugar beet pulp, are arsenic (As), cadmium (Cd), copper (Cu), lead (Pb), zinc (Zn), iron (Fe) and mercury (Hg) [45,46]. In general, pectins bind, by this order, to Pb^{2+} >> Cu^{2+} > Co^{2+} (cobalt) > Ni^{2+} (nickel) >> Zn^{2+} > Cd^{2+} but pectin affinity for cations differs among sources. Sugar beet pectin revealed higher affinity for Cu^{2+} and Pb^{2+} [37,69]. However, a different study reports that both citrus and sugar beet bind, by this order, to Cu^{2+} ~ Pb^{2+} >> Zn^{2+} > Cd^{2+} ~ Ni^{2+} [70].

There are few studies about heavy metal tolerance in yeasts and their mechanisms of action. Nevertheless, a few reports permit to determine which species are more resistant to the different metals. In the case of Zn, *Meyerozyma guilliermondii* (50 mM), *Rhodotorula aurantiaca, Torulaspora delbrueckii* (35 mM), *Debaryomyces hansenii, Candida krusei*, *Cryptococcus laurentii* and *Pichia anomala* (20 mM), are the most resistant strains [71,72]. *Rhodotorula glutinis* (15-35 mM), *Candida albicans, S. cerevisiae, Wickerhamomyces anomalus (Pichia anomala), Wickerhamomyces ciferrii* (3.5 mM) are the most tolerant to Cd [38,71]. *Cryptococcus laurentii* (10-15 mM), *Metschnikowia pulcherrima* and *Pichia americana* (10 mM), *Debaryomyces hansenii* and *P. guilliermondii* (5 mM) resist to Cu [71,72]. *Candida krusei*, *Candida lambica*, *Metschnikowia pulcherrima* and *Williopsis saturnus* (5 mM) tolerate Ni [71]. Also, *Candida albicans* presents a slight growth at 750 µg/mL (10 mM) As [73]. Nevertheless, the tolerance to heavy metals seems to be dependent from the metal itself and the stain, since there is a great variability among strains of the same species [71,72].

The pesticides (mostly fungicides, herbicides and insecticides) used in cultivation vary among the countries, but their maximum residues levels allowed are regulated [74]. In European Union, there are 481 pesticides approved to be used in sugar beet cultivation. Most of them present a maximum residue level (highest pesticide residue value tolerated in food when Good Agriculture Practices are followed) of 0.01 mg/kg, which is the lowest one approved by default, still mancozeb level is 2 mg/kg, and 2,4-D level is 0.05 mg/kg [74,75]. A Serbian study reveal that the 23 insecticides, 17 fungicides

and 18 herbicides utilized in this country follow Good Agriculture Practices values, and present lower levels than the maximum residue levels allowed in this country [76].

Mancozeb and 2,4-D mechanism of tolerance in *S. cerevisiae* were already studied [68]. In the case of mancozeb, the mechanisms involve the increase in expression of genes related with oxidative stress and antioxidant response, mitochondrial function, vacuolar H^+-ATPase function, protein synthesis, folding and degradation (proteasome), MDR transporters (Tpo1 and Flr1), lipids and ergosterol synthesis, and cell wall alterations [68]. For 2,4-D, the mechanisms are similar, since involve overexpression of genes related with oxidative stress and antioxidant response, vacuolar H^+-ATPase activation, carbon and nutrient sources metabolism, amino acids homeostasis and MDR transporters (Tpo1, Pdr5, and Pdr18) [68].

Overall, it seems that sugar beet present variable compositions in terms of heavy metals and pesticides, that are not always assessed, representing a potential problem for yeasts fermentations and metabolism.

2.4. Production of added-value products by non-conventional yeasts in pectin-rich residues

There is a wide range of products synthesized by different non-conventional yeast species in pectin-rich substrates reported so far in the literature. Predictively, the value of products is inversely proportional to the its volume of production [13], as it is shown in Figure 7. Production volume and product value are important to define the optimal production conditions, as well as to choose the most suitable yeast species as cell factory.

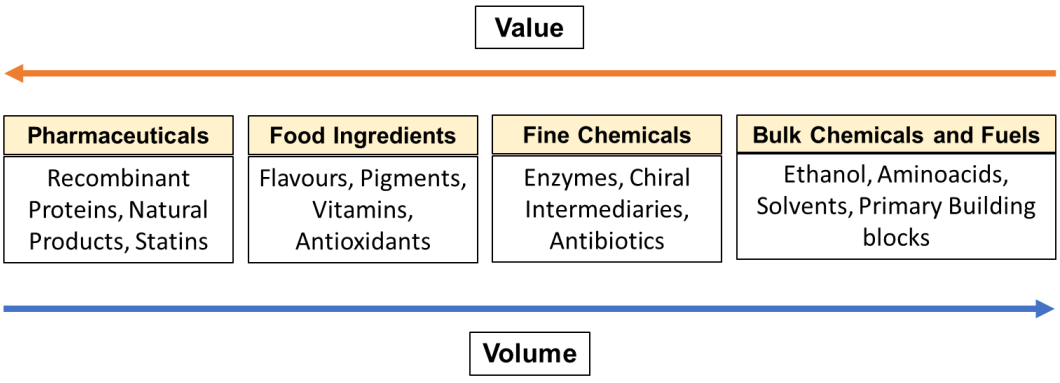

Figure 7 – Examples of biotechnologically relevant products, from high added-value, produced in lower amounts, to low added-value products, produced in large amounts. Based on [13].

The most relevant products produced from pectin-rich residues by non-conventional yeast species will be addressed below, especially bioethanol, sugar alcohols, carotenoids and enzymes. An overview of all products found to be produced in pectic residues is depicted in Table 3.

Table 3 – Added-value products generated by non-conventional yeasts from pectin-rich residues.

Species	Strain	Products	Pectin-rich residues	References
Kluyveromyces marxianus	CCEBI 2011	Bioethanol	Sugar beet molasses	[20]
	CCEBI 2011	Polygalacturonase	Sugar beet molasses	[20]
	ATCC 10022	Fruity-like aroma compounds (high ester and alcohols content)	Sugar beet molasses + sugar cane bagasse	[77]
Scheffersomyces (Pichia) stipitis	NRRL Y-7124	Bioethanol	Sugar beet pulp hydrolysate	[78]
Pichia kudriavzevii	-	Bioethanol	Kinnow mandarin (*Citrus reticulata*) peels	[79]
Meyerozyma guilliermondii	NRRL Y-2075	Bioethanol	Soybean hull hydrolysate	[80]
	NRRL Y-2075		Soybean hull hydrolysate	[18]
	NRRL Y-2075	Xylitol	Soybean hull hydrolysate	[18]
Clavispora lusitaniae	-	Naringinase (complex α-rhamnosidase and β-glucosidase)	Kinnow mandarin (Citrus) juice	[21]
Metschnikowia pulcherrima	MACH1	Chitinase	Apple juice	[22]
***Rhodotorula* spp.**	PW34	Chitinase	Apple juice	[22]
	-	Carotenoids	Apple pomace	[81]
Rhodotorula glutinis	DBVPG 3853	Carotenoids	Sugar beet molasses	[24]
	TR29	Total lipids and fatty acids (oleic, stearic, palmitic and palmitoleic acids)	Sugar beet molasses	[19]
Rhodotorula mucilaginosa	NRRL-2502	Carotenoids (β-carotene, torulene and torularhodin)	Sugar beet molasses	[23]
Xanthophyllomyces dendrorhous (Phaffia rhodozyma)	2A2N	Carotenoids (astaxanthin)	Sugar beet molasses	[25]
Trichosporon penicillatum	SNO-3	Protopectin-solubilizing enzyme	*Citrus unshiu* (Citrus) peel	[82]
Torula (Candida) utilis	CCT3469	Lignocellulosic enzymes: pectinase, manganese-dependent peroxidase, cellulase, xylanase	Apple pomace	[83]
	DSM 70163	Single cell protein (SCP)	Sugar beet pulp and molasses	[84]
	-	Animal feed nutrient enrichment (proteins, fat, fibres, vitamins and minerals)	Apple pomace	[85]
	-		Apple pomace	[86]
Candida tropicalis	DSM 70 15	Single cell protein (SCP)	Sugar beet pulp and molasses	[84]
Candida parapsilosis	DSM 70125	Single cell protein (SCP)	Sugar beet pulp and molasses	[84]
Candida solani	ATCC 14440	Single cell protein (SCP)	Sugar beet pulp and molasses	[84]
***Kloeckera* sp.**	-	Animal feed nutrient enrichment	Apple pomace	[85]
Aerobasidium pullulans	-	Exo-Polygalacturonase	Tomato pomace	[87]
Debaryomyces hansenii	NRRLY-7483	Arabitol	Soybean flour and soybean hull hydrolysates	[17]
Hanseniaspora uvarum	-	Volatile fruity-like aroma compounds (acetic acid esters)	Apple pomace	[88]
	-	Bioethanol	Apple pomace	[88]
Hanseniaspora valbyensis	-	Volatile fruity-like aroma compounds (acetic acid esters)	Apple pomace	[88]
	-	Bioethanol	Apple pomace	[88]

2.4.1. Bioethanol

Bioethanol (also called ethyl alcohol, chemical formula C_2H_5OH or EtOH) is the most studied and produced renewable biofuel as an alternative to fossil fuels, derived from the fermentation of several feedstocks. It is mostly used in transportation sector [5,18]. It is environmentally friendly, since the CO_2 produced is naturally absorbed and reused by plants, presenting benefits in greenhouse gases

reduction [5,77]. It can be used as pure ethanol itself, or blended with gasoline, which is widely implemented in several countries, allowing to reduce exhausted fumes emissions [5].

First generation bioethanol is mostly produced from starch (corn, wheat, barley, cassava, potato) and sugar-containing feedstocks (sugarcane, sugar beet, sweet sorghum, fruits). Brazil produces 100% of bioethanol from sugarcane, while the United States produce 98% from corn [5,31]. In Europe, 48% is produced from wheat and 29% from sugar beet [5,31]. Sugar beet has been growing, since ethanol yield per hectare obtain is three time higher than wheat [5,31]. Due to problems with seasonal availability, land and plant nutritional requirements together with agricultural practices, costs and especially social problems [16,77], there was the need for the development of second generation bioethanol processes, in which ethanol is derived from lignocellulosic and agro-industrial wastes [77]. Examples of possible substrates are sugar beet pulp, sugar cane bagasse, corn stover, wheat straw, corn cob, rice husk and straw, soybean and cereals hull, grasses and switchgrass, forest-based woody wastes, municipal solid wastes, and crude glycerol [18,31]. There is also a third generation bioethanol, produced by algae, but its production is not viable yet [31].

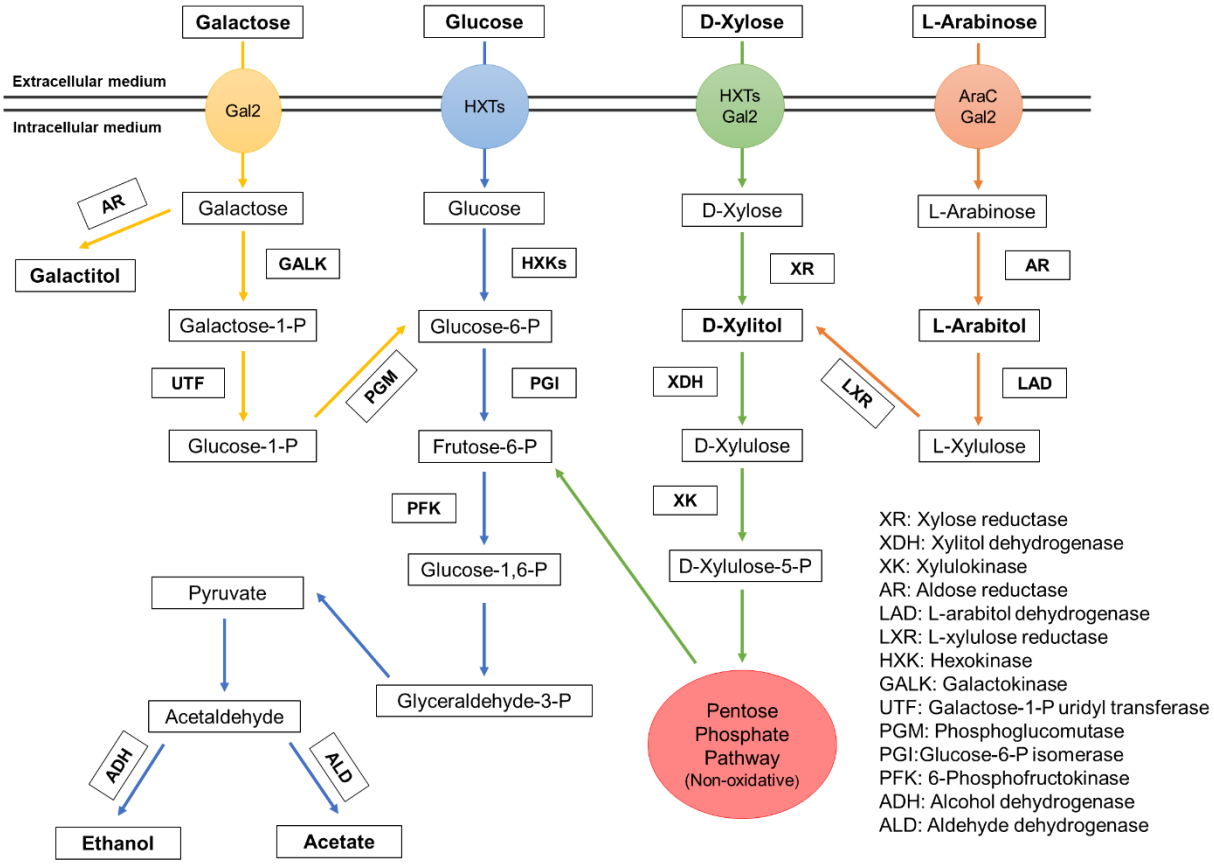

Figure 8 – Yeast metabolic pathways of glucose (blue arrows), galactose (yellow arrows), xylose (green arrows) and arabinose (orange arrows). Gal2, HXTs and AraC are sugar membrane transporters. Adapted from [78].

Yeasts produce most of the ethanol in the market from hexoses fermentation, namely glucose, fructose and galactose. Still, several yeast species are also able to ferment xylose, namely *K. marxianus* [79] and *M. guilliermondii* [80], with this last species even being reported to ferment arabinose [81]. Pyruvate (originated from direct glycolysis from glucose, or intermediaries of glycolysis

derived from pentose phosphate pathway in case of xylose and arabinose, and from Leloir pathway in the case of galactose) is transformed into acetaldehyde by pyruvate decarboxylase (PDC), being then transformed to ethanol by alcohol dehydrogenase (ADH), leading to the release of energy (2 ATP) [82,83]. The pathways for each sugar metabolization in yeasts are depicted in Figure 8.

S. cerevisiae cannot grow in lignocellulosic and pectin-rich substrates fermentation, since it is unable to metabolize xylose and arabinose. There are several examples of NCY reported to produce ethanol from pectin-rich residues. *Kluyveromyces marxianus* fermented sugar beet molasses, under aeration conditions [20]. *Meyerozyma guilliermondii* generated ethanol from soybean hull hydrolysate, at higher yields when first detoxified [84]. The same species attained higher ethanol concentration in this substrate under anaerobic conditions, but it did not metabolize xylose [18]. *Pichia stipitis* was grown in detoxified SBP hydrolysates, and its maximum ethanol concentration was obtained in hydrolysate supplemented with xylose [77]. *Pichia kudriavzevii* produced ethanol in Kinnow mandarin peel pre-hydrolysate, during simultaneous saccharification and fermentation. Still, despite this species potential to ferment glucose, sucrose, fructose and galactose, it cannot use not xylose, arabinose and GalA [85]. *Hanseniaspora uvarum* and *Hanseniaspora valbyensis* strains produced as much ethanol as *S. cerevisiae* (all strains presented similar yield) from apple pomace [86].

2.4.2. Sugar alcohols

Sugar alcohols are a family of polyols that include xylitol, arabitol, ribitol, mannitol, erythritol and galactitol [87]. These compounds are used as food sweeteners and in pharmaceutical industries [88,89]. They are equivalent to sucrose in sweet, but have lower caloric content, and are metabolized by an insulin-independent pathway, which is a good alternative for diabetic patients. Sugar alcohols have anticariogenic effects, since they do not endure oral bacteria growth, and are utilized in mints, candies, chewing gums and toothpastes. Additionally, xylitol is a precursor chemical in the synthesis of other compounds (ethylene glycol, propylene glycol, glycerol, xylaric acid, hydroxyl furan,1,2-propanediol), and polymers. L-arabitol showed capacity to reduce substantially human adipose tissue, and to prevent fat accumulation in the digestive tract [88–90]. Galactitol is also utilized in polymers, for tissue engineering and drug delivery [87]. Both xylitol and arabitol are mostly produced chemically at an industrial scale to fulfil its requirements. So, their synthesis by yeasts is considered as a desirable alternative and has been growing lately, since it is more environmental friendly, and in the case of arabitol, less expensive [88–90].

Xylitol is originated in the first step, through reduction of xylose by an NAD(P)H-dependent xylose reductase (XR) [88,91]. The same happens with L-arabitol, which is originated by NAD(P)H-dependent L-arabinose reductase (aldose reductase) (AR) in the first step of arabinose pathway (Figure 8). Arabitol is further converted into xylitol, which is an intermediate compound in the both xylose and arabinose pathways [89]. Galactitol is produced from galactose through an aldose reductase, but in a distinct pathway than the galactose fermentation metabolism [87]. The conversion pathways are represented in Figure 8. As aldose reductases prefer NADPH as cofactor, whereas polyol dehydrogenases are dependent on NAD^+, sugar alcohols are mostly accumulated under low oxygen

conditions, due to redox imbalance [18,88,89]. In the conversion of sugars to its sugar alcohols, glucose catabolite repression is the major drawback, in co-substrate presence [17,88,90].

It is also possible to produce D-arabitol (food sweetener; anticariogenic agent; precursor of arabinoic and xylonic acids, propylene and ethylene glycol, xylitol, enantiopure compounds, immunosuppressive glycolipids, herbicides and anti-pathogenic drugs) and xylitol from glucose and glycerol [88,89]. Also, D-arabitol production from xylose was described for some species, as *Rhodosporidium toruloides* [87].

Despite *S. cerevisiae* possession of native xylose and arabinose metabolic pathways, the lower expression of these enzymes do not allow growth in media with these sugars as sole carbon sources [92,93]. In fact, the aldose reductase encoded by the *GRE3* gene is known to have both D-xylose and L-arabinose reductase activities. Still, xylitol and arabitol can be produced in lower amounts [92,93]. Several non-conventional yeast species are able to utilize these sugars and convert them into their respective polyols, namely *K. marxianus, M. guilliermondii, R. mucilaginosa*, *Debaryomyces hansenii* and *Candida tropicalis* [88,89]. *M. guilliermondii* is one of the most effective yeasts in xylitol production from xylose, and it is reported to originate this polyol from pectin-rich residues, namely soybean hull hydrolysates (composed by 30% pectin) [18]. *Debaryomyces hansenii,* on the other hand, produces arabitol from soybean flour and soybean hull hydrolysates [17]. Despite reports of galactitol production in galactose medium by yeasts, namely *Rhodosporidium toruloides* , no reports of production in pectin-rich residues were found [87].

2.4.3. Carotenoids

Carotenoids are tetraterpenoids (highly unsaturated isoprene derivatives). These lipid-soluble pigments class is the most broadly disperse in nature, having yellow, orange, pink and red colours. They are found naturally in eggs, fruit and vegetables, fishes and oil. Carotenoids are synthesized by some species of plants, yeasts, filamentous fungi, prototrophic bacteria, algae and lichens [23,94–96]. In non-photosynthetic organisms, they confer protection against photooxidative stress derived from radiation (intensive light) exposure, so yeasts produced them to grow in light and aerated conditions [94,95]. Carotenoids are divided into 2 groups: carotenes, that include β-carotene and torulene; and xanthophylls, as astaxanthin, canthaxanthin and torularhodin [94,96].

Due to the increase in carotenoids global market and in the desire of natural compounds, the interest in their production by microorganisms has grown, despite they are still being mostly chemically synthesized [94–96]. Carotenoids applicability is extended to the most diverse areas: in food industry, as colouring agents of margarine, soft drinks, and baked goods; as precursors of vitamin A, being the main source in human food and animal feed; in ingredients in poultry, fish, and molluscs feed; as antioxidants in food additives, diet supplements, multivitamin preparations; in cosmetic and pharmaceuticals, as dyes and antioxidants, having photoprotective properties that allow to strengthen immune system, accelerating wounds healing and counteracting eye disorders and age-related degeneration, reducing aging and cellular and tissue damage; as agents protecting the skin against

harmful ultraviolet radiation; as anti-cancer prevention agents, related with their antioxidant properties; as antibacterial and antifungal agents [23,94–96].

Carotenes biosynthetic pathways are represented in Figure 9. The pathway starts with the conversion of acetyl-CoA into to 3-hydroxy3-methylglutaryl-CoA, leading to geranylgeranyl pyrophosphate (GGPP) after several intermediary reactions. Neurosporene is transformed into lycopene or β-zeacarotene, after several intermediate compounds' formation. Both compounds lead to γ-carotene generation, which is the precursor of both β-carotene and torulene, while torularhodin is obtained in torulene transformation. These are the most common pathways, occurring in most yeasts, like *Rhodotorula*. Torulene can also be produced from 3,4-dehydrolycopene in some yeast, like *Sporidiobolus pararoseus* [94]. The genes that code for phytoene synthase (crtB), lycopene cyclase (crtY), and phytoene desaturase (crtI) were identified in *R. mucilaginosa*, and it was found that they are located in relatively close in the genome, while geranyl pyrophosphate synthase gene is located on separate contigs [94].

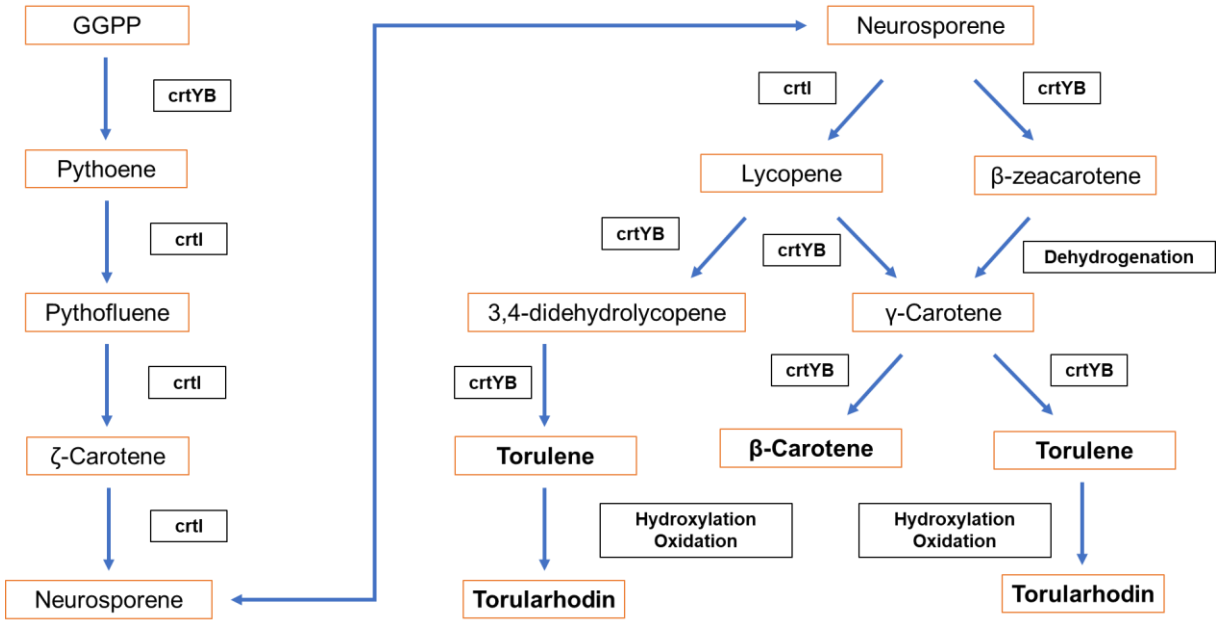

Figure 9 – Proposed carotenes biosynthetic pathways in yeasts. The pathways start in the precursor geranylgeranyl pyrophosphate (GGPP). Gene crtYB codes for bifunctional lycopene cyclase and phytoene synthase, whereas gene crtI codes for a phytoene desaturase. Adapted from [94].

Yeast genera *Rhodotorula*, *Rhodosporidium* and *Sporobolomyces* are known for producing mostly β-carotene, torulene and torularhodin. *R. mucilaginosa* and *R. glutinis* are major producers and the most studied species, and their carotenoids showed to be great antioxidant, anti-cancer and antimicrobial properties. The genus *Xanthophyllomyces*, namely *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*), is the great producer of astaxanthin [3,23,95,96]. Carotenoids production by yeasts presents advantages in terms of high biomass growth rate, ease cultivation and scale up, and growth in several carbon sources [96]. Carotenoids can be generated by yeasts from oats, wheat, barley, corn, rice, lipids and related substances, glycerol, cellobiose, grape must, cheese whey and specially, sugar cane and sugar beet molasses [23,96]. In pectin-rich substrates, *R. mucilaginosa* and *R. glutinis* were able to produce carotenoids, namely β-carotene, torulene, and mostly torularhodin, from sugar

beet molasses [23,24]. A *Rhodotorula* strain was reported to produce higher biomass and carotenoids yield from apple pomace, and addition of ferrous ammonium sulphate led to the highest carotenoids [97]. Furthermore, a *Xanthophyllomyces dendrorhous* carotenoid-hyperproducing mutant produced astaxanthin from sugar beet molasses [25].

2.4.4. Enzymes

Enzymes are used in the most diverse areas of Biotechnology, such as food and feed industries, pulp and paper processing, textiles, detergents, fine chemicals, aromatic and flavouring compounds production, biofuels, pharmaceuticals, bioremediation, cosmetics and personal care products [1]. Production of enzymes by yeasts from agro-industrial residues is still one of the most relevant applications for these substrates, specifically the production of pectinases.

Pectinases are used in extraction and clarification of fruit juices, maceration of vegetables, oil extraction and animal feed, being extremely useful in food and sugar industries [98], but also in textiles and pectin wastewaters treatment [97]. Polygalacturonases (PGs) or hydrolytic depolymerases are pectinolytic enzymes that hydrolyse D-GalA polymers [29,97]. *K. marxianus* secretes an endogenous polygalacturonase (PG) from glucose medium and sugar beet molasses, although with lower activity [20]. Other pectinases are protopectinases (solubilise protopectin to pectin), and pectinesterases (de-esterify and remove methoxy groups) [29]. *Trichosporon penicillatum* produced a protopectin from citrus peels. Almost all pectin was extracted from peel without its maceration [99]. Moreover, pectinases and lignocellulosic enzymes (pectinase, manganese-dependent peroxidase, cellulase, xylanase) were produced in apple pomace, by *Torula (Candida) utilis* [100].

Additionally, there are other yeast enzymes produced in pectic extracts, including naringinase, produced by a *Clavispora lusitaniae* in citrus juice. Metal ions Mg and Zn stimulated this enzyme activity. This enzyme is important in beverages industries, for aroma production and in pharmaceuticals, in antibiotics production and sterol biotransformation. Naringinase hydrolyses glycosides, and the result products have antioxidant, anti-inflammatory, antiulcer, hypocholesterolaemic, antimutagenic, neuroprotective and antiviral properties [21]. Also, chitinases, which has antifungal activity (post-harvest biocontrol), were produced by *Metschnikowia pulcherrima* and *Rhodotorula* sp. in apple juice medium [22].

2.4.5. Other interesting added-value products

Yeasts can also produce from pectic residues aroma compounds and oils. Volatile or non-volatile aromatic compounds improve products' organoleptic properties and are very valuable in chemical, food, cosmetic and pharmaceutical industries [101]. They comprise 25% of global market of food additives [86]. In addition to *K. marxianus* production of volatile fruit-like aroma compounds, in sugar beet plus sugar cane residues [101], *Hanseniaspora uvarum* and *Hanseniaspora valbyensis* produce volatile fruity-like aroma compounds, with high acetic acid esters content, from apple pomace [86].

High temperatures and limited nitrogen availability are stress conditions that appear to be important for promotion of volatiles production [101].

Fatty acids and oil are important for biodiesel production. The interest in biodiesel have been growing in the last years, since it is a renewable and environmentally friendly option [102]. Single-cell oils and lipids, in particular fatty acids, produced by yeasts having agro-industrial wastes as carbon sources, are good choices for the production of biodiesel [19], and can also be used as substitutes for vegetable oils and fats (as cocoa butter) [102]. *R. glutinis* produced lipids from sugar beet molasses. From total lipids obtained, fatty acids composition was mostly oleic acid ($C_{18:1}$), palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$), and palmitoleic acid ($C_{16:1}$), with a total C16 and C18 content of 92.3%, being a suitable biodiesel substrate [19].

Yeasts are rich sources of proteins, minerals and vitamins, desired in nutritional enrichment. Single cell protein (SCP) or yeast components can easily be produced from agro-industrial wastes. They are extremely useful for food and feed nutritional enrichment, which is important in the reutilization of these wastes substrates, but also in food and nutritional shortages [1,97]. *Torula utilis, Candida tropicalis, Candida parapsilosis* and *Candida solani* are sources of SCP from sugar beet pulp and molasses [103]. *Kloeckera* sp. and *Torula utilis* produce proteins, fat, fibres, vitamins and minerals for animal feed nutrient enrichment from apple pomace [104,105]. Fermentation of apple pomace by *Torula utilis,* followed by *Kloeckera* sp. was used as rat feed, but only a mixture of fermented apple pomace with commercial rat feed led to better acceptability and digestibility [104]. Strains of *Torula utilis* fermented hydrated apple pomace with ammonium sulphate. After simultaneous saccharification and fermentation, higher protein content and mineral (Zn, Cu, Mn and Fe) enrichment were notorious [105].

2.5. Non-conventional yeast species with potential for the synthesis of added-value bioproducts

Non-conventional yeasts present great added-value production potential, not even in pectin-rich wastes, but in the most diverse substrates. Some of the species that present higher biotechnological interest are *Kluyveromyces marxianus*, *Meyerozyma guilliermondii* and *Rhodotorula mucilaginosa*. The most relevant features of these species and the products synthesized by these yeasts are detailed below.

2.5.1. *Kluyveromyces marxianus*

Kluyveromyces marxianus is one of the most studied and used non-conventional yeast species in biotechnological industries. It was first isolated from grapes by Marx and later described in 1888 by E. C. Hansen, which named the isolate *Saccharomyces marxianus* [106]. It is frequently found in dairy products (fermented cheeses, milk or yogurts), several fresh and fermented fruits and vegetables and residues of sugar processing industries [1,15,107]. It is considered a GRAS organism [107].

K. marxianus is a respiro-fermentative species that generates energy from the TCA cycle by oxidative phosphorylation and ethanol fermentation. *K. marxianus* shows similarities with *S. cerevisiae*, being reported as both Crabtree-positive and Crabtree-negative, which is still controversial [107]. Crabtree effect is the ability of fermentation in high concentration glucose medium and presence of oxygen. In these conditions, Crabtree positive yeasts, like *S. cerevisiae,* can perform fermentation and respiration simultaneously [82].

This species is described to utilize several carbon sources, including lactose, glucose, fructose, galactose, xylose, arabinose, inulin, cellobiose, glycerol, malic acid, lactic acid and others [15,106,108]. Several strains of *K. marxianus* are reported to grow in medium with xylose, xylitol, arabinose, lactose, cellobiose or glycerol [108]. The utilization of lactose make it an interesting yeast in dairy food industry and it explains the isolation from these sources [1]. Also, this species is described by fermentation of agro-industrial substrates as sugar cane, corn silage juice, molasses, lignocellulosic hydrolysates, whey powder and waste streams [15,108].

One of *K. marxianus* most important traits is its thermotolerance, being capable of growing until a maximum temperature of 52ºC and it ferments at high rates in the range 40ºC - 45ºC. Also, *K. marxianus* presents high secretion capacity [106–108].

K. marxianus is able to synthesize a vast range of valuable industrial products being one of the most used non-conventional yeasts. This species is well known for its ability to produce bioethanol at high temperatures. Several strains produced ethanol from glucose and xylose, at temperatures ranging from 30ºC to 45ºC [79,108]. A study reports fermentation of glucose, xylose, mannose, galactose, but not arabinose, under shaking conditions, while no pentose fermentation occurred in static conditions [109]. Ethanol was also produced in corncob with both aerating and non-aerating conditions [110]. Additionally, there are reports of galactose fermentation in aerobic conditions [111], lactose and cheese whey [107].

The production of xylitol and arabitol by *K. marxianus* is a desired trait. A study reports a maximum xylitol production from xylose medium at 40ºC, decreasing at 30ºC and 45ºC [109]. Xylitol production was also reported in corncob at 40ºC, and in glucose and xylose co-fermentation, with both aeration and no aeration conditions [91,110]. Production of arabitol was also seen in arabinose medium, at 30ºC and 40ºC [110].

K. marxianus strains produce several aromatic compounds, like fruit esters, carboxylic acids, ketones, furans, alcohols and aldehydes, monoterpene alcohols, isoamyl acetate and the isoamyl alcohol 2-phenylethanol [106]. In cassava bagasse, 11 compounds were identified, especially acetaldehyde and ethyl acetate [112]. In a glucose medium, *K. marxianus* produced alcohols, acids esters and aldehydes, and the higher concentrations were attained for isoamyl alcohol, 2-phenylethyl alcohol, and isobutyric acid [113].

The ability of *K. marxianus* to synthesize enzymes of several groups make it a desired cell factory in several industries. *K. marxianus* secretes endogenous polygalacturonases (PG) from glucose medium, and sugar beet molasses [20], and also fructose, sucrose, galactose and xylose in anaerobic

conditions [114]. Thermostable β-galactosidases (used for the removal of lactose from dairy products) can be produced by *K. marxianus* in lactose or galactose media [115]. Also, a thermostable β-xylosidase, utilized in grape juice extraction and wine aroma production, can be synthesized from glucose, xylose, cellobiose, sucrose and lactose media [116]. Inulinases were produced from inulin or sucrose medium, being used for the production of fructose syrup [117]. More recently, carboxypeptidases and aminopeptidases were produced in glucose, by strains isolated from meat and cheeses substrates, to which these enzymes confer taste and texture [118,119]. Moreover, β-glucosidase and phosphatases, have also been reported to be produced by *K. marxianus* strains [106].

Besides their endogenous proteins, *K. marxianus* is also used as host for heterologous protein production. Some examples of these proteins are lactate dehydrogenase, which lead to generation of lactate, utilized in food industry, and thermostable endo-β-1,4-glucanase, cellobiohydrolase and β-glucosidase, leading to the conversion of cellulosic materials into ethanol [26,120]. Furthermore, an *Aspergillus oryzae* α-amylase was successfully expressed in *K. marxianus* [108].

K. marxianus is also useful in the removal of textile dyes and copper ions from wastewaters and cleaning of paper industry sludges, having molasses as carbon sources [106,107]. It is used as baker's yeast and it was tested as an anticholesterolemic agent in rats [121]. Studies show that some strains can also be a source of protein-rich biomass, as an alternative to yeast lysates from *S. cerevisiae*, and of oligonucleotides (flavour enhancers), oligosaccharides (prebiotics) and oligopeptides (immunologic stimulators added to dairy products after whey protein hydrolysis) or can function as natural bioemulsifiers [40,106].

2.5.2. *Meyerozyma guilliermondii*

Meyerozyma guilliermondii (previously known as *Pichia guilliermondii* and the anamorph form being known as *Candida guilliermondii*) biotechnological potential was first assessed in the end of the 1970s in the former Soviet Union and the German Democratic Republic. Nowadays, its industrial interest in production of sweeteners, vitamins, biofuels, enzymes, aroma and pharmaceutical compounds is being investigated [90].

M. guilliermondii is an aerobic Crabtree-negative yeast. Still, it exhibits respiro-fermentative metabolism, reported in a few studies [122]. This species is unable to growth on strict anaerobiosis [90,122]. Strains of *M. guilliermondii* are spread in a variety of environments, being isolated from plants, fruits, soil (oil-containing soil), sea water, lake water, insect exudates, processed food products and animals [81,90,123]. Despite being an opportunistic pathogen, it belongs to GRAS organisms and can be used for research and industry [40].

Different strains present dissimilar capacity of sugar metabolization. In general, *M. guilliermondii* is capable of fermenting and assimilate diverse sugars, namely glucose, sucrose, arabinose, xylose, ribose, rhamnose, galactose, cellobiose and many others, but they are unable to use lactose, starch and inositol [81,90,123]. This species can also grow in glycerol, succinate or citrate (respiratory

substrates) and to utilize hydrocarbons (natural mixtures and n-hexadecane) [40]. Growth in xylose, arabinose, maltose, glycerol, and whey is also reported [81,124].

This species optimal growth is achieved at 30ºC, and presents high osmotolerance and tolerance to the inhibitors present in hemicellulosic bagasse hydrolysates, that can be used as growth substrates, due to the fast metabolization of xylose and arabinose [81,84].

Several *M. guilliermondii* strains can ferment glucose, xylose and even arabinose, present in hydrolysates [81,123]. Good fermentative efficiency is observed in xylose medium [80]. Despite ethanol production in soybean hull hydrolysates [18,84], a strain isolated from sugarcane juice revealed xylose and arabinose-fermentation ability, and it produced ethanol from sugarcane bagasse [81].

M. guilliermondii is extremely efficient in the conversion of xylose to xylitol, and there are also several reports of arabitol production from arabinose by this species. Both xylitol and arabitol are produced when oxygen-limited conditions started, in xylose and arabinose medium [125]. Also, at low oxygen levels, high xylitol production yields were obtained in xylose medium, xylose plus acetic acid, furfural and 5-hydroxymethylfurfural medium [84,126], and in sugarcane bagasse hydrolysates [18,81]. *M. guilliermondii* is able to produce it in arabinose medium, with high concentrations [89,127]. A combination of arabinose medium with corn fibre hydrolysate allowed the simultaneous xylose and arabinose metabolization into xylitol and arabitol, but at low production yields [127].

Fatty acids and oil are also synthesized by some strains. *M. guilliermondii* originates lipids and single cell oil in oil-producing medium containing inulin or glucose, producing mainly palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$), and the majority of lipids can be transformed into biodiesel [102]. Lipids production from glucose and glycerol, derived from oil-based biodiesel factory, also showed great biodiesel production potential [128].

The production of riboflavin (vitamin B2) is characteristic of this species. Riboflavin is used in human nutrition, as treatment for several diseases, in animal feed supplementation and as a yellow colorant in some beverages [90,123]. *M. guilliermondii* is capable of overproduce riboflavin in iron-limiting media. This mechanism is not fully understood, but most biosynthetic enzymes are overexpressed under iron deficiency [40,90].

M. guilliermondii is also a producer of industrially relevant enzymes. This species is one of the principal producers of inulinase and rhamnosidase. An exo-inulinase was produced from oil-producing medium containing inulin, sucrose, glucose or fructose [102]. This species can also synthesize α-amylase, which is induced in the presence of starch, in glucose and starch medium [129]. An intracellular α-rhamnosidase, produced by *M. guilliermondii* in rhamnose medium, is used for obtaining L-rhamnose and in grape processing for wine making [130].

The antifungal activity of *M. guilliermondii* is widely used in the protection of plants from postharvest fruit rotting. It presents multiple modes of action, like attach of the hyphae and competition for nutrients [90]. *M. guilliermondii* synthesises cell wall-degrading enzymes, like exo-1,3-β-glucanase, which play an important role in fungi inhibition, in medium with fungi cell walls, glucose and sucrose [131]. Also,

tomatoes inoculated with *M. guilliermondii* at 20ºC demonstrated an activation plant defence enzymes (peroxidase, polyphenol oxidase, superoxide dismutase, catalase, phenylalanine ammonia lyase, chitinase and b-1,3-glucanase) [132].

Several aromatic compounds are reported to be formed by *M. guilliermondii*: esters, alcohols, ketones, alkanes, acids and other aroma substances, from fermentation of grapes [133]. This species is also reported as a great producer of 2-phenylethanol production from whey and glucose [124]. *M. guilliermondii,* produced monoterpenols (geraniol, linalool, nerol), 2-phenylethanol and geranic acid, from glycosidic precursors from Muscat grape juice at 40ºC [130].

2.5.3. *Rhodotorula mucilaginosa*

Rhodotorula mucilaginosa (previously called *Rhodotorula rubra*) is an obligate aerobic yeast, generally non-fermentative. This species is found in a wide variety of environments, such as food and beverages, like living rotten fruits and other plants, juices and ciders, peanuts, soil, processed and dairy products (milk, cheese, sausages), air, fishes and sea food, fresh waters (lakes, rivers), sea waters (coastal and deep waters) and even in cold environments, ultra-acidic waters and uranium leachates [43,134]. It is reported as animal and human opportunistic pathogenic species, being isolated from faeces, skin and digestive tract, causing lung and skin infections and meningitis [43,134], which may compromise its applications.

Different strains present variable sugar assimilation capacity. In general, *R. mucilaginosa* can metabolize glucose, xylose, arabinose, galactose, mannose, ethanol and glycerol [134,135]. There are references to a highly efficient galacturonic acid metabolism, although its functioning remains unknown [136]. This is an extremely important feature, since pectin-rich residues contain a large fraction of this acid sugar, but generally it is not metabolized by yeasts. There are also reports of growth in medium containing glucose, xylose, maltose, galactose, sucrose or glycerol, and whey [124].

R. mucilaginosa presents good growth at 30ºC, presenting variable growth at 35ºC, at which some strains even impaired growth [134,137]. Cells produce pink pigments, which are carotenoids, that confer them colour, and the corresponding colonies are also very mucoid [43,124,134].

R. mucilaginosa presents interesting features that, despite being an opportunistic pathogen, are useful for biotechnology and industry. There are few reports on its fermentative ability, still one study states the production of ethanol from glucose, mannose and galactose medium, xylitol from xylose and arabitol from arabinose, and higher conversion levels into ethanol and xylitol were attained through fermentation of lignocellulosic hardwood and softwood hydrolysates [135]. Concerning the presence of acetic acid and furfural, this last compound enhanced the production of xylitol, while increasing concentrations of acetic acid (until 20 g/L) improved ethanol yield, but xylitol yield decreased [138].

R. mucilaginosa is one of the greater producers of carotenoid pigments, namely β-carotene, torulene and torularhodin [3,23]. Carotenoids production in glucose, whey lactose medium, and sugar beet molasses were observed, and it was found that increasing aeration and increasing glucose or molasses sucrose concentration led to higher production. [23]. In another study, carotenoids

production in glucose, namely β-carotene, torulene and torularhodin, was favoured by metal ions addition (Fe^{2+}, Mg^{2+}, Ca^{2+}) [96]. When food wastes were used as substrates, ketchup permitted to produced higher total carotenoids, followed by sugarcane molasses [96]. Growth in glucose medium indicate that the nature of the nitrogen sources chosen is a relevant factor for intracellular accumulation of carotenoids [139].

This species secretes cold-active pectinases, in citrate pectin medium, specifically pectin methyl esterase, endopolygalacturonase, and exopolygalacturonase, being relevant for wine making and juice industry [140]. *R. mucilaginosa* extracellular acetylxylan esterase, useful for removal of acetyl groups from lignocellulosic residues, can be produced in glycerol medium [141]. Also, a phytase, used for degrading phytates (strong chelating agents of minerals, reducing their uptake by intestines) present in human and animal food (cereals and vegetables), was synthesized from glucose medium [142].

R. mucilaginosa accumulates single cell oil and fatty acids, showing potential for biofuels production [143]. There is one report describing fatty acids production from molasses states that the majority of fatty acids were C16 and C18, showing great biodiesel potential [143]. In cassava starch hydrolysate, a higher oil concentration was produced compared with glucose, xylose and sucrose medium, with the best nitrogen source being yeast extract; most of the fatty acids produced were C16 and C18 [144]. In similar tests in inulin hydrolysate and extract of Jerusalem artichoke tubers hydrolysate, the accumulation of fatty acids was slightly higher in the first substrate, and C16 and C18 were the most accumulated lipids [145].

R. mucilaginosa plays a role in bioremediation and biocontrol. It can be applied for the removal of phenolic compounds, totally degrading protocatechuic, vanillic and p-coumaric acids and tyrosol, and reducing gallic acid and catechol, being able to decrease the chemical oxygen demand and phenols content of olive mill wastewaters [146]. This species was also used as antifungal agent for the removal of postharvest grey and blue mould, and as anti-natural decay agent from apples, reducing apples' lesions diameter and decay incidence caused by moulds [147].

3. Materials and Methods

3.1. Strains and culture media

Nineteen yeast strains isolated either in this study or selected from IST yeast collection were screened to determine the potential for bioconversion of pectin-rich residues. Six strains were selected: *Rhodotorula mucilaginosa* IST390 and *Kluyveromyces marxianus* IST389 were isolated from hydrated sugar beet (SBP) in this work and in the work of MSc student Paula Semedo, whereas, *Meyerozyma guilliermondii* IST369 and *Rhodotorula mucilaginosa* IST423 were taken from the IST collection. The reference industrial strain *Saccharomyces cerevisiae* Ethanol Red and the type strain *Kluyveromyces marxianus* CBS712 (PYCC 3886) were also tested for comparative analysis. *Saccharomyces cerevisiae* CEN.PK122 was also utilized as control in toxicity assays.

Strains of yeasts isolated in this work and from yeast collection were grown in yeast peptone dextrose (YPD) agar plates, containing 1 %(w/v) yeast extract (Difco), 2 %(w/v) bactopeptone (Difco), 2 %(w/v) glucose (Sigma-Aldrich), and 2 %(w/v) agar at 30ºC, pH 5.0. For the screening assay of growth ability in different carbon sources, pre-cultures were performed in liquid yeast peptone glycerol (YPG) composed by 1% (w/v) yeast extract, 2 %(w/v) bactopeptone and 1.8 %(v/v) glycerol at 30ºC, pH 5.0, with orbital shaking (250 rpm), in aerobic conditions. Yeast growth profiles were monitored in minimal media (MM) containing 0.17 %(w/v) yeast nitrogen base (YNB) (Difco) without ammonium sulphate ($(NH_4)_2SO_4$) and amino acids supplemented with 0.265 %(w/v) $(NH_4)_2SO_4$ and 20 g/L (2% (w/v)) of glucose, xylose or arabinose (Sigma-Aldrich), or on the SBP hydrolysate, at pH 5.0, all at 30ºC and orbital agitation. Aerobic and microaerophilic assays were performed in SBP hydrolysates, at 30ºC, pH 5.0, at 130 rpm. The hydrolysates were prepared, sterilized and provided by Professor Wolfgang Liebl Lab (TUM), the German partner of the EraNet Project YEASTPEC. Their composition is detailed in the Results section, Table 5. The influence of acetic acid in yeast growth was tested in minimal media (MM) with 20 g/L (2% (w/v)) of glucose or arabinose (Sigma-Aldrich), supplemented with 0, 10, 20, 35 or 50 mM acetic acid, at 30ºC, pH 5.0, 130 rpm. The influence of temperature and pH was tested in SBP hydrolysates at pH 4.5 and 5.0, at 30ºC and 35ºC. For carotenoid production, cultures of *Rhodotorula mucilaginosa* strains were carried out in MM with 20 g/L glucose, YPD or in SBP hydrolysate H11, 30ºC, pH 5.0, 250 rpm.

3.2. Isolation and identification of yeast stains potentially interesting for pectin-rich residues bioconversion

3.2.1 Isolation of yeast stains from SBP

The isolation of yeasts with potential for bioconversion of pectin sugars was achieved by hydration of dry sugar beet pulp (SBP) (50 g of dry pulp in 500 mL water, corresponding to 10 %(w/v)), obtained from the Belgian sugar company Tiense Suiker (https://www.tiensesuiker.be), supplemented with peptone (10 g/L) and chloramphenicol (100 µg/mL), and incubated during 3 weeks at 30ºC, 250 rpm. Each two days, samples were collected. For macerated cherries and fresh grapes, they were washed in sterile water, and samples of the water extract were collected. 100 µL of these samples were

spread on YPD agar plates, supplemented with 100 µg/mL chloramphenicol. The utilization of chloramphenicol allowed the control of growth of the bacteria also present in the samples used for yeast isolation. Plates were incubated for 2 to 4 days, at 30ºC. Single colonies morphologically distinct were obtained from different samples, and were streaked on new YPD agar plates, incubated for 2 to 4 days at 30ºC.

3.2.2. Molecular identification of the yeast isolates

Total yeast DNA from the isolates was extracted with 1:1 volume (200-300 µL) of phenol/water plus the corresponding volume of glass beads to disrupt the cells. After 2 minutes vortex, the mixtures were centrifuged (14000 rpm, 5 minutes, 4ºC) in a microcentrifuge MiniSpin Plus (Eppendorf). The supernatant was resuspended in 1:1 volume (300 µL) phenol/aqueous phase, vortexed by 10 seconds and centrifuged in the same conditions. The upper phase was collected, and 1:1 volume (300 µL) ether/aqueous phase was added, being vortexed for 20 seconds and centrifuged. After collection of the lower phase, DNA was precipitated with 1mL of absolute ethanol, incubated at 20ºC for 15 minutes and centrifuged. The pellet was washed with 0.5 mL of 70 %(v/v) ethanol, dried using a SpeedVac Concentrator Plus (Eppendorf) (45ºC, 15 minutes), and resuspended in 100 µL of sterile water. Quantification of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer. Amplification of yeast ribosomal DNA was performed by PCR with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-GCATATCAATAAGCGGAGGA-3') [148], which amplify rDNA of ITS 1 and 2 regions, respectively, and primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGT TTCAAGACGG-3') [148], that amplify large-subunit 26S D1/D2 region from rDNA. The reactions of 50 µL contained 1X Phusion HF Buffer, 200 µM of each dNTP, 1.0 µM of each primer (ITS1 + ITS4 or NL1 + NL4), 3% DMSO, 0.5 mM $MgCl_2$, 1.0 U Phusion DNA Polymerase, 75 ng/µL of template (gDNA) and sterile water. The PCR program used was: initial denaturation (98°C, 30 sec); 35 cycles of denaturation (98°C, 10 sec), annealing (52°C, 20 sec) and extension (72°C, 30 sec); final extension step (72°C, 8 min). PCR products were separated by electrophoresis (110 V, 400 mA, 100 W) in 0.8% agarose gel for 50 minutes, DNA bands were cut in the transilluminator, and purified with NZYGelpure kit (NZYTech). Quantified DNA by NanoDrop spectrophotometry was sequenced by STABVIDA, Lda. The sequencing results were submitted to BLASTN analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), being compared to the ones deposited in the National Center for Biotechnology Information (NCBI) database, to identify the yeasts species. After correct identification, aliquots of yeast cultures in YPD medium with 20 % glycerol were stored at -80ºC.

3.3. Screening of yeast strains for growth ability in distinct carbon sources

Nineteen yeast strains were tested for growth in several carbon sources. Yeast strains were first batch cultured in liquid YPG, at 30ºC, pH 5.0, 250 rpm. Tests were performed in liquid MM with 20 g/L of glucose, xylose or arabinose (Sigma-Aldrich), or in the SBP hydrolysate H6. SBP hydrolysates were received after filtration, being sterile and not containing solid residues. The initial acidic pH of the hydrolysate was increased to 5.0 with 10M NaOH solution. Yeast strains were cultivated in cotton

plugged test tubes with 4 mL for 42h, at pH 5.0, 30ºC and 250 rpm, with initial optical density (OD) 0.25 for single carbon source media and 0.5 for SBP hydrolysate. Culture OD (absorbance at 600 nm) were measured at 0 h, 21 h and 42 h of cultivation.

3.4. Growth and metabolic activity of the selected yeast strains in SBP hydrolysates

3.4.1. Aerobic and microaerophilic assays

Selected yeast strains were tested for aerobic growth and microaerophilic fermentations. Strains were cultured in the respective diluted SBP hydrolysates, with and without supplementation with 2 g/L urea, diluted with distilled water (ratio 1:1), in 50 mL flasks, at 30ºC, pH 5.0, with orbital shaking (130 rpm). Initial acidic pH of the hydrolysates was increased to 5.0 with 10M NaOH solution. For aerobic growth curves, strains were inoculated in 25 mL SBP hydrolysates with and without supplementation with 2 g/L urea, with initial optical density (OD) at 600nm (absorbance at 600 nm) of 0.5. The absorbance was measured in a U-2001 (Hitachi) spectrophotometer. Cultivations were performed in cotton plugged 50 mL flasks, at 30ºC, pH 5.0, 130 rpm for a minimum of 168 h and a maximum of 216 h. Culture OD_{600nm} were measured and 400 µL samples were collected at several time points until the end of the growth. Final OD_{600nm} and pH were measured, and 1 mL samples collected. The maximum specific growth rates (µ) were calculated in the exponential phase of growth based on each strain growth curves, using the formula:

$$\mu = \frac{\ln (OD_{600nm} - OD_{initial\ 600nm})}{\Delta t}$$

For microaerophilic assays, yeast strains were inoculated in 20 mL SBP hydrolysates with and without supplementation with 2 g/L urea, with initial OD_{600nm} 4, and cultured in sealed 30 mL flasks, with magnetic agitation (130 rpm), at 30ºC, pH 5.0 for 168 h. For monitoring CO_2 release, flaks were weighted at several time points. Final OD_{600nm} and pH were measured, and 1 mL samples collected.

3.4.2. Determination of carbon sources consumption and ethanol and arabitol production by HPLC

The collected samples were centrifuged (10000 rpm, 3 minutes) in a microcentrifuge MiniSpin Plus (Eppendorf) and 100 µL of supernatant were pipetted into a high-performance liquid chromatography (HPLC) vial and diluted in 900 µL 5 mM H_2SO_4. The concentration of glucose, arabinose, galactose, galacturonic acid, acetic acid, ethanol and arabitol in each sample was determined by HPLC (Hitachi LaChrom Elite), using a column Aminex HPX-87H (Bio-Rad). Sugars and alcohols were quantified through refractive index detection, while the acids were quantified through UV/visible detection. The elution of the compounds was performed at 65ºC and 0.6 mL/min flow rate, with 5 mM H2SO4, for 30 minutes. The respective concentrations were calculated through calibration curves constructed for each compound.

In aerobic assays, volumetric consumption rates were calculated as the ratio of g/L of each substrate per its time of consumption, whereas maximum production rates (productivity) of ethanol and arabitol were calculated as the ratio of maximum or final g/L of product per time of production [149,150], as indicated below:

$$\text{Consumption rate (g/L/h)} = \frac{\text{Final substrate - Initial substrate present (g/L)}}{\text{Consumption time (h)}}$$

$$\text{Production rate (Productivity) (g/L/h)} = \frac{\text{Maximum product produced - Initial product present (g/L)}}{\text{Production time (h)}}$$

Maximum ethanol yields were determined as the ratio of g/L of maximum ethanol produced per g/L of total reducing sugars consumed (considering glucose and galactose), for each strain, while maximum arabitol yields were determined as the ratio of g/L of maximum arabitol produced per g/L of total arabinose consumed, for each strain [135,149]. The respective formulas are:

$$\text{Maximum ethanol yield (g/g)} = \frac{\text{Maximum ethanol - Initial ethanol present (g/L)}}{\text{Total reducing sugars consumed (g/L)}}$$

$$\text{Maximum arabitol yield (g/g)} = \frac{\text{Maximum arabitol (g/L)}}{\text{Total arabinose consumed (g/L)}}$$

3.5. Carotenoids production assays

3.5.1. Culture of *Rhodotorula mucilaginosa* strains

Rhodotorula mucilaginosa IST390 and *Rhodotorula mucilaginosa* IST423 were first cultured in MM with 20 g/L glucose, YPD medium (100 mL flasks) and in diluted SBP hydrolysate 11 (1:1) (50 mL flasks), at 30ºC, pH 5.0, 250 rpm. Then, yeasts were inoculated in same MM media (100 mL flasks) and SBP hydrolysate (50 mL flasks), at 30ºC, pH 5.0, 250 rpm, for 120 h, being the initial OD_{600nm} 0.5. Final OD_{600nm} was also measured.

3.5.2. Extraction and quantification of total carotenoids

The extraction and quantification of carotenoids was performed as described before [23], with some modifications. Cells were centrifuged (8000 rpm, 3 minutes) in a centrifuge 5804 R (Eppendorf), to remove supernatant, and the pellet was washed three times with distilled water. Light exposure was prevented using aluminium foil. Biomass pellets were frozen at -20ºC and lyophilized in Scanvac CoolSafe Freeze Dryer (LaboGene), for 3 days. Before extraction, cells dry weight was determined. Then, the pellet was resuspended in 2 mL acetone, and cells were disrupted using zirconia beads, releasing intracellular carotenoid to acetone phase. This suspension was vortexed for 5 minutes and centrifuged (8000 rpm, 3 minutes). The supernatant was collected, and 2 mL acetone was added, repeating this step until total pellet colourless was achieved. Acetone extracts were collected, and all acetone evaporated using RapidVap Vacuum Dry Evaporator (Labconco). Dried carotenoids were resuspended in 1 mL acetone and their absorbance was measured at 452 nm (β-carotene maximum absorbance wavelength in acetone phase), with an extinction coefficient $E^{1\%}_{450}$ = 2500 [151], in a spectrophotometer. Total carotenoids concentration was calculated using the expression:

$$\text{Total carotenoids (µg/g dry biomass)} = \frac{Abs_{452nm} \times 10^4 (\mu g/ml)}{2500 \times \text{cells dry weight (g/ml)}}$$

3.6. Effect of stress factors in yeast growth profiles

3.6.1. Influence of acetic acid in glucose and arabinose growth profiles

Rhodotorula mucilaginosa IST390, *Kluyveromyces marxianus* IST389, *Meyerozyma guilliermondii* IST369 and *S. cerevisiae* Ethanol Red were tested in minimal medium with glucose or arabinose supplemented with increasing concentrations of acetic acid, to know the influence of acetic acid in yeast growth profiles in the different sugars. Yeast pre-inocula were prepared by growth in MM with 10 g/L glucose or arabinose, in 100 mL flasks at 30ºC, pH 5.0, 130 rpm. The main culture was inoculated in the same medium supplemented with 0, 10, 20, 35 and 50 mM acetic acid, in 100 mL flasks at 30ºC, pH 5.0, 130 rpm, for 168 h. A stock solution of 5 M acetic acid at pH 4.5 was utilized to perform the correct dilutions in the medium. Culture OD_{600nm} were measured at different times points. The assays were performed at least in duplicate. After the construction of the growth curves, the maximum specific growth rates (μ) in exponential phase were calculated.

3.6.2. Influence of temperature and pH in SBP hydrolysates growth curves

Rhodotorula mucilaginosa IST390, *Meyerozyma guilliermondii* IST369, *Kluyveromyces marxianus* IST389, *Kluyveromyces marxianus* CBS712, *S. cerevisiae* Ethanol Red and *S. cerevisiae* CEN.PK122 were pre-grown in SBP hydrolysate H13, pH 4.5, 250 rpm, at both 30ºC and 35ºC. The inocula were performed in 96-well microplates, in 200 μL of SBP hydrolysates H11 and H13, at pH 4.5 and 5.0. The assays were performed with lidded plates, at both 30ºC and 35ºC, with an initial OD 0.1. Growth profiles were determined in a Multi-mode Microplate Reader FilterMax F5 (Molecular Devices), through Kinetics mode, during 40 h. ODs were constantly measured at each 15 minutes, during 40 h, with orbital agitation between reads.

4. Results

4.1. Isolation and identification of yeast strains for bioconversion of pectin-rich agro-industrial residues

In order to isolate and identify yeast strains with potential for the valorisation of pectin-rich residues, the isolation of yeasts was performed from samples of 10 %(w/v) hydrated sugar beet pulp (SBP) (50 g of dry pulp to 500 mL of water), received from Belgian sugar company Tiense Suiker (https://www.tiensesuiker.be) , that were supplemented with 100 µg/mL chloramphenicol and 10 g/L peptone. The utilization of chloramphenicol allows the prevention of bacterial growth, also present in the samples used for yeast isolation. These mixtures were incubated during 3 weeks at 30ºC, 250 rpm. Each two-days of incubation, culture samples were collected and spread on YPD agar plates with 100 µg/mL chloramphenicol. In addition, samples of macerated and fresh fruits, namely cherries and grapes, washed in sterile water, were also used for yeast isolation. Despite their lower pectin content, those samples are still interesting yeast sources considering the project objectives. These samples were also spread on YPD agar plates with 100 µg/mL chloramphenicol. Plates were incubated for 2 to 4 days at 30ºC.

Single colonies morphologically distinct were obtained from different samples and were streaked on new YPD agar plates. Total DNA from these yeast isolates was extracted, precipitated and quantified. Specific regions of ribosomal DNA that are very conserved among strains of the same species was amplified using universal primers widely used in species identification for ITS 1 and 2 regions and large-subunit D1/D2 region rDNA [148], allowing their specific identification. After purification and quantification of amplified DNA, this was sequenced, and the sequences submitted to BLASTN analysis in NCBI (National Center for Biotechnology Information) database. This isolation work was performed in collaboration with the MSc student Paula Semedo.

In this work, strains from 5 different species were identified and deposited in IST Yeast Collection with a specific identity number. The ID of each strain, their environmental origin, rDNA region used for species identification and identity percentage obtained in BLASTN analysis (% of identity with rDNA sequences deposited in NCBI database are detailed in Table 6. The DNA sequences obtained for each strain are showed in Supplementary Materials Table S1.

From hydrated SBP with peptone samples, 3 distinct colonies were identified. One colony was distinguished by its dull light-cream colour and smooth and flat texture and identified as *Pichia kudriavzevii*, through IST1/ITS2 rDNA sequencing, with a 99 % identity (Table 6). Another colony was cream-coloured, shining and rugose with slight raised centre, further identified as *Clavispora lusitaniae*. The last strain was identified as *Rhodotorula mucilaginosa,* a dark pink-coloured strain, very mucous and glistening, with a smooth and flat appearance. Both strains were identified based on the sequences of their D1/D2 rDNA region sharing 99 % identity with DNA sequences from yeast strains deposited in the NCBI database.

From cherries and grapes, two strains were identified, one from each sample. From macerated cherries, one strain was identified as *Metschnikowia pulcherrima*, by sequencing of its IST1/IST2

rDNA region, with 96 % identity. Their colonies exhibited a reddish colour which released the pigment to the medium, smooth and shining, with a raised centre and thin edge ring. The other strain, identified as *Hanseniaspora opuntiae*, showed a very apiculate centre, with smooth and glistening aspect, and cream colour. It was identified through D1/D2 rDNA sequencing, with 99 % identity.

All the isolated and identified strains were deposited in IST Yeast Collection. The isolates and IST Collection strains of non-conventional yeasts were subjected to screening to evaluate their ability to grow in single carbon sources and in SBP hydrolysates with complex sugar mixtures.

Table 4 – Isolated and identified yeast strains of different species. Strains were isolated from pectin-rich sources, namely sugar beet pulp and fruits (cherries and grapes) and identified following BLASTN analysis. The respective species, strain denomination, origin of isolation, rDNA region sequenced and percentage of identity with database sequences (BLASTN query) are detailed below.

Origin of isolation	Sequenced region	% Identity (BLASTN query)	Species and Strains
Hydrated SBP + peptone	ITS1/ITS2	99%	*Pichia kudriavzevii* IST381
Macerated cherries	ITS1/ITS2	96%	*Metschnikowia pulcherrima* IST383
Hydrated SBP + peptone	D1/D2	99%	*Clavispora lusitaniae* IST388
Hydrated SBP + peptone	D1/D2	99%	*Rhodotorula mucilaginosa* IST390
Washed mature white grapes	D1/D2	99%	*Hanseniaspora opuntiae* IST408

4.2. Screening of non-conventional yeasts growth ability in distinct carbon sources

The performance of 19 yeast strains to catabolise glucose, arabinose and xylose and grow in sugar beet pulp hydrolysate was first screened. The SBP hydrolysates studied as substrate for yeast growth were prepared through enzymatic hydrolysis and supplied by Professor Wolfgang Liebl Lab (TUM), the German partner of the EraNet Project YEASTPEC. The hydrolysates were received after filtration, that allowed sterilization of medium and removal of solids in suspension, make it possible to follow growth profiles by turbidity in this substrate. The composition of each hydrolysate tested in this work is detailed in Table 5.

Table 5 – Composition of SBP hydrolysates utilized in this study. Sugar beet pulp hydrolysates were prepared by enzymatic hydrolysis and supplied by the German partner of the EraNet Project YEASTPEC. The composition of hydrolysates H6, H8, H11 and H13 is shown. GalA: Galacturonic acid.

SBP hydrolysates compositions	Glucose (g/L)	Galactose (g/L)	Xylose (g/L)	Sucrose (g/L)	Arabinose (g/L)	GalA (g/L)	Acetic Acid (mM)	Ethanol (% V/V)	Methanol (% V/V)
H6	8.30	-	0.050	1.00	9.90	3.90	33.3	-	-
H8	3.72	1.62	-	-	9.10	8.81	35.3	0.0856	0.290
H11	10.4	5.18	0.718	-	12.9	11.6	33.5	0.0828	0.0427
H13	4.73	3.45	0.326	-	14.1	7.26	43.8	0.0207	0.231

For this screening, also performed in collaboration with the MSc student Paula Semedo, yeasts were pre-inoculated in glycerol media, to avoid the possible glucose catabolite repression [152], at 30ºC, pH 5.0 and 250 rpm, in aerobic conditions. The cultures were performed in MM with 20 g/L glucose, arabinose or xylose, and in SBP hydrolysate H6 (Table 5), at 30ºC, pH 5.0, 250 rpm. The assays started with an optical density (OD) of 0.25 for single sugar media and of 0.5 for the hydrolysate, once

it was suspected to include growth inhibitory compounds, like acetic acid. Higher OD increases the number of cells, allowing higher variability, facilitating the growth of the most robust cells. These growth experiments were performed in cotton plugged test tubes with a small volume (4 mL), since it was a large screening, and this allowed to get preliminary information about yeast growth profiles saving time and resources. The list of yeast strains tested in this study and the respective origin of isolation are detailed in Table S2 of Supplementary Materials. The subsequently studied strains were chosen based on the OD_{600nm} values measured at 21 h and 42 h, as well as on the literature data, especially considering carbon sources catabolic performance, stress resistance and added-value compounds production described for the different yeast species. The obtained OD values are displayed in Table 6.

Table 6 – Screening of the ability of the different yeast strains isolated in this work and chosen from the IST culture collection to grow in the SBP hydrolysate and MM containing 20 g/L of glucose, arabinose or xylose. The cultures were performed at pH 5.0, 30ºC and 250 rpm and the growth performances were assessed by OD measurements at 600nm. Values highlighted in dark green represent highly elevated OD, light green represent elevated OD, yellow represent medium growth and red represent non-significant growth.

Species and strains	Glucose			Arabinose			Xylose			SBP Hydrolysate H6		
	0h	21h	42h	0h	21h	42h	0h	21h	42h	0h	21h	42h
Pichia anomala IST317	0.25	10.2	13.5	0.25	0.33	0.33	0.25	0.55	0.66	0.50	7.55	11.2
Pichia anomala IST358	0.25	11.0	14.5	0.25	0.32	0.32	0.25	0.56	0.84	0.50	7.43	11.1
Pichia kudriavzevii IST381	0.25	9.75	13.0	0.25	0.39	0.32	0.25	0.71	0.46	0.50	8.02	12.7
Pichia kudriavzevii IST405	0.25	9.80	13.1	0.25	0.52	1.12	0.25	0.53	0.24	0.50	8.33	13.2
Kluyveromyces marxianus IST382	0.25	8.76	8.43	0.25	1.99	4.34	0.25	1.14	3.42	0.50	8.26	11.1
Kluyveromyces marxianus IST389	0.25	8.35	8.96	0.25	1.78	4.38	0.25	1.52	7.18	0.50	8.63	12.0
Metschnikowia pulcherrima IST383	0.25	3.02	4.76	0.25	0.47	0.94	0.25	0.78	1.20	0.50	7.99	11.5
Candida intermedia IST385	0.25	8.34	10.8	0.25	0.31	0.31	0.25	3.51	8.04	0.50	7.85	12.7
Clavispora lusitaniae IST388	0.25	7.46	10.1	0.25	0.88	2.08	0.25	1.48	2.88	0.50	6.79	11.3
Rhodotorula mucilaginosa IST390	0.25	7.60	8.08	0.25	0.85	2.02	0.25	1.46	4.94	0.50	10.0	16.2
Rhodotorula mucilaginosa IST392	0.25	5.78	9.30	0.25	0.81	1.40	0.25	0.58	1.86	0.50	9.73	14.0
Meyerozyma guilliermondii IST369	0.25	12.2	15.9	0.25	2.14	4.82	0.25	3.06	15.1	0.50	10.8	18.6
Meyerozyma guilliermondii IST335	0.25	8.58	11.2	0.25	1.80	5.36	0.25	2.46	9.16	0.50	10.1	17.9
Hanseniaspora opuntiae IST406	0.25	3.27	2.86	0.25	0.27	0.30	0.25	0.24	0.25	0.50	3.03	2.34
Hanseniaspora opuntiae IST408	0.25	3.42	6.56	0.25	0.35	0.48	0.25	0.40	0.40	0.50	3.12	7.08
Saccharomyces cerevisiae Ethanol Red	0.25	6.74	8.30	0.25	0.47	0.54	0.25	0.53	0.62	0.50	8.34	11.7
Saccharomyces cerevisiae H4531	0.25	5.44	5.44	0.25	0.43	0.43	0.25	0.28	0.28	0.50	6.49	10.2
Saccharomyces cerevisiae T18	0.25	5.42	5.62	0.25	0.55	0.90	0.25	0.50	0.74	0.50	5.58	9.82
Saccharomyces cerevisiae T18:Haa1	0.25	3.35	4.14	0.25	0.41	0.36	0.25	0.44	0.40	0.50	3.01	4.08

Highly elevated OD Elevated OD Moderate OD Low OD

The OD values were displayed in a heat map. The strains that exhibit a majority of green values in all growth substrates were considered relevant, once they reached high final biomass values. The strains with a majority of red values were excluded, because they do not appear to be efficient to metabolize the carbon sources usually present in SBP hydrolysates. When more than one strain of the same species was tested, the strain showing better results was chosen for further experiments. According to OD values and all data collected, the most interesting non-conventional yeast strains for our study were considered to be *K. marxianus* IST389*, M. guilliermondii* IST369 and *R. mucilaginosa* IST390. These species are described to metabolize and ferment carbon sources present in this hydrolysate,

being interesting for the production of bioethanol, biodiesel, enzymes, aromatic compounds, pigments, among other applications [23,43,81,106,107,123,135]. Additionally, four strains of *S. cerevisiae* were also tested as controls. *S. cerevisiae* Ethanol Red presented the highest OD in all conditions, being also chosen to be submitted to further studies. This yeast strain is an industrial strain used in bioethanol production, having high-ethanol tolerance and relative temperature resistance (optimal temperature of 34ºC) and it is suitable for fermentation of sugar juices, molasses and hydrolysates [14].

In addition to these selected yeast strains, other strains were subsequently added to the study. This is the case of *R. mucilaginosa* IST423, the type strain *K. marxianus* CBS712, and *S. cerevisiae* CEN.PK 122.

Table 7 – Selected yeast strains to be studied in this work. Strains were isolated and identified in the context of YEASTPEC project or chosen from the IST collection. Reference strains of *S. cerevisiae* and type strain of *K. marxianus* species were also used. Their isolation origin is also indicated.

Source	Strains and Species	Origin of isolation
Reference strains	*Kluyveromyces marxianus* CBS712 (PYCC 3886)	-
	Saccharomyces cerevisiae Ethanol Red	-
	Saccharomyces cerevisiae CEN.PK122	-
IST collection	*Meyerozyma guilliermondii* IST369	Beer barrel 5 (B5)
	Rhodotorula mucilaginosa IST423	Lupine processing effluent
Isolated in YEASTPEC project	*Kluyveromyces marxianus* IST389	Hydrated SBP + Peptone
	Rhodotorula mucilaginosa IST390	Hydrated SBP + Peptone

4.3. Yeasts' performance in SBP hydrolysates

4.3.1. Aerobic cultures in SBP hydrolysate H8 and H8 + 2 g/L Urea

Meyerozyma guilliermondii IST369, *Kluyveromyces marxianus* IST389, *Rhodotorula mucilaginosa* IST390, *Kluyveromyces marxianus* CBS712 and *S. cerevisiae* Ethanol Red were further evaluated for performance in aerobic conditions in the sterile SBP hydrolysate H8 (Table 5). Urea supplementation (2 g/L) as nitrogen source, was tested in one half of the assays, even though SBP hydrolysates are expected to be a rich media composed by a mixture of essential nutrients, yet detailed carbon and nitrogen composition is not completely defined and variable. Yeasts were first batch cultured in diluted hydrolysate (1:1), to previously adapt the yeasts to the medium, without causing the anticipated stress especially due to the presence of acetic acid and methanol. The culture assays were performed in cotton plugged 50 mL flasks, to enable oxygen and CO_2 exchanges, at 30ºC, pH 5.0, 130 rpm. This agitation was selected to be in accordance with other assays performed by Belgium YEASTPEC project partner. Cultures were performed during a maximum of 216 h, and the initial OD was 0.5, to assure a proper growth in a presumably toxic medium. The growth curves obtained for both H8 and H8 + 2 g/L urea cultivations are displayed in Figure 10. After obtaining the growth curves, maximum specific growth rates were calculated (Table 8).

M. guilliermondii IST369 was the strain that exhibited the highest final biomass of all strains in both conditions (Figure 10A and B), showing a 2-fold higher final OD compared with the other yeast strains. All strains exhibited a higher final biomass in H8 + 2 g/L urea, except *K. marxianus* strains, which show higher OD values in H8 without urea than in urea presence. The highest maximum specific growth rates, during exponential growth phase, were exhibited by *S. cerevisiae* Ethanol Red (Table 8A and B).

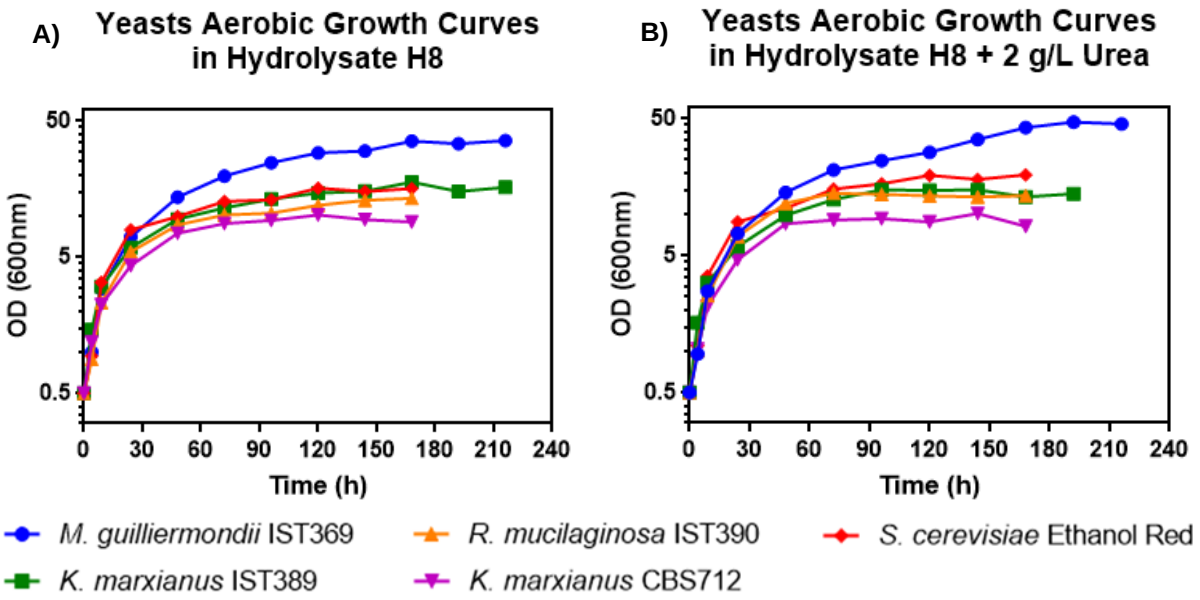

Figure 10 – Growth curves of the selected yeast strains in SBP hydrolysate H8 (A) and H8 + 2 g/L urea (B). Growth profiles were based on OD measurements of *M. guilliermondii* IST369 (dark blue circle), *K. marxianus* IST389 (green square), *K. marxianus* CBS712 (purple inverted triangle), *R. mucilaginosa* IST390 (orange triangle), and *S. cerevisiae* Ethanol Red (red diamond), at 30ºC, pH 5.0 and 130 rpm.

During growth, samples were collected at several time points, and then submitted to HPLC analysis, in order to determine the consumption of carbon sources and the possible production of added-value products, including ethanol. After obtaining these profiles, consumption or production rates were calculated, as well as the products' yields. The consumption and production profiles of each strain in H8 and H8 + 2 g/L urea are showed in Figures 11 to 15, and products' yields, consumption and production rates are shown in Table 8A and B.

Glucose was the first sugar to be completely catabolized by yeasts. It was fully consumed in 24 h by *K. marxianus* IST389 (Figures 12A and C) and *S. cerevisiae* Ethanol Red (Figures15A and C), in both conditions. These strains exhibited the higher consumption rates (Table 8 A and B). *M. guilliermondii* IST369 (Figure 11A and C) and *K. marxianus* CBS712 (Figure 13A and C) complete glucose metabolization in 24 h, in H8 + 2 g/L while in H8, it took more time (Figure 11C), showing similar consumption rates (Table 8A and B). *R. mucilaginosa* IST390 took 48 h to metabolize all glucose in H8 with urea (Figure 14A), and more than 72 h to exhaust this sugar in H8 without urea, presenting the lower consumption rates.

Galactose started to be utilized when glucose concentration decreased in the medium. Once again, *K. marxianus* IST389 (Figure 12A and C) and *S. cerevisiae* Ethanol Red (Figure 15A and C) used

Table 8 – Maximum specific growth rates (μ), consumption rates of glucose, galactose, arabinose and acetic acid, maximum production rates and yields of ethanol and arabitol, in both SBP hydrolysate H8 (A), H8 + 2 g/L urea (B) and H11 + 2 g/L urea (C). Maximum specific growth rates are in h^{-1}, consumption rates in g/L of carbon source / time of consumption, maximum production rates in g/L of product / time of production and product yields are represented in g of product (ethanol or arabitol) / g of consumed substrate to achieve that production (glucose or glucose and galactose for ethanol production, and arabinose for arabitol production).

A) H8	**Specific growth rate (μ) (h^{-1})**	**Glucose consumption rate (g L^{-1} h^{-1})**	**Galactose consumption rate (g L^{-1} h^{-1})**	**Arabinose consumption rate (g L^{-1} h^{-1})**	**Acetic acid consumption rate (g L^{-1} h^{-1})**	**Maximum ethanol production rate (g L^{-1} h^{-1})**	**Maximum ethanol yield (g/g)**	**Ethanol consumption rate (g L^{-1} h^{-1})**	**Maximum arabitol production rate (g L^{-1} h^{-1})**	**Maximum arabitol yield (g/g)**
***M. guilliermondii* IST369**	0.085	0.13	0.039	0.048	0.015	0.027	0.38	0.050	0.030	0.10
***K. marxianus* IST389**	0.086	0.33	0.11	0.017	0.038	0.042	0.32	0.035	0.010	0.81
***K. marxianus* CBS712**	0.072	0.15	0.064	0.047	0.038	0.11	0.33	0.018	0.031	0.77
***R. mucilaginosa* IST390**	0.074	0.042	0.026	ND	0.028	0.056	0.42	0.020	ND	ND
***S. cerevisiae* Ethanol Red**	0.091	0.32	0.10	ND	0.022	0.14	0.44	0.051	ND	ND
B) H8 + 2 g/L Urea	**Specific growth rate (μ) (h^{-1})**	**Glucose consumption rate (g L^{-1} h^{-1})**	**Galactose consumption rate (g L^{-1} h^{-1})**	**Arabinose consumption rate (g L^{-1} h^{-1})**	**Acetic acid consumption rate (g L^{-1} h^{-1})**	**Maximum ethanol production rate (g L^{-1} h^{-1})**	**Maximum ethanol yield (g/g)**	**Ethanol consumption rate (g L^{-1} h^{-1})**	**Maximum arabitol production rate (g L^{-1} h^{-1})**	**Maximum arabitol yield (g/g)**
***M. guilliermondii* IST369**	0.083	0.16	0.094	0.051	0.025	0.083	0.39	0.036	0.018	0.12
***K. marxianus* IST389**	0.086	0.37	0.11	0.017	0.061	0.074	0.34	0.036	0.011	0.90
***K. marxianus* CBS712**	0.070	0.14	0.065	0.043	0.054	0.070	0.31	0.019	0.032	0.80
***R. mucilaginosa* IST390**	0.079	0.11	0.029	ND	0.029	0.095	0.47	0.022	ND	ND
***S. cerevisiae* Ethanol Red**	0.095	0.35	0.099	ND	0.029	0.13	0.39	0.056	ND	ND
C) H11 + 2 g/L urea	**Specific growth rate (μ) (h^{-1})**	**Glucose consumption rate (g L^{-1} h^{-1})**	**Galactose consumption rate (g L^{-1} h^{-1})**	**Arabinose consumption rate (g L^{-1} h^{-1})**	**Acetic acid consumption rate (g L^{-1} h^{-1})**	**Maximum ethanol production rate (g L^{-1} h^{-1})**	**Maximum ethanol yield (g/g)**	**Ethanol consumption rate (g L^{-1} h^{-1})**	**Maximum arabitol production rate (g L^{-1} h^{-1})**	**Maximum arabitol yield (g/g)**
***M. guilliermondii* IST369**	0.10	0.41	0.11	0.087	0.013	0.26	0.40	0.053	0.020	0.22
***K. marxianus* IST389**	0.12	0.89	0.22	0.044	0.013	0.24	0.37	0.062	0.043	0.86
***K. marxianus* CBS712**	0.090	0.66	0.069	0.055	0.013	0.21	0.35	0.052	0.045	0.90
***R. mucilaginosa* IST390**	0.078	0.099	0.025	ND	0.026	0.035	0.15	0.025	ND	ND
***R. mucilaginosa* IST423**	0.049	0.11	0.026	ND	0.021	-	-	0.027	ND	ND
***S. cerevisiae* Ethanol Red**	0.12	0.90	0.072	ND	0.015	0.27	0.41	0.084	ND	ND

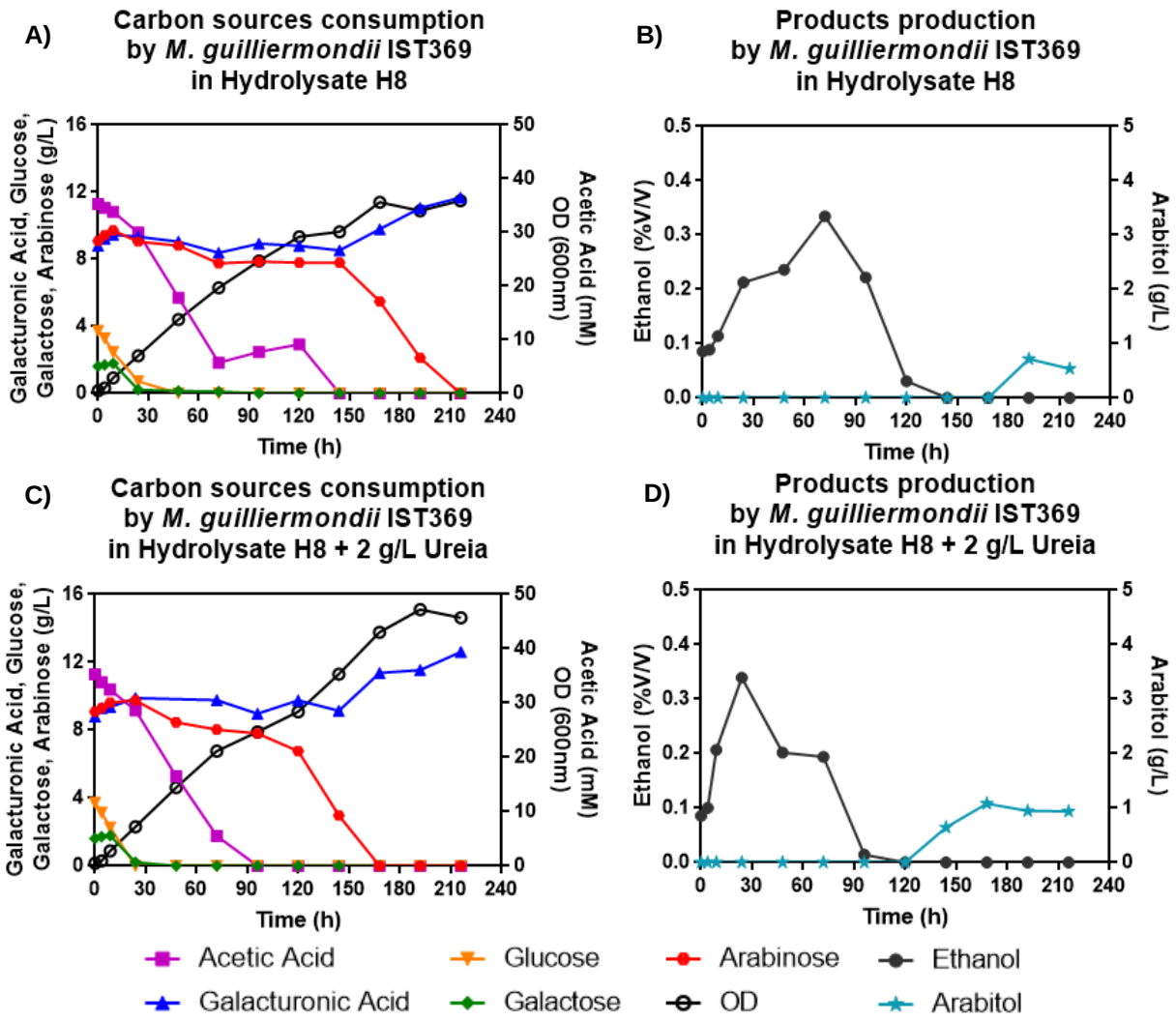

Figure 11 – Carbon sources consumption (A, C) and ethanol and arabitol production (B, D) profiles obtained in SBP hydrolysate H8 (A, B) and H8 + 2 g/L urea (C, D) by *M. guilliermondii* IST369. The samples were collected from the yeast cultures at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144, 168 h, 192 h and 216 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

galactose with faster rate than the other strains (Table 8 A and B), consuming it in less than 24 h. *K. marxianus* CBS712 metabolized galactose in more than 24 h (Figure13A and C). *M. guilliermondii* IST369 metabolized in less than 24h, and in non-supplemented H8, it took approximately 72h (Figure11A and C). *R. mucilaginosa* IST390 presented the lowest rates of consumption, fully catabolizing galactose in 48 h in H8 + 2 g/L urea, and in 72 h in H8 (Figure 14A and C).

Ethanol was the product obtained from glucose or glucose and galactose. Still, a lower amount was already present in the hydrolysate (Table 5), and it must be taken into consideration to accurately determine the amount produced by yeasts and respective production yield. The ethanol production yields were calculated based on the grams of ethanol produced, per grams of glucose or glucose and galactose catabolized to attained maximum production. Theoretical ethanol yield (maximum ethanol yield possible to obtain) from both glucose and galactose is 0.51 g ethanol/g sugar [153].

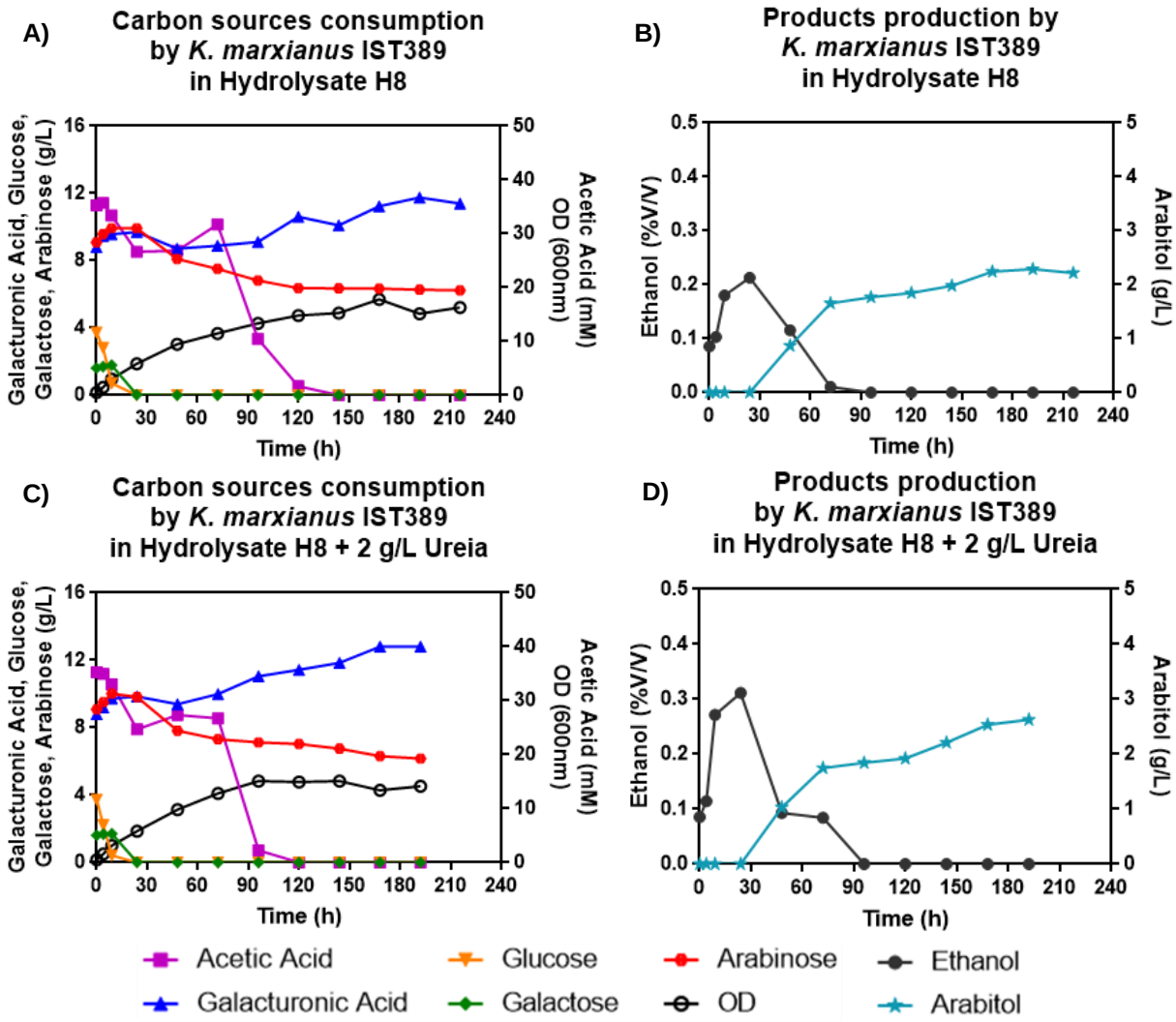

Figure 12 – Carbon sources consumption (A, C) and ethanol and arabitol production (B, D) profiles obtained in SBP hydrolysate H8 (A, B) and H8 + 2 g/L urea (C, D) by *K. marxianus* IST389. The samples were collected from the cultures at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144, 168 h, 192 h and 216 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

S. cerevisiae attained a maximum of 0.2 %(v/v) in 9 h, in both H8 conditions (Figure 15B and D), presenting the highest production rates and production yields (Table 8). The yields were 86 % and 76 % of theoretical value, respectively. However, *M. guilliermondii* IST369 was the strain that produced the highest concentration of ethanol, 0.3 %(v/v) (Figure 11B and D). In H8, maximum ethanol production was reached after 72 h, but in H8 + 2 g/L urea, it only took 24 h, accomplishing a 3 times higher rate, with yields of conversion of about 75 % of the theoretical value. Both *K. marxianus* strains produced almost 0.1 %(v/v) in H8, but CBS712 (Figure13B) achieved it earlier than IST389 (Figure 12B). Ethanol production yield of CBS712 was 65 % of theoretical value, while yield of IST389 was 59%. In H8 with urea, IST389 produced 0.2%(v/v) in 24 h (Figure 12D), while CBS712 only generated 0.08 %(v/v) in 9 h (Figure 13D), present similar production rates (Table 8 A and B). The yields of production corresponded to 67 % and 61 % of theoretical yield.

Despite *R. mucilaginosa* being generally described as non-fermentative [134], ethanol was produced in this hydrolysate. IST390 produced 0.06 %(v/v) in H8 (Figure 14B), and 0.1 %(v/v) in H8 with urea (Figure 14D), showing the highest production rates (Table 8). Also, IST390 presented highest yields of conversion, being comparable to *S. cerevisiae* (82 % and 92 % of theoretical value). Still, ethanol concentrations generated by this strain were far from the highest obtained.

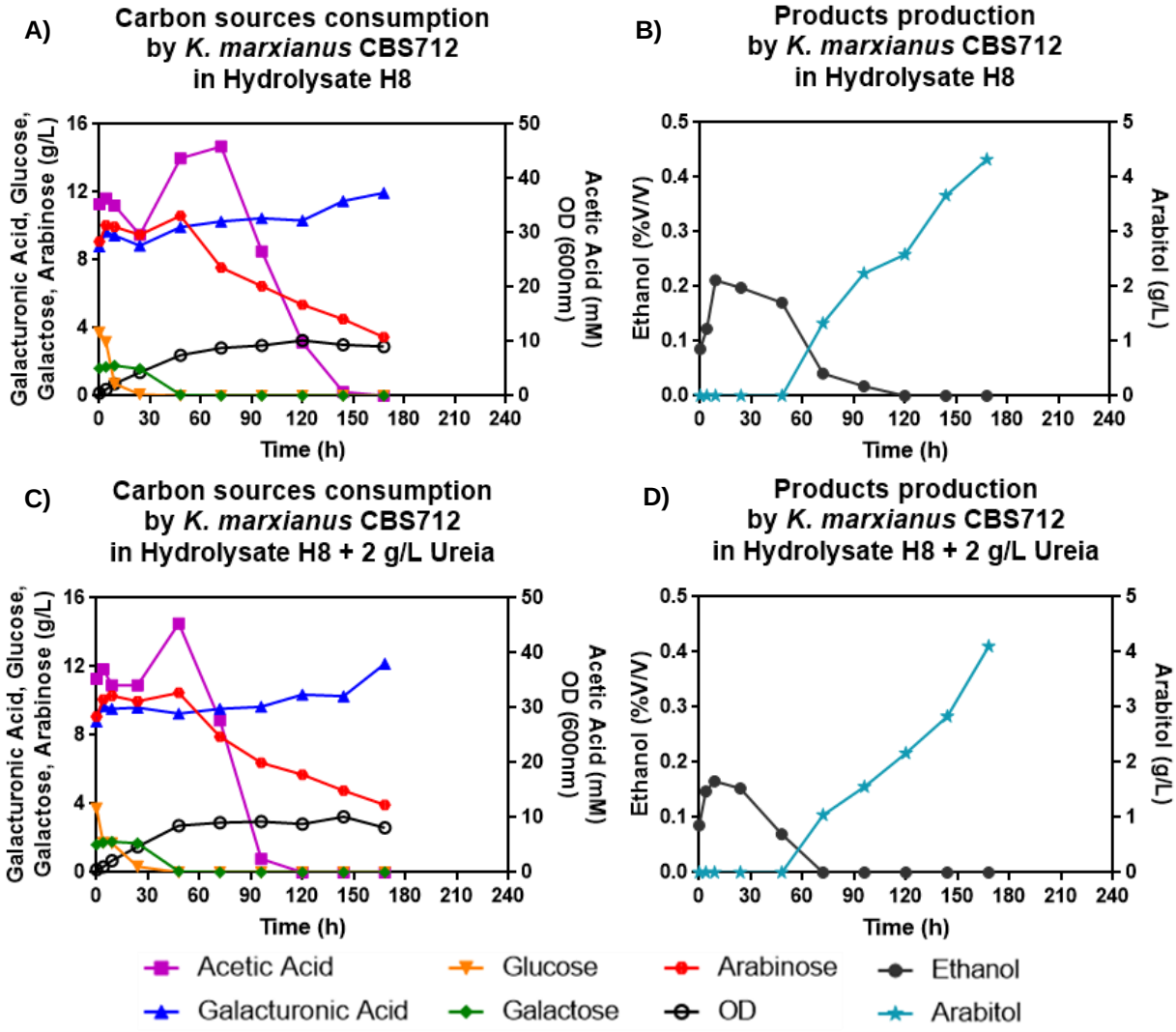

Figure 13 – Carbon sources consumption (A, C) and ethanol and arabitol production (B, D) profiles obtained in SBP hydrolysate H8 (A, B) and H8 + 2 g/L urea (C, D) by *K. marxianus* CBS712. The samples were collected from the yeast cultures at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

After reaching the maximum values, ethanol was further metabolized as an additional carbon source. *S. cerevisiae* catabolized it faster than the other strains (Table 8A and B), exhausting it in approximately 24 h. *M. guilliermondii* produced a higher ethanol concentration than *K. marxianus* IST389 in H8, but they both took more than 48 h to fully metabolize ethanol. These two strains presented similar amount of ethanol produced in H8 with urea, and consumed it in approximately 72 h, showing similar profiles of consumption (Figures 11D and 12D). *K. marxianus* CBS712 and *R. mucilaginosa* strains presented lower consumption rate. CBS712 produced 1.6 times more ethanol in

H8, metabolizing it in almost 96 h. In presence of urea, ethanol was utilized in 48 h. IST390 took more than 24 h in H8 with urea, and 72 h hours in H8, to fully respirate ethanol.

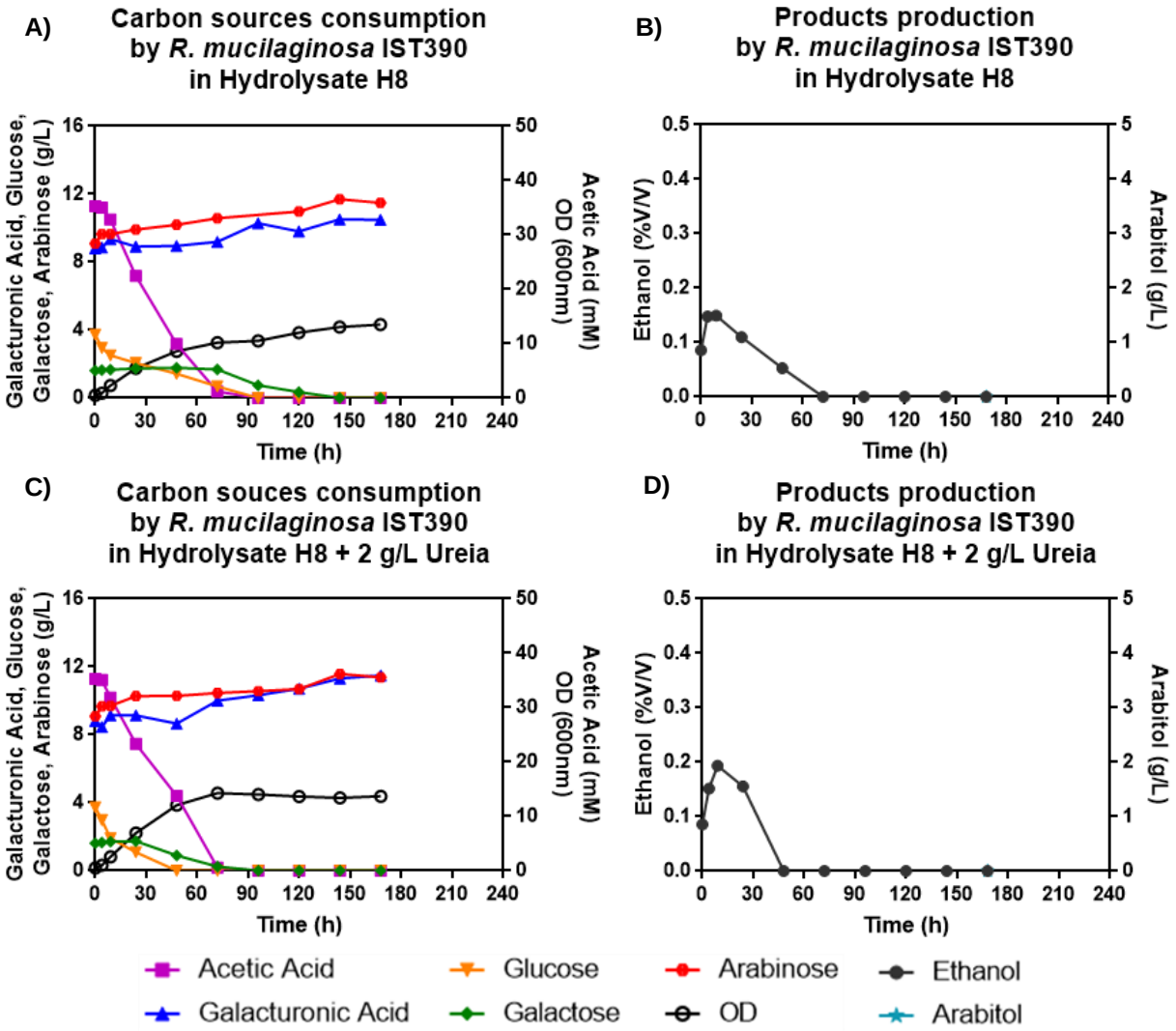

Figure 14 – Carbon sources consumption (A, C) and ethanol and arabitol production (B, D) profiles obtained in SBP hydrolysate H8 (A, B) and H8 + 2 g/L urea (C, D) by *R. mucilaginosa* IST390. The samples were collected from the yeast cultures at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

Acetic acid is a toxic compound for yeasts at high concentrations, depending on the pH. Still, it is also produced as a fermentation by-product, and it is a respirable carbon source [44]. As the pH 5.0 is higher than pKa, more than half is in the deprotonated non-toxic form, acetate. Different metabolization profiles were presented for the different strains, affecting the rate of consumption. In the case of *K. marxianus* and *S. cerevisiae* strains, after an initial period of slight consumption, acetic acid was produced and accumulated in the next 24 h to 48 h. Only after this period, acetic acid was completely metabolized, which was taken into consideration in rates calculations.

K. marxianus strains have the highest average consumption rates (Table 8A and B), being faster in H8 + 2 g/L urea condition. After acetic acid production, IST389 consumed it in approximately 48 h in H8, and 24 h in H8 + 2 g/L urea (Figure12 A and C). CBS712 took approximately 72 h, but it previously

produced more acetic acid than IST389 (Figure 13A and C). *S. cerevisiae* Ethanol Red metabolized acetic acid in approximately 96 h. In H8 without urea, acetic was produced and then consumed, while in H8 with urea, no production was detected, despite a stabilization of 24 h. *M. guilliermondii* and *R. mucilaginosa* strains did not produce acetic acid in the beginning, starting to consume it after 4 h of growth in H8. IST369 catabolized acetic acid in about 144 h in H8, but between 72h and 120h, acetic acid concentration increased. In H8 with urea, acetic acid started to be consumed immediately, ending in approximately 96 h. IST390 took this same time in both conditions. So, at concentrations present in the hydrolysate, acetic acid is an important respirable carbon source.

Arabinose was the last carbon source, with the exception of GalA, that was consumed. Only *M. guilliermondii* and *K. marxianus* were able to utilize this sugar. In the case of *M. guilliermondii*, despite the slight consumption after galactose metabolization, the majority of arabinose consumption occurred after 120 h of culture, when acetic acid had been already metabolized. *K. marxianus* strains did not completely utilize arabinose in during their respective growth time, and it seemed to be simultaneously metabolized with acetic acid. Still, K marxianus CBS712 presented and higher rate of consumption (Table 8A and B).

Associated with arabinose catabolization, there is the production of arabitol, an interesting added-value product. Production rates and yields were calculated (Table 8). Production yield of arabitol is calculated as grams of arabitol produced, per grams of arabinose consumed during that time of production. In the case of *M. guilliermondii,* arabitol was only detected when arabinose was almost consumed. The maximum arabitol detected in H8 was 0.7 g/L (Figure 11B). In H8 with urea, 1.1 g/L were reached (Figure 11D), when all arabinose was exhausted. After the maximum was attained, a decrease in arabitol concentration was observed. *K. marxianus* IST389 attained a maximum of 2.3 g/L and 2.6 g/L after 168 h, in the medium H8 and H8 + 2 g/L urea, respectively. This strain exhibited the greatest conversion yields (Table 8A and B). CBS712 presented the higher concentrations, 4.3 g/L and 4.1 g/L after 168 h.

It was possible to observe that *R. mucilaginosa* and *S. cerevisiae* strains exhibited an increase in arabinose concentration, and that some arabitol is present, but analytical methods resolution do not allow to separate these compounds due to their concentration and similar retention time. Also, some evaporation of the medium may also be contributing to this increase.

None of the yeast strains examined in this work was able to catabolize galacturonic acid when present in the mixture of sugars and acetic acid present SBP hydrolysates, even *R. mucilaginosa*, which is described as having an efficient galacturonic acid metabolic pathway [136]. Still, this strain seems to stop growing prematurely, after galactose consumption.

Additionally, the final pH was measured in the end of these fermentations (Table 9). An increase was registered for all strains, from pH 5.0 to a minimum of 6.8 in *M. guilliermondii* and a maximum of 9.0 in *S. cerevisiae* cultures. The increase in the pH, especially in urea supplemented medium, may be related with deacidification of the medium by acetic acid consumption and the production of some unidentified product of metabolism.

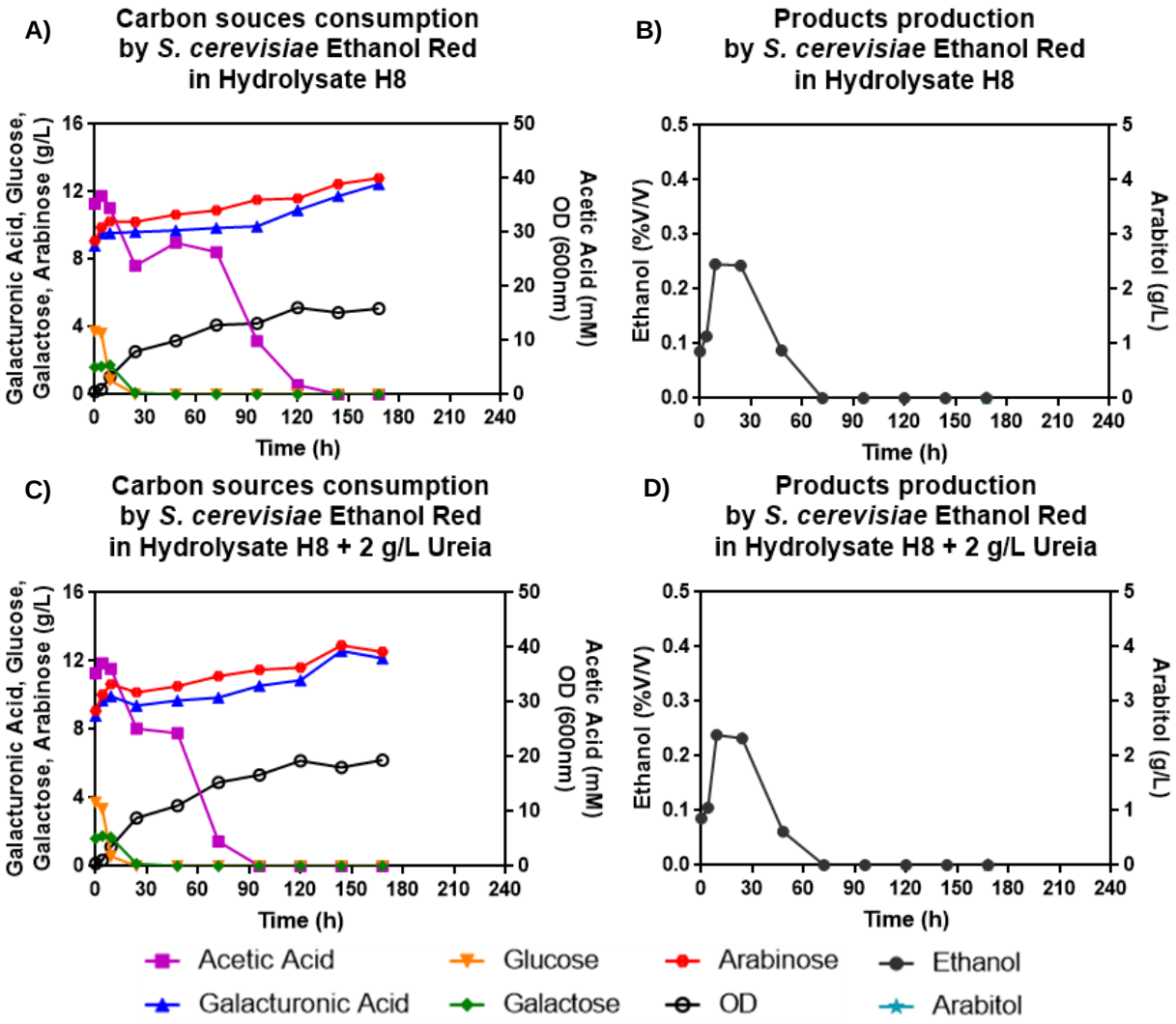

Figure 15 – Carbon sources consumption (A, C) and ethanol and arabitol production (B, D) profiles obtained in SBP hydrolysate H8 (A, B) and H8 + 2 g/L urea (C, D) by *S. cerevisiae* Ethanol Red. The samples were collected from the yeast cultures at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

In general, supplementation with urea as an additional nitrogen source increased final biomass, carbon sources consumption and ethanol and arabitol maximum concentration and yields, except in the case of *K. marxianus* CBS712, in which some parameters are favoured in H8, as the higher ethanol production values.

A sequential carbon sources metabolization was observed, with some differences between strains. In the case of *S. cerevisiae* and *K. marxianus*, the sequence is glucose > galactose > ethanol > acetic acid/arabinose, in case of *K. marxianus* strains. *M. guilliermondii* preferentially consumed glucose/acetic acid > galactose > ethanol > arabinose. *R. mucilaginosa* IST390 catabolized glucose/ acetic acid/ethanol > galactose. The rate of consumption is associated with this sequential carbon sources utilization, with preferred sugars being consumed faster than the least desired ones. Nevertheless, it is necessary to emphasize that the low concentrations of carbon sources influence the

observed profiles, that may not be exactly the same ones that would be observed in the presence of higher initial concentrations.

Table 9 – Final pH obtained by each strain (IST369, IST389, IST390, IST423, CBS712 and Ethanol Red) in the end of aerobic cultures in all SBP hydrolysates conditions (H8, H8 + 2 g/L urea and H11 + 2 g/L urea).

Final pH Aerobic Cultures	H8	H8 + 2 g/L Urea	H11 + 2 g/L Urea
***M. guilliermondii* IST369**	6.8	8.8	7.6
***K. marxianus* IST389**	8.8	8.2	8.3
***K. marxianus* CBS712**	8.6	8.5	8.2
***R. mucilaginosa* IST390**	8.0	8.6	8.7
***R. mucilaginosa* IST423**	-	-	8.6
***S. cerevisiae* Ethanol Red**	8.8	9.0	8.4

4.4.2. Aerobic cultures in SBP hydrolysate H11 + 2 g/L Urea

After studying the aerobic cultures in SBP hydrolysate H8, a similar study was performed in SBP hydrolysate H11. The major differences between hydrolysates H8 and H11 are the increase in glucose and galactose concentrations in the hydrolysate (Table 5), which is desirable since all the yeast strains prefer these sugars. These assays were only performed with supplementation of 2 g/L urea, since it seemed to be advantageous for the yeasts' hydrolysates faster metabolization. The growth curves were performed in the same conditions than the previous ones. Cells were batch cultured in diluted hydrolysate (1:1) for yeasts adaptation, being then cultivated in H11, in cotton plugged 50 mL flasks, at 30ºC, pH 5.0, 130 rpm, during 168 h, with initial OD 0.5. The growth curves of the H11 + 2 g/L urea cultivation are showed in Figure 16, and the maximum specific growth rates calculated (Table 8C). Since *R. mucilaginosa* IST423 was posteriorly added to IST Collection, this strain was only tested in H11 assays, since it was isolated from an interesting source, lupine processing effluent (Table 7).

M. guillermondii IST369 once again exhibited the higher final biomass, almost 3-fold higher than the other strains, while *K. marxianus* CBS712 showed the lower OD. *S. cerevisiae* Ethanol Red and *K. marxianus* IST389 exhibited the highest maximum specific growth rates (Table 8C). Additionally, *R. mucilaginosa* IST423 exhibited a higher final OD than *R. mucilaginosa* IST390. Still, IST423 was the strain with the lowest maximum specific growth rate. The profiles of growth and maximum biomass attained are similar between the two hydrolysates, only with slightly higher OD for *K. marxianus* strains. Also, the maximum specific growth rates are higher, except the ones exhibited by *R. mucilaginosa* strain, which are similar to the previous ones. Despite higher glucose concentration, this fact does not seem to improve maximum specific growth rates as happens for the other strains, revealing a naturally slow growth.

Glucose was rapidly catabolized by *K. marxianus* (Figure 18A and C), *S. cerevisiae* (Figure 20A) and *M. guilliermondii* IST369 in 24 h. (Figure 17A. *S. cerevisiae* presented the fastest consumption rate (Table 8C). The rates increased approximately 3-fold for Ethanol Red, IST369, and IST389, and more than 4-fold for CBS712, compared with H8. *R. mucilaginosa* IST423 consumed glucose in 96 h (Figure 19C), while IST390 took 120 h (Figure 19A). These strains present the lower consumption rates,

similar to H8, showing once again that higher substrate concentrations do not improve glucose consumption, which may be related with metabolic expenses for production of pigments.

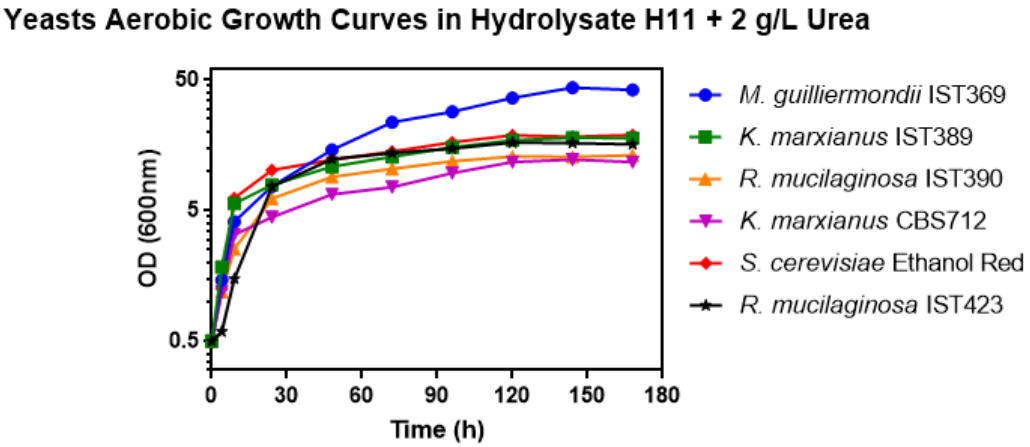

Figure 16 – Growth curves of the studied yeasts in SBP hydrolysate H11. Growth profiles were based on OD measurements of *M. guilliermondii* IST369 (dark blue circle), *K. marxianus* IST389 (green square), *K. marxianus* CBS712 (purple inverted triangle), *R. mucilaginosa* IST390 (orange triangle), *R. mucilaginosa* IST423 (black star) and *S. cerevisiae* Ethanol Red (red diamond), at 30ºC, pH 5.0 and 130 rpm.

Galactose metabolization followed glucose consumption. *K. marxianus* IST389 (Figure 18A) still has the faster consumption rate, which is 2 times higher than in H8 (Table 8). *M. guilliermondii* IST390 (Figure 17A) took more than 48 h to consume galactose, at a greater rate than in H8. *K. marxianus* CBS712 and *S. cerevisiae* Ethanol Red catabolized galactose in 72 h (Figure 18C and 20A), at comparable rates. *R. mucilaginosa* strains presented the lowest rates of consumption, similar to the ones in H8, consistently to what is observed for glucose. Galactose was not completely utilized, with approximately 1 g/L remaining in the medium (Figures 19A and C) and only was effectively metabolized after glucose and acetic acid catabolization.

S. cerevisiae, M. guilliermondii and *K. marxianus* reached the maximum ethanol production in 24 h. Ethanol Red produced a maximum of 0.8 %(v/v), presenting the maximum concentration obtained (Figure 20B). The rate of production exhibited a 2-fold increase, and the yield of production was 80 % of the theoretical value. IST369 (Figure 17B) and IST389 (Figure 18B) produced 0.7 %(v/v), while CBS712 (Figure 18D) produced 0.67 %(v/v), attaining approximately 3 times higher rates than in H8, but equivalent maximum production yields. However, *R. mucilaginosa* IST390 produced a much low ethanol amount (Figure 19B), at a 2.7-fold decreased rate comparing with H8 with urea. The yield was 29 % of theoretical, 3 times lower than formerly. No ethanol was produced by IST423 (Figure 19D). Despite low fermentation ability on H11 (contrarily to H8), both *R. mucilaginosa* strains produce higher pigmentation.

In terms of ethanol consumption as a carbon sources, *S. cerevisiae* utilized it faster than the other strains, with 1.5-fold increased consumption rate, being fully metabolized in 96 h. *K. marxianus* strains showed 1.7- and 2.7-times augment in consumption rates by IST389 and CBS712, respectively. *M. guilliermondii* metabolized all ethanol in 120 h, at similar rate than in H8. *R. mucilaginosa* IST390 and IST423 present ted the lower consumption rates. IST390 consumed the ethanol produced in approximately 36 h, while IST423 only utilized the all ethanol present in 24 h.

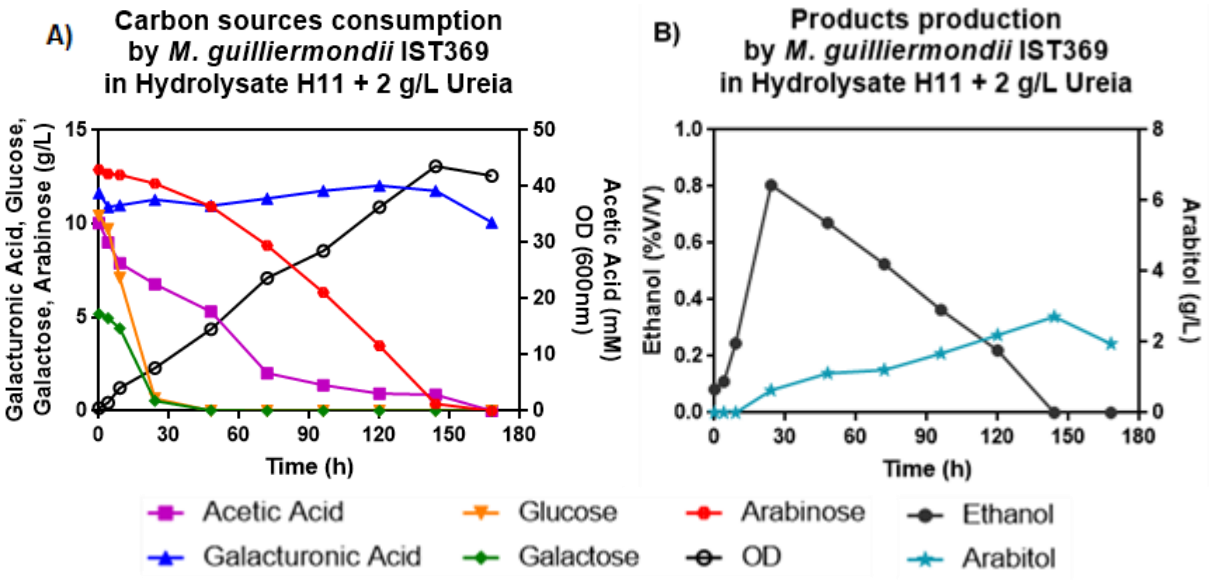

Figure 17 – Carbon sources consumption (A) and ethanol and arabitol production (B) profiles obtained in SBP hydrolysate H11 by *M. guilliermondii* IST369. The samples were collected from the cultivations at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

Acetic acid started to be consumed together with glucose by *M. guilliermondii* and *R. mucilaginosa* strains, in the beginning of the cultures, while the production observed in H8 was not verified. *S. cerevisiae, M. guilliermondii* and *K. marxianus* showed comparable consumption rates, since metabolized it in 144 h to 168 h (Figures 17A, 18A and C, 20 A). This represented a 4.7-fold and 4.2-fold reduction in consumption rate for IST389 and CBS712, respectively compared with H8 with urea. *R. mucilaginosa* strains were the fastest consumers of acetic acid, at similar rates than in H8 (Table 8C). Both IST390 and IST423 completely utilized it in 96 h, fully respiring acetic acid even before completing glucose metabolization.

M. guilliermondii was the strain that presented faster arabinose consumption rate (1.7-fold augment comparing with H8), fully utilizing arabinose in less than 144 h (Figure 17A). Arabitol reached a maximum of 2.7 g/L, a much higher concentration than in H8 (Figure 17B). Once again, *K. marxianus* strains did not completely metabolize arabinose during culture time. Still, IST389 presents a 2.7-fold increase of arabinose consumption rate (Figure 18A), while CBS712 rate was similar to the values in H8 assays (Figure 18C). In terms of arabitol, 6.0 g/L was achieved from IST389 (Figure 18B), and 6.3 g/L from CBS712 (Figure 18D), which was the strain that produced the highest arabitol concentration. The rates of production were the highest achieved, representing a 3.9-fold and 1.4-fold increase.

R. mucilaginosa and *S. cerevisiae* strains did not utilize arabinose, but a significant increase in concentration was observed. Due to the resolution of the analytical methods, it was not possible to separate arabinose and arabitol, and correctly quantify them. Also, evaporation of the medium may cause some concentration increase.

There are several reports claiming *Meyerozyma (Pichia) guilliermondii* arabitol production. In arabinose medium, arabitol production yields varied from 0.21 g/g to 0.71 g/g, increasing with the

arabinose concentration increase [89,125,127]. In corn fiber, 2.6 g/L arabinose were produced, with a yield of 0.53 g/g [127]. In all conditions, higher yields than the ones registered in this work were attained. *K. marxianus* was able to produce arabitol in 20 g/L arabinose medium achieving production yields of approximately 0.27 g/g at 30ºC (4.8 g/L), and 0.48 g/g (8.7 g/L) at 40ºC [110], much lower than the ones obtained in this study. *R. mucilaginosa* produced arabitol in arabinose medium, also presenting a low production yields (0.2 g/g) [135]. Nevertheless, low oxygen (until 200 rpm) seem to be important production factors [89].

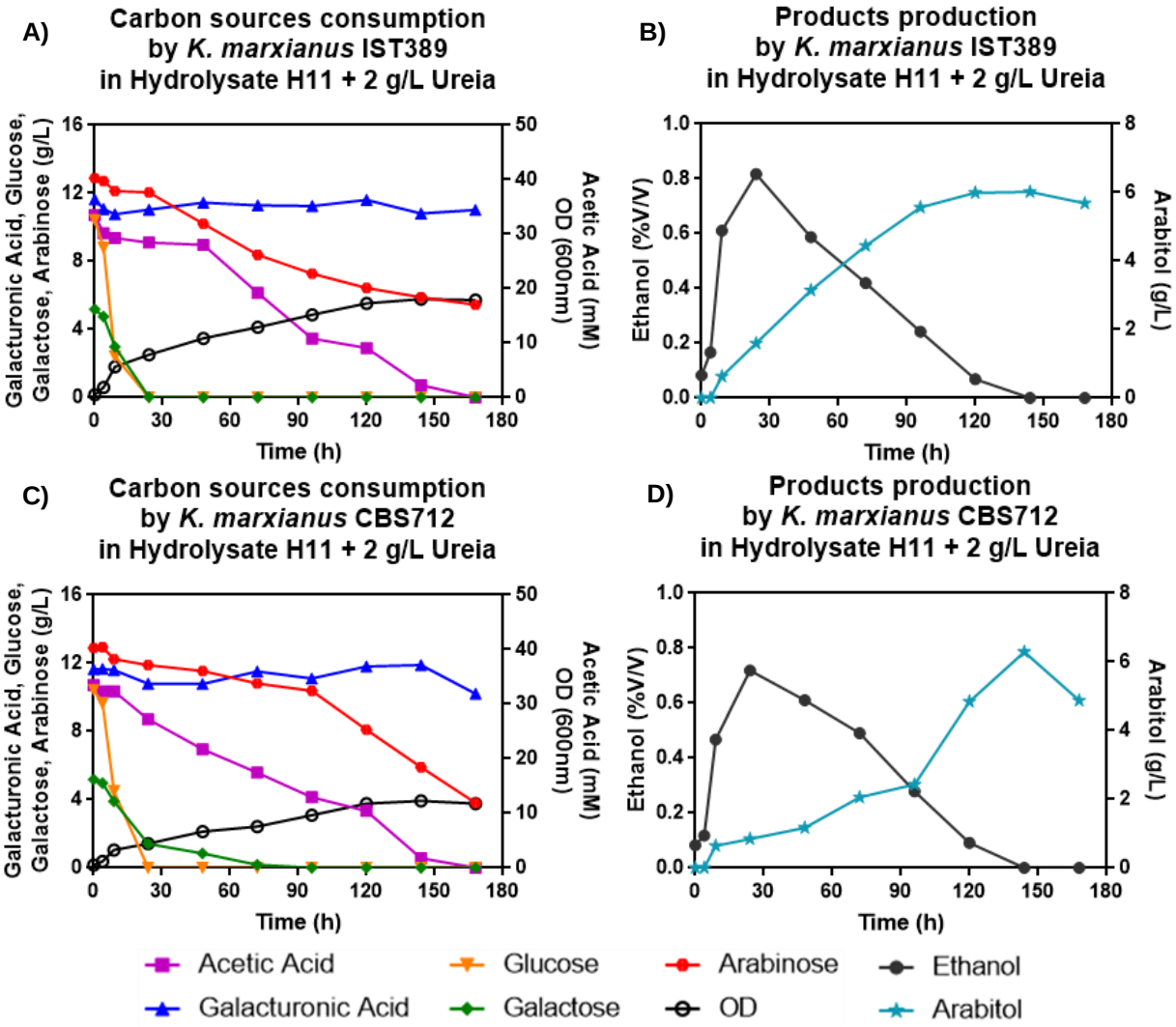

Figure 18 – Carbon sources consumption (A, C) and ethanol and arabitol production (B, D) profiles obtained in SBP hydrolysate H11 by *K. marxianus* IST389 (A, B) and *K. marxianus* CBS712 (C, D). The samples were collected from the yeast cultures at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

Once more, galacturonic acid was not utilized by these yeasts, being stabilized from the beginning of cultures. However, after 144 h of growth, both *R. mucilaginosa* strains seem to start slowly metabolizing arabinose and GalA. Since *R. mucilaginosa* strains show low maximum specific growth rate and consumption rates, culture conditions and duration may not be the most appropriate.

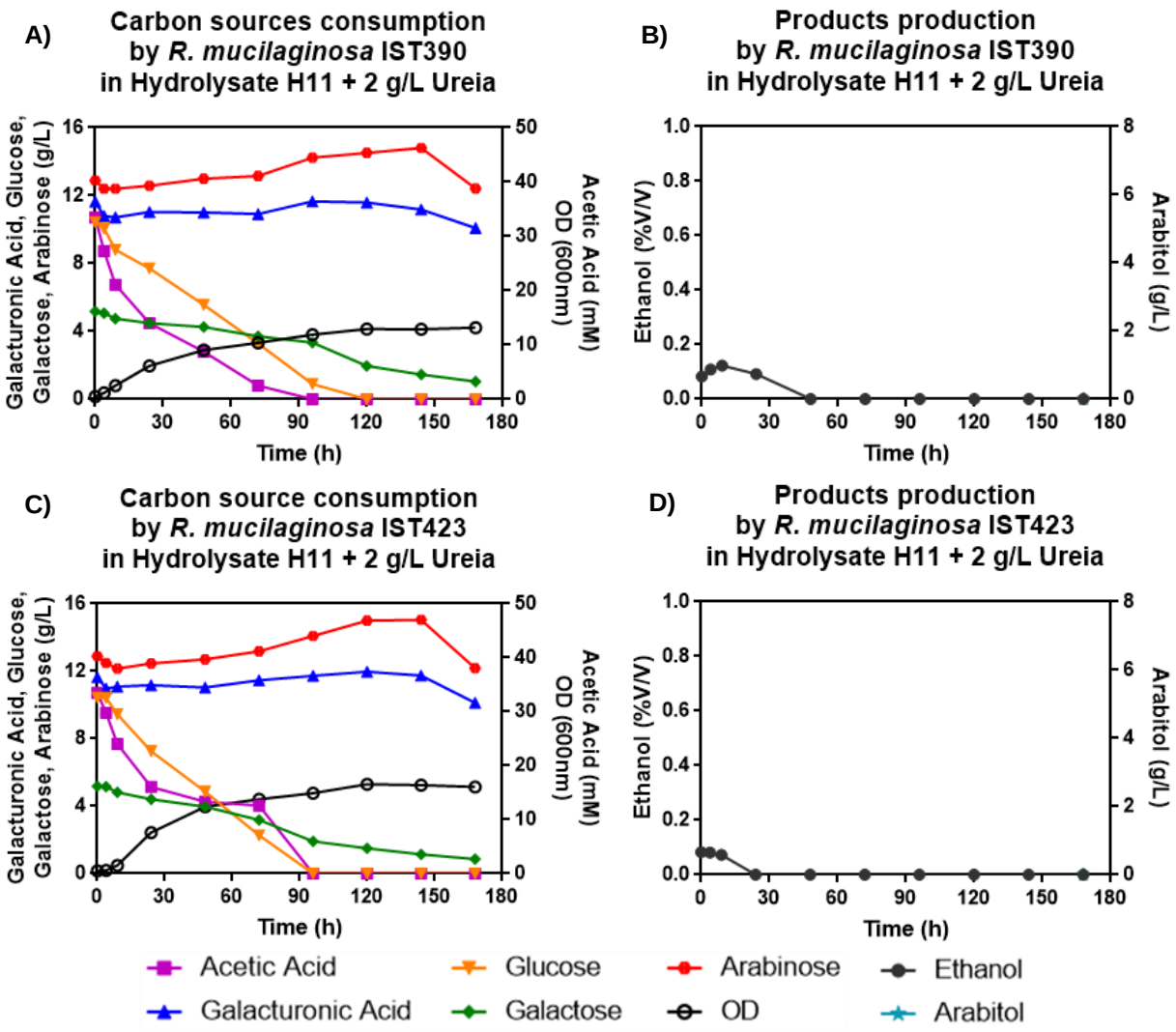

Figure 19 – Carbon sources consumption (A, C) and ethanol and arabitol production (B, D) profiles obtained in SBP hydrolysate H11 by *R. mucilaginosa* IST390 (A, B) and *R. mucilaginosa* IST423 (C, D). The samples were collected from the cultivations at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

As observed in H8, there was again an increase in pH (Table 9) from 5.0 to values between 7.6 registered for *M. guillermondii* IST369, and 8.7 for *R. mucilaginosa* IST390, the yeast strain that grow more slowly and stop consuming acetic acid earlier. It is reported that the pH of the medium increased constantly from pH 5.5 until more than 9, when *M. guilliermondii* grew in acetic acid medium as a sole carbon source [154]. Also, in arabinose medium, pH increased from 4.0 to 5.4 in *M. guilliermondii* culture, and from pH 5.0 to 6.2 in *Candida entomaea* culture, when arabitol was produced, decreasing when arabitol was consumed [127]. So, pH increase seems to be related with medium deacidification and some products' production.

In H11, yeasts' performance was significantly better in terms of maximum specific growth rates, consumption and ethanol and arabitol production rates, with higher maximum concentrations, except acetic acid consumption rates, which were similar between the two hydrolysates. This increase may be related with higher initial carbon sources concentrations, while acid concentration is approximately

the same. Also, in the case of *R. mucilaginosa* strains, the obtained results are similar between the two hydrolysates, showing naturally low specific growth and consumption rates in these culture conditions. Ethanol and arabitol production yields are similar to the ones obtained in H8, which shows that despite higher substrate concentrations, the yields may be due to same culture conditions, in which yeasts convert proportional amounts of substrate into product.

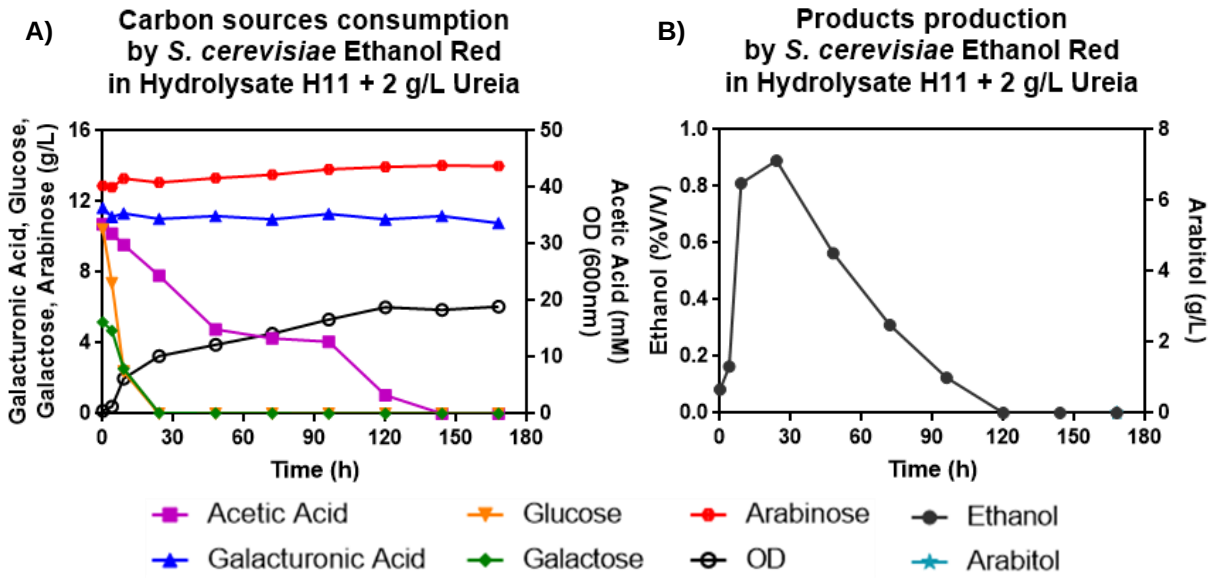

Figure 20 – Carbon sources consumption (A) and ethanol and arabitol production (B) profiles obtained in SBP hydrolysate H11 by *S. cerevisiae* Ethanol Red. The samples were collected from the yeast cultures at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed squares), ethanol (grey circles) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

The sequential consumption of carbon sources in H11 was slightly different than in H8, which may be dependent on the initial carbon sources concentrations, since high concentration is a major parameter influencing consumption profiles. Even the higher concentrations of H11 compared with H8 are still low to precisely determine consumption profiles. It was revealed that the preferential catabolization by the *M. guilliermondii* was glucose/acetic acid > galactose > ethanol/arabinose, while by *S. cerevisiae* was glucose > acetic acid/galactose > ethanol. In *K. marxianus* strains the preference was glucose > galactose > acetic acid/ ethanol/ arabinose and in *R. mucilaginosa* strains, glucose/acetic acid/ethanol > galactose was preferred, being the only species presenting equal consumption profile in H8 and H11. Ethanol, but especially acetic acid, are important respirable substrates in the SBP hydrolysate that are preferred by yeasts over arabinose, and by some of the yeasts tested, also over galactose.

4.4.3. Microaerophilic assays in SBP hydrolysates H8 and H11

Complementary to the cultures performed in oxygenated conditions, microaerophilic conditions were also tested. Yeasts were first grown as for aerobic growth, in diluted hydrolysates (1:1), to adapt the yeasts to the medium. Then, fermentations were performed in 20 mL SBP hydrolysate H8, H8 + 2 g/L urea or H11 + 2 g/L urea (Table 5), in sealed 30 mL flasks, to avoid oxygen entry and to permit only

CO_2 release, at 30ºC, pH 5.0, 130 rpm magnetic agitation, in the medium, during 168 h. The agitation was defined based on the conditions applied in assays performed by Belgium YEASTPEC project partner. The quantification of CO_2 released is a way of monitoring the fermentation performance, since CO_2 and ethanol are produced in 1:1 ratio during fermentation [155]. Initial OD was 4.0, to assure sufficient biomass to ferment in these very low oxygen conditions. Fermentation performance of selected strains in H8, H8 + 2 g/L urea and H11 + 2 g/L urea are depicted in Figure 20A to C. As in aerobic assays, *R. mucilaginosa* IST423 only tested in H11 assays, since this strain was posteriorly added to IST Collection (Table 7).

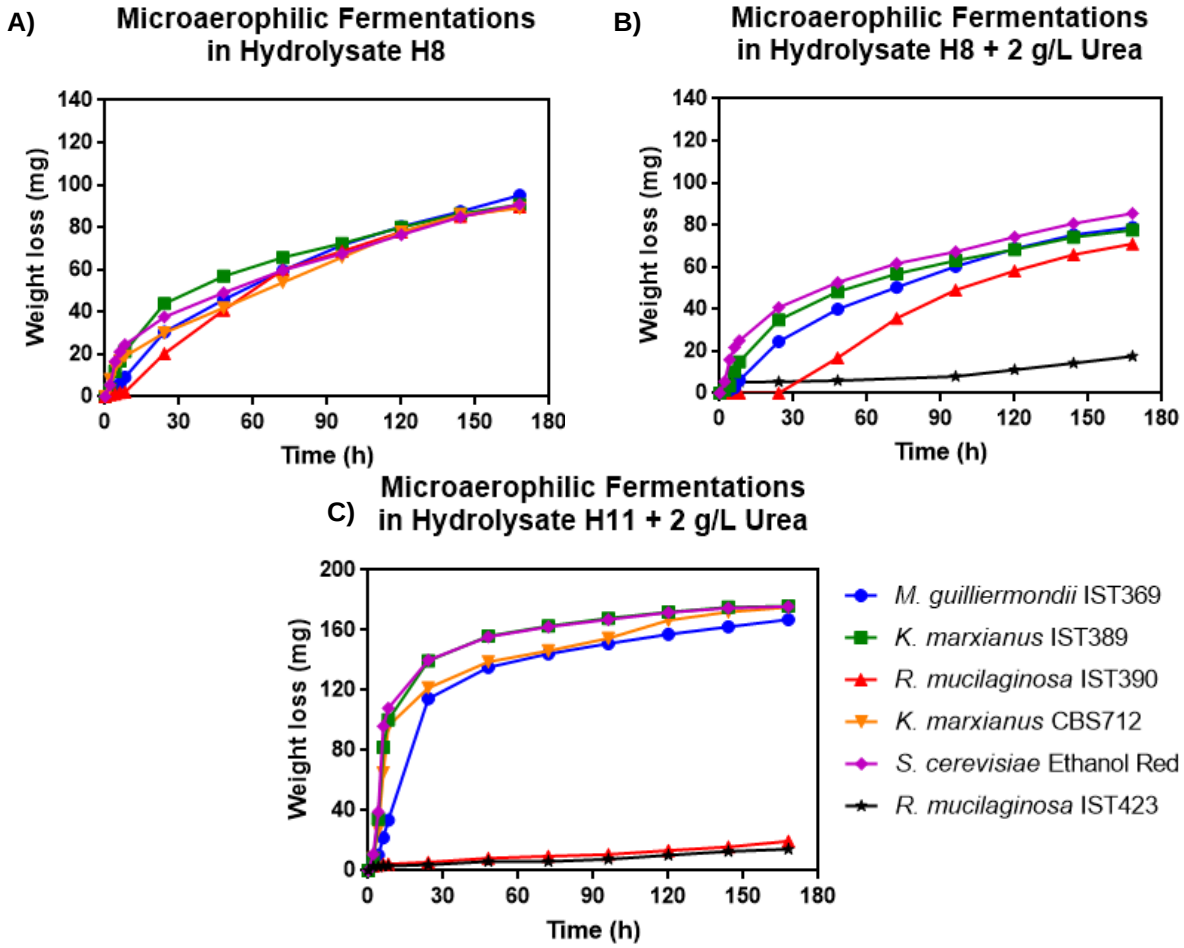

Figure 21 – Fermentation profile of the selected yeasts in SBP hydrolysate H8 (A), H8 + 2 g/L urea (B) and H11 + 2 g/L urea. Theses profiles were based on CO_2 released, assessed by flasks weight loss, by *M. guilliermondii* IST369 (dark blue circle), *K. marxianus* IST389 (green square), *K. marxianus* CBS712 (purple inverted triangle), *R. mucilaginosa* IST390 (orange triangle), *R. mucilaginosa* IST423 (black star) and *S. cerevisiae* Ethanol Red (red diamond), at 30ºC, pH 5.0 and 130 rpm.

In H8 assays (Figure 21A), all strains reached almost the same final weigh loss. Still, in the first hours, weigh loss was higher in *K. marxianus* IST389. In H8 with urea, *S. cerevisiae* presented similar lost than in H8, while the other strains showed lower weight loss than in H8. *R. mucilaginosa* IST390 only started to ferment after 8 h in H8, and 24 h in H8 with urea. Apparently, in microaerophilia, urea does not favour yeast behaviour as in aerobiosis does. In H11 with urea (Figure 21C), *S. cerevisiae* and *K. marxianus* showed the faster and equivalent loss, being lower for *M. guilliermondii*. The weight loss is approximately 2 times higher. Still, *R. mucilaginosa* strains showed almost no weight loss, suggesting

that very low ethanol was produced, as occurred in H11 aerobic growth. These values represent a 7-fold lower weight loss for IST390. Additionally, as CBS712 culture in H8 + 2 g/L urea was contaminated, the obtained results were removed from the study.

In the final of fermentations, after 168 h, a sample of each flask was collected for HPLC analysis and compared with the sample taken at initial timepoint. The graphical depiction is showed in Figure 22.

Glucose (Figure 22A) and galactose (Figure 22B) were completely catabolized by all strains in all conditions after 168 h, except by *R. mucilaginosa* strains in H11, that only metabolized a small amount of these carbon sources. This is consistent with the fact that these strains are not fermenting H11 hydrolysate, which was also observed in aerobic assays. Also, *S. cerevisiae* Ethanol Red did not completely metabolize galactose in both H8 conditions, while the same happened to *M. guilliermondii* IST369 in H11, which may be due to lower fermentation rates than in glucose.

Ethanol (Figure 22E) was the major product obtained in both H8 and H11, with final concentrations higher than in aerobic cultures. *M. guilliermondii* IST369 attained higher ethanol concentrations in H8 without and with urea, 0.4 %(v/v) and 0.5 %(v/v). *K. marxianus*, *S. cerevisiae* and *R. mucilaginosa* strains produced equivalent ethanol amounts. *S. cerevisiae* was the only strain that reached higher ethanol concentration in H8 than in H8 with urea. Still, the other strains weight loss was higher in H8, but the ethanol concentration attained was higher in H8 with urea. In H11 medium, *K. marxianus* IST389 was the strain that produced the higher ethanol concentration, 1.0 %(v/v). *R. mucilaginosa* strains presented the lowest ethanol concentrations, as it happened during H11 aerobic cultivations. IST390 achieved approximately 8.5-fold less ethanol concentration than in H8 assays. These ethanol values are only slightly higher than the ones obtained in aerobic conditions in H8 and H11.

Both acetic acid (Figure 22D) and galacturonic acid profiles (Figure 22G) are distinct between both H8 and H11 + 2 g/L urea assays. In H8 assays, all strains showed production of acetic acid, being *K. marxianus* strains the greater producers in both H8 conditions. This increased concentration may be associated with ethanol fermentation, or some concentration effect due evaporation. This same fact may explain the increase in GalA concentration. However, acetic acid and GalA concentrations maintained approximately the same initial concentration in H11. The results of acetic acid and GalA presented the same concentration effect and stabilization than in respective aerobic assays.

Differently from what was observed during aerobic cultures, the only strain that showed arabinose metabolization and arabitol synthesis in H8 was *K. marxianus* IST389 (Figure 22C and F). However, arabinose catabolization by *M. guilliermondii* was observed in H11, but arabitol production was much lower. *K. marxianus* IST389 was the greater producer of arabitol, with 2.57 g/L achieved, slightly higher than in H8, but at a lower yield.

In the end of fermentations, final pH and final OD were also measured, being showed in Table 10. Final OD and pH show that no significant alterations compared with the initial ones were registered. However, Ethanol Red OD increased to 8.1 in H11. Still, this is consistent with the fact that biomass is only produced in aerobic conditions and not microaerophilia, and that pH increased in aerobic assays cultures was due to acetic acid metabolization and products generation.

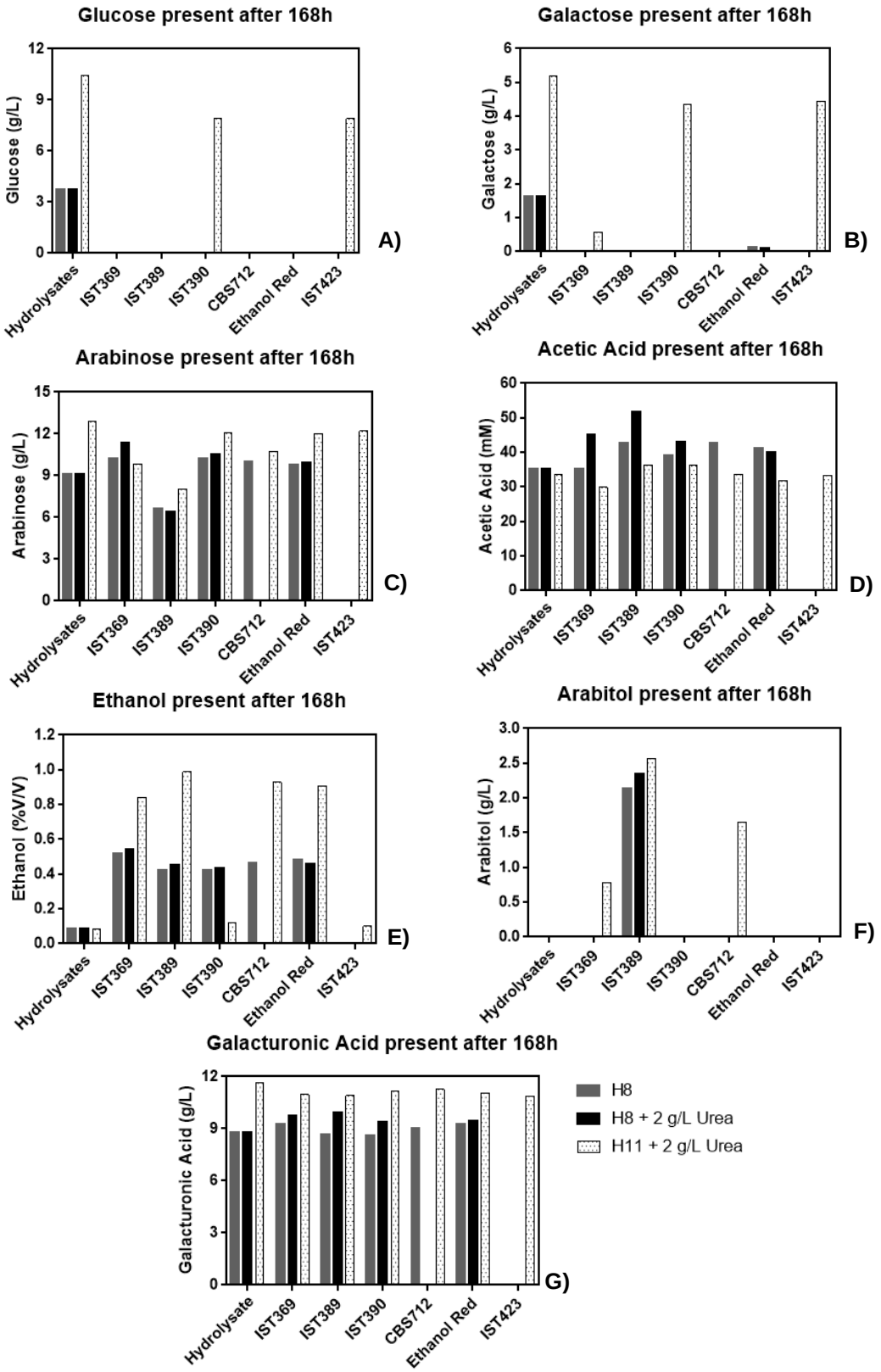

Figure 22 – Compounds present after 168 h of culture in SBP hydrolysate H8 (grey bars), H8 + 2 g/L urea (black bars), and H11 + 2 g/L urea (white dotted bars) metabolized *M. guilliermondii* IST369, *K. marxianus* IST389, *K. marxianus* CBS712, *S. cerevisiae* Ethanol Red, *R. mucilaginosa* IST390 and *R. mucilaginosa* IST423. The samples were compared with timepoint 0. Compounds analysed were glucose (A), galactose (B), arabinose (C), acetic acid (D), ethanol (E), arabitol (F) and galacturonic acid (G).

Table 10 – Final OD and pH obtained by each strain (IST369, IST389, IST390, IST423, CBS712 and Ethanol Red) in the end of microaerophilic cultures in all SBP hydrolysates conditions (H8, H8 + 2 g/L urea and H11 + 2 g/L urea).

Microaerophilic Assays	Final OD			Final pH		
	H8	H8 + 2 g/L Urea	H11 + 2 g/L Urea	H8	H8 + 2 g/L Urea	H11 + 2 g/L Urea
***M. guilliermondii* IST369**	4.4	3.2	4.9	5.1	5.0	5.0
***K. marxianus* IST389**	4.6	5.1	6.4	4.9	4.9	4.7
***K. marxianus* CBS712**	3.7	-	5.5	4.9	-	4.8
***R. mucilaginosa* IST390**	6.3	6.9	3.2	5.0	5.1	5.1
***R. mucilaginosa* IST423**	-	-	3.5	-	-	5.0
***S. cerevisiae* Ethanol Red**	4.6	5.3	8.1	5.0	5.0	4.9

4.5. Carotenoid production by *R. mucilaginosa* strains

High pigment production was observed in *R. mucilaginosa* strains when these cells were grown aerobically, and specific assays were performed to determine their production capacity in SBP hydrolysate. Mineral medium with glucose and rich YPD medium were used as controls. To prepare the inocula, yeast strains were batch cultured in MM with 20 g/L glucose, YPD and diluted (1:1) SBP hydrolysate H11, to adapt cells to the respective media of main growth. Cells of *R. mucilaginosa* strains were inoculated in the same media and in SBP H11 (Table 5), at 30ºC, pH 5.0 and 250 rpm in cotton plugged flasks of 100 mL for synthetic media and 50 mL for H11, to assure sufficient oxygenation, since illuminated and aerated conditions are desirable [95]. These cultivations last 120 h, as suggested by the observed biomass pink colour during aerobic growth in H11. After biomass harvest, it was lyophilized and weighted. The final concentration of dry biomass obtained is shown in Table 11. Carotenoids were extracted with zirconia beads and successive acetone washes, until pellet maximum colourless was attained and maximum pink pigment possible was in acetone phase. Total carotenoid concentration were quantified, by measuring the absorbance at 452nm, corresponding to the maximum absorbance wavelength for β-carotene, which was described as the most abundant carotenoid produced by *R. mucilaginosa* [23]. Pictures exemplifying the processes of production and extraction of carotenoids from *R. mucilaginosa* IST390 and IST423 are showed in Figure 23. The total carotenoids concentration reached in each growth medium under standard conditions by strains IST390 and IST423 is displayed in Table 12.

Table 11 – Final biomass of *R. mucilaginosa* IST390 and IST423 obtained in MM with glucose, and in YPD and SBP hydrolysate H11. These values were obtained from cultures grown at 30ºC, pH 5.0 and 250 rpm, for 120 h, in 50 mL of minimal and rich media in 100 mL flasks, and in 25 mL of SBP hydrolysate H11 in 50 mL flasks in the case of SBP H11.

Final Biomass (g dry biomass)	*R. mucilaginosa* IST390	*R. mucilaginosa* IST423
MM (20 g/L Glucose)	0.334	-
YPD	0.545	0.547
SBP hydrolysate H11	0.125	0.178

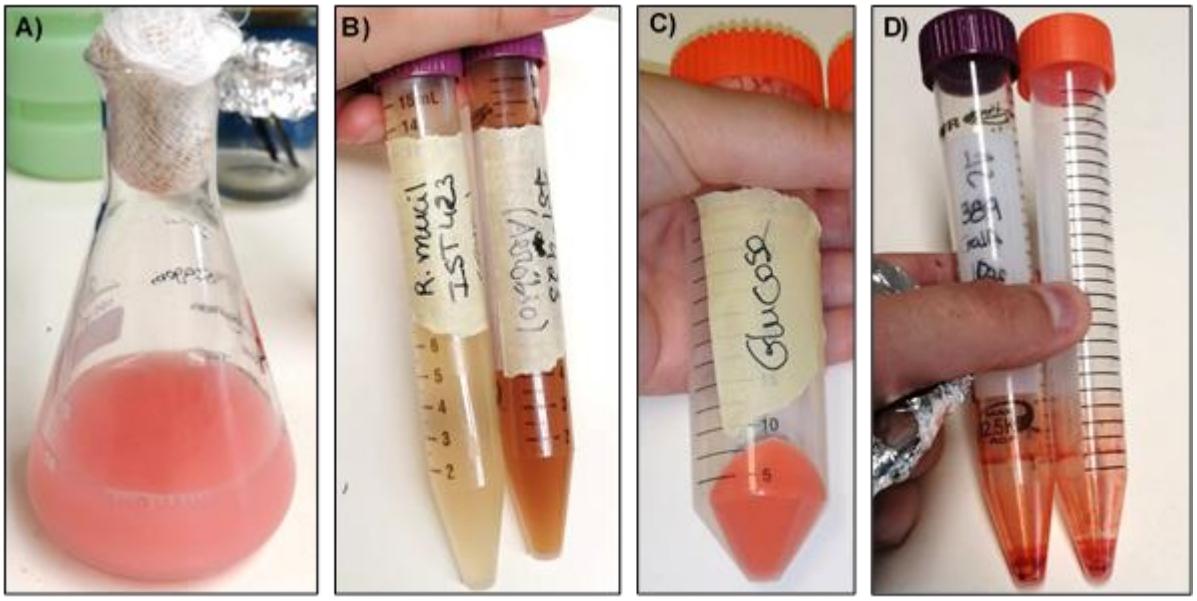

Figure 23 – Production and extraction of carotenoids from *R. mucilaginosa* strains. A) Culture of *R. mucilaginosa* IST390 in MM with 20 g/L glucose. B) Differences between *R. mucilaginosa* IST423 cultures in H11 in aerobic (right Falcon tube) and microaerophilic conditions (left Falcon tube). C) Biomass pellet of IST390 cells before lyophilization. D) Final carotenoids extraction step, after acetone evaporation.

The final dry biomass of *R. mucilaginosa* IST390 and IST423 was much higher in YPD than in MM, since YPD is a rich medium while in SBP hydrolysate H11 biomass reached the lowest values (Table 11). Despite biomass production by IST423 in MM with glucose, the biomass did not present a pink colour, but instead a cream colour suggesting absence or not significant carotenoid production in this condition.

Concerning carotenoid production, *R. mucilaginosa* IST390 produced only 0.112 mg total carotenoids/g dry biomass, in minimal medium MM with glucose, whereas IST423 did not produce detectable levels of carotenoids in this medium (Table 12). In YPD, IST390 synthesized 0.148 mg/g, while 0.312 mg/g were produced in H11, which correspond to a 2.1-fold increase in the concentration of carotenoids from the rich medium YPD to the SBP hydrolysates. Compared with IST390, strain IST423 produced lower levels of carotenoids in both YPD and SBP hydrolysate but confirming maximum production levels in SBP hydrolysates.

Table 12 – Total carotenoids produced per biomass units by *R. mucilaginosa* IST390 and IST423 in MM with glucose, YPD and SBP hydrolysate H11. These values were obtained from cultures grown at 30ºC, pH 5.0 and 250 rpm, for 120 h. The concentration is in mg of carotenoids per g of dry biomass.

Total carotenoids (mg/g)	*R. mucilaginosa* IST390	*R. mucilaginosa* IST423
MM (20 g/L Glucose)	0.112	-
YPD	0.148	0.116
SBP hydrolysate H11	0.312	0.206

Although preliminary, these results show that *R. mucilaginosa* IST390 is a greater carotenoid producer compared with IST423, consistent with the more intense colour exhibited by IST390 cells prior to

carotenoids extraction. A notorious carotenoids production improvement was obtained in the hydrolysate compared with minimal and rich media, independently of the concentration of biomass produced. Minimal medium and rich medium only contained glucose as carbon source, despite the presence of yeast extract and peptone in YPD. Nevertheless, SBP H11 is a complex mixture of carbon sources. By observation of carbon sources consumption profile in H11 aerobic growth, carotenoids are mainly being produced from glucose and acetic acid catabolization. Acetic acid is apparently the best carbon source for carotenoid production, as indicated by the results of growth in MM with arabinose medium supplemented with increasing concentration of acetic acid (Figure 24).The objective of this experiment and the corresponding growth curves can be seen in section 4.6 of Results, Figure 26, showing that the biomass attained by the different cultures after 96 h of growth is not significantly different.

Figure 24 – *R. mucilaginosa* IST390 production of carotenoids observed in MM with 10 g/L arabinose supplemented with increasing concentrations of acetic acid (0 mM, 10 mM, 20 mM and 35 mM) after 96 h. These assays were performed at 30ºC, pH 5.0, 130 rpm.

Several studies report the production of carotenoids by *R. mucilaginosa* strains. A study performed in food wastes, namely ketchup, sugarcane molasses and health drink, showed similar carotenoids concentration than the ones obtained in this study (376.5 µg/g in ketchup, 268.6 µg/g in molasses and 245.0 µg/g in health drink) [96], compared with the 312 µg/g obtained in the present study by IST390 from H11, without optimization. The results obtained reveal the production potential of both stains, as well as sugar beet pulp potential as suitable substrates for carotenoids production, besides ethanol and arabitol production.

4.6. Effect of potential stress factors present in SBP hydrolysates in yeast growth profiles

4.6.1. Effect of increasing concentrations of acetic acid in the growth of different yeast species in glucose or arabinose

Pectin-rich hydrolysates contain several components that may have potential toxic effects in the yeast cells impairing their growth, fermentative and production abilities. Among them, the expected presence of acetic acid is thought to be one the most relevant inhibitors.

To determine acetic acid effect in the growth of the examined yeasts, cultures in minimal medium with glucose or arabinose supplemented with increasing concentrations of acetic acid were performed. Those were the sugars present in the hydrolysates that were proven to be metabolized by the non-conventional yeasts examined. As such, it was considered of interest to assess that influence of yeast

growth in both carbon sources. To prepare the inocula, *R. mucilaginosa* IST390, *K. marxianus* IST389, *Meyerozyma guilliermondii* IST369 and *S. cerevisiae* Ethanol Red were initially grown in MM with 10 g/L glucose or 10 g/L arabinose. Then, yeasts were inoculated in the same media, that contains approximately the same concentration of those sugars that are present in SBP hydrolysate H11, supplemented with different acetic acid concentrations (0, 10, 20, 35 and 50 mM acetic). Acetic concentrations only reached 50 mM, because SBP hydrolysates studied contained between 30 mM and 40 mM acetic acid. The cultures occurred at 30ºC, pH 5.0, 130 rpm, for 168 h, in aerobic conditions. The growth curves for each yeast strain are in Figures 25 and 26. The final ODs obtained by each strain are shown in Table 13. The maximum specific growth rates in the exponential phase were calculated and compared for the different the acid concentrations (Figure 27).

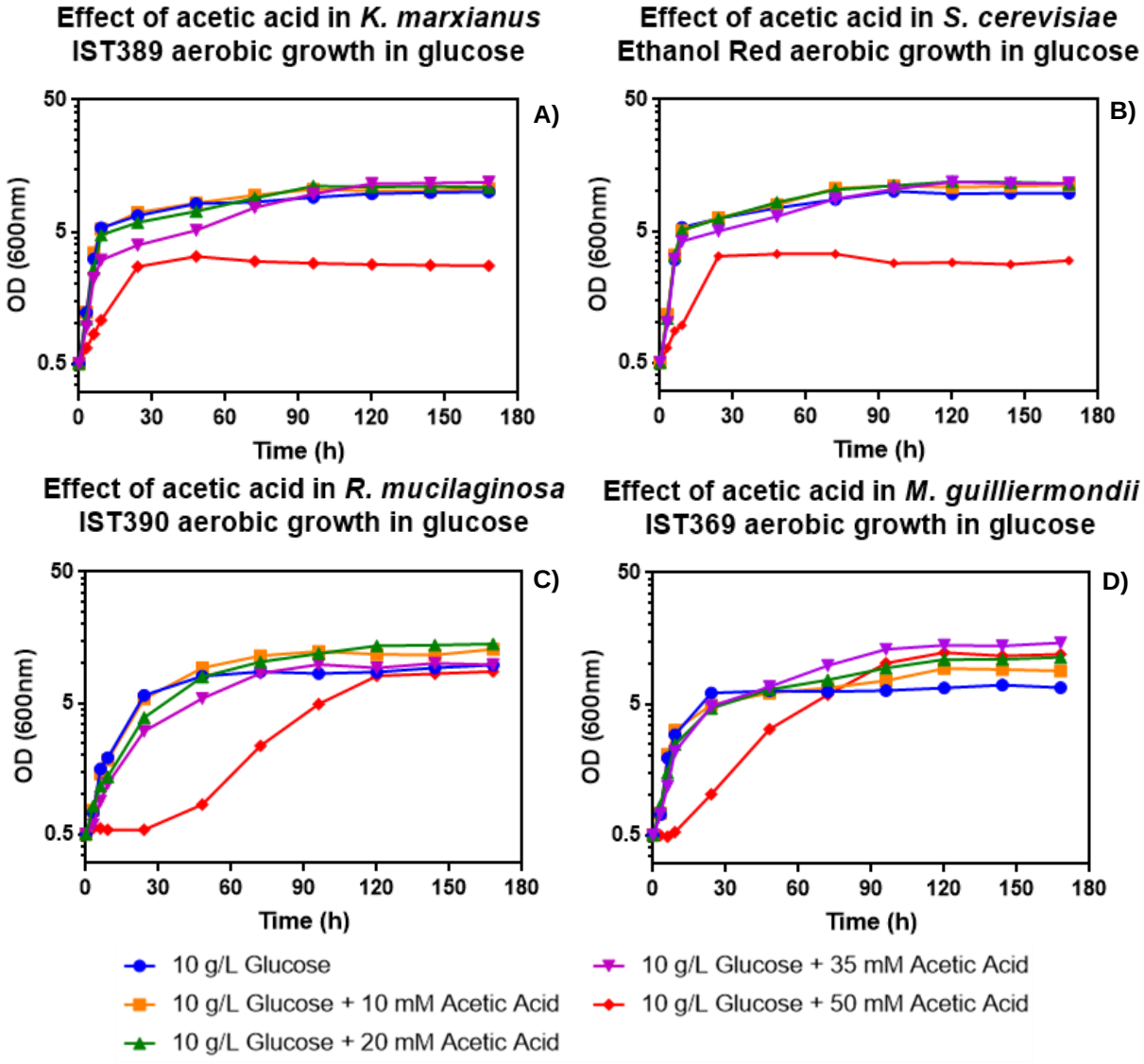

Figure 25 – Growth curves of the selected yeasts strains in MM medium with glucose and increasing concentrations of acetic acid. Growth profiles were based on OD measurements of *K. marxianus* IST389 (A), *S. cerevisiae* Ethanol Red (B), *R. mucilaginosa* IST390 (C) and *M. guilliermondii* IST369 (D) cultures at 30ºC, pH 5.0 and 130 rpm. The conditions studied were 10 g/L glucose (blue circle), 10 g/L glucose + 10 mM acetic acid (orange square), 10 g/L glucose + 20 mM acetic acid (green triangle), 10 g/L glucose + 35 mM acetic acid (purple inverted triangle) and 10 g/L glucose + 50 mM (red diamond).

K. marxianus IST389 and *S. cerevisiae* Ethanol Red exhibit the same profile of growth in all glucose plus acetic acid conditions until 35 mM (Figure 25 A and B). Only for 50 mM acetic acid a latency phase is visible, lasting for 3h, and the maximum specific growth rate (Figure 27A) suffer a significant (3-fold) decrease. Both strains attained similar OD (Table 13), increasing from in 0 mM to 35 mM acetic acid conditions, but at 50 mM, they reached the lowest final OD. *R. mucilaginosa* IST390 (Figure 25C) presents the highest final ODs with 0 mM, 10 mM and 20 mM acetic acid, with no latency phase observed. At 35 mM, a 3 h latency phase was observed, while with 50 mM, latency last for 24 h, also showing a reduction in final OD values (Table 13). *M. guilliermondii* IST369 achieved the highest OD at 35 mM and 50 mM (Figure 25D, Table 13A), only showing a 9 h latency in this last medium.

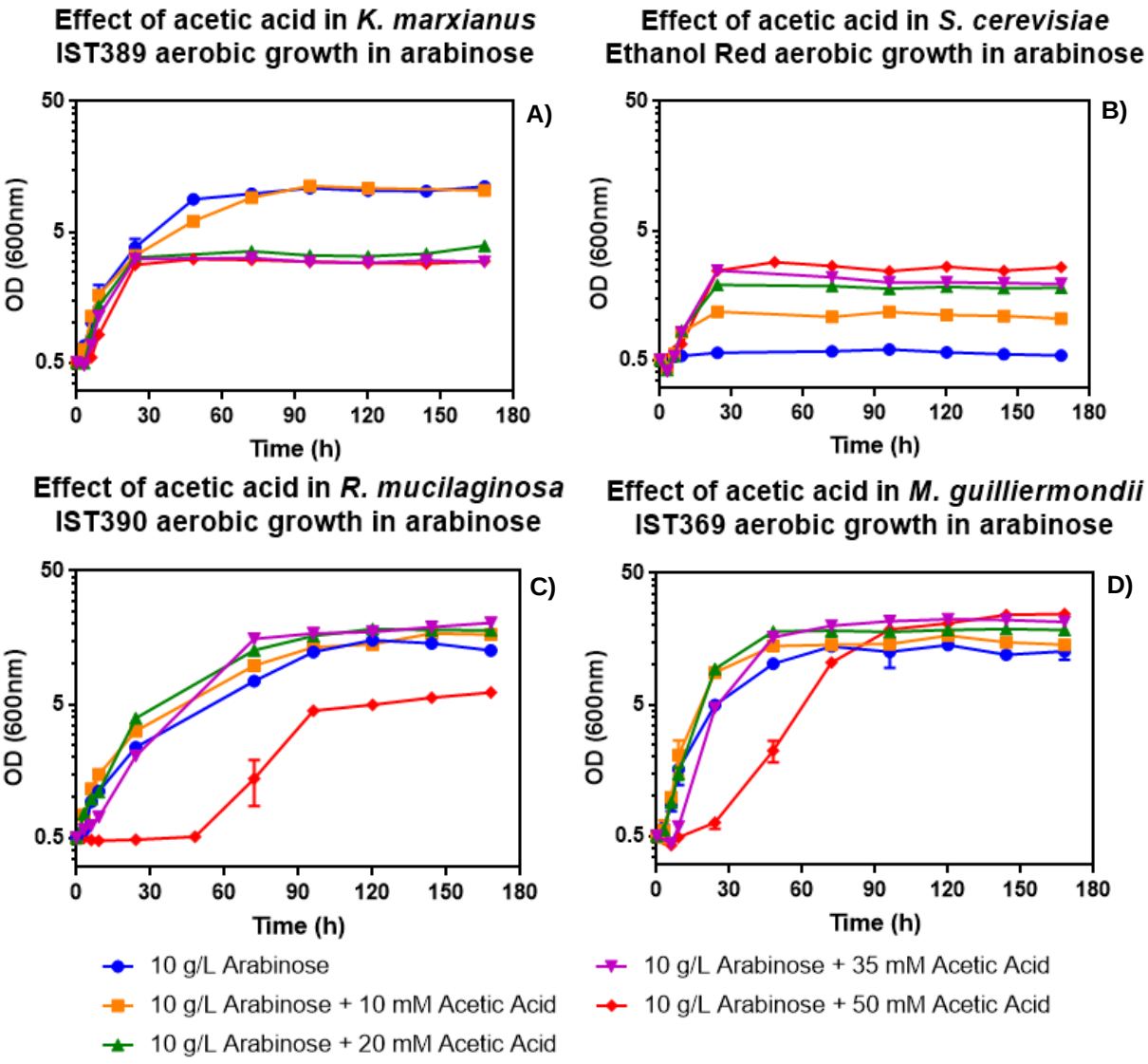

Figure 26 – Growth curves of the selected yeasts strains in MM medium with arabinose and increasing concentrations of acetic acid. Growth profiles were based on OD measurements of *K. marxianus* IST389 (A), *S. cerevisiae* Ethanol Red (B), *R. mucilaginosa* IST390 (C) and *M. guilliermondii* IST369 (D) cultures at 30ºC, pH 5.0 and 130 rpm. The conditions studied were 10 g/L arabinose (blue circle), 10 g/L arabinose + 10 mM acetic acid (orange square), 10 g/L arabinose + 20 mM acetic acid (green triangle), 10 g/L arabinose + 35 mM acetic acid (purple inverted triangle) and 10 g/L arabinose + 50 mM (red diamond).

As expected, *S. cerevisiae* Ethanol Red (Figure 26B) did not grow in medium containing only arabinose. In presence of acetic acid, it presented a 6 h latency phase, which was a little higher in 50 mM acetic acid. Maximum OD successively increased from 10 mM to 50 mM (Table 13B). Since *S. cerevisiae* does not grow in arabinose, this increasing growth is related with acetic acid metabolization.

In arabinose, *R. mucilaginosa* IST390 (Figure 26C) and *M. guilliermondii* IST369 (Figure 26D) present the highest final OD, increasing from 0 mM to 35 Mm acetic. With 50 mM acetic acid, IST390 produced 3 times less biomass than at 35 mM, while IST369 reached the highest OD of all strains. This strain showed a latency of 3 h in 10 mM and 20 mM acetic acid, 6 h in 35 mM, and as in glucose, a 9 h latency in 50 mM. Maximum specific growth rates show 2-fold decrease in comparison with glucose at 0 mM, a 1.6 reduction at 10 mM, and 1.3 times reduction at 20m mM and 35 mM. *R. mucilaginosa* only shows a latency of 48 h in arabinose with 50 mM acetic acid, 24 h more than in glucose medium. The maximum specific growth rates observed in this strain are similar in both glucose and arabinose media. Also, *K. marxianus* (Figure 26A) presented same growth impairment that in glucose, except in arabinose it is not only verified at 50 mM, but also at 20 mM and 35 mM acetic acid, showing low maximum ODs.

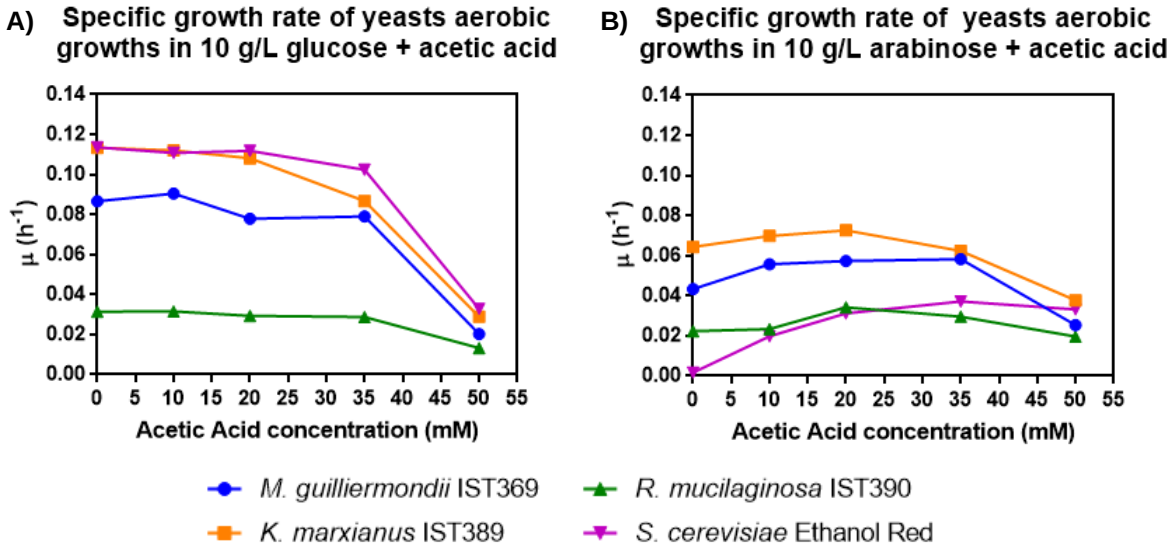

Figure 27 – Maximum specific growth rates (μ) of chosen yeasts in MM with glucose (A) or arabinose (B), in relation with the increasing acetic acid concentration. These values were calculated based on growth curves, in Figures 25 and 26, of *M. guilliermondii* IST369 (dark blue circle), *K. marxianus* IST389 (orange square), *R. mucilaginosa* IST390 (green triangle) and *S. cerevisiae* Ethanol Red (purple inverted triangle).

S. cerevisiae presented the highest tolerance to acetic acid, since it was increasingly growing from this carbon source. *M. guilliermondii* and *R. mucilaginosa* showed to be the most sensible yeasts to acetic acid, which is more accentuated in arabinose medium. Still, at 50 mM acetic acid, after cells adaptation, high biomass production was achieved.

Acetic acid concentration until 35 mM seem to improve the maximum specific growth rates in arabinose, that only decreases at 50 mM. This emphasizes the importance of acetic acid as a carbon source, only causing growth inhibition of the yeasts tested at 50 mM, at pH 5.0, in both glucose and

arabinose. Nevertheless, maximum specific growth rates are lower in arabinose than in glucose, except in the case of *R. mucilaginosa*, showing lower maximum specific growth rates in the absence of stress. Only at 50 mM acetic acid, similar maximum specific growth rate values are observed. Higher concentrations until 35 mM lead to higher biomass, except in the case of *K. marxianus*, in arabinose medium, given that 20 mM already seem to affect biomass production. Apparently, acetic acid affects yeast growth in both glucose and arabinose but exerts a higher inhibitory effect in this last carbon source.

Table 13 – Final OD reached by each strain (IST369, IST389, IST390 and Ethanol Red) in MM with glucose (A) or arabinose (B) plus incresing concentrations of acetic acid. The cultures occurred at 30ºC, pH 5.0, 130 rpm, for 168 h, in aerobic conditions, with increasing concentrations of acetic acid (0 mM, 10 mM, 20 mM, 35 mM and 50 mM).

A)	**Final OD in 10 g/L Glucose + Acetic Acid**				
Acetic Acid (mM) / **Strains**	**0 mM**	**10 mM**	**20 mM**	**35 mM**	**50 mM**
***M. guilliermondii* IST369**	6.95	9.30	11.30	14.60	12.30
***K. marxianus* IST389**	10.05	10.60	11.10	11.85	3.24
***R. mucilaginosa* IST390**	9.75	12.85	14.15	10.05	8.68
***S. cerevisiae* Ethanol Red**	10.05	11.15	11.80	11.85	3.34
B)	**Final OD in 10 g/L Arabinose + Acetic Acid**				
Acetic Acid (mM) / **Strains**	**0 mM**	**10 mM**	**20 mM**	**35 mM**	**50 mM**
***M. guilliermondii* IST369**	14.20	16.73	18.50	22.33	24.5
***K. marxianus* IST389**	11.12	11.2	3.92	3.15	3.10
***R. mucilaginosa* IST390**	15.10	17.00	18.30	20.40	6.14
***S. cerevisiae* Ethanol Red**	0.60	1.18	1.90	2.45	2.84

4.6.2. Comparison of yeast growth in SBP hydrolysates H11 and H13 at different pH and temperatures

SBP hydrolysates are complex mixtures of nutrients but also possess several compounds that may be inhibitors, besides acetic acid, methanol, heavy metals and pesticides can also be present. Process conditions, such as increased process to values close to the range of optimal/supraoptimal temperatures and decreased medium pH when weak acids are present in the medium, may increase their toxicity. At pH 4.5, acetic acid is below its pKa, contrarily to pH 5.0, and more than half of acetic acid is in the undissociated form, which is the toxic form.

In this part of the work, a rapid comparison of the growth profiles was performed in SBP hydrolysates H11 and H13 (Table 5) at 30ºC and 35ºC and pH 4.5 and 5.0. The major differences between these two hydrolysates are the glucose concentration (H11 has the double of glucose compared with H13) and acetic acid concentration (33.5 mM in H11, and 43.8 mM in H13). *S. cerevisiae* CEN.PK122 was added to this assays, since *S. cerevisiae* CEN.PK is tolerant to acetic acid [156].

After being previously cultivated in H13, at pH 4.5, 250 rpm, at both 30ºC and 35ºC, yeasts were inoculated in 96-well microplate, in both H11 and H13, at pH 4.5 and 5.0, and cultured at respective temperatures, during 40 h, since stationary phase had been already attained at this timepoint, with an initial OD 0.1. Lower initial ODs are important for these assays, to avoid rapid saturation causing reading's errors. The growth profiles of each strain at 30ºC are depicted in Figure 28, and at 35ºC are showed in Figure 29.

It is worth noting that growth conditions in these microplate kinetics assays are limited. Fast growth saturation was observed, which may be related with oxygen exhaustion. Since these assays were performed during 40 h, most of the carbon sources were probably not metabolized. So, the growth profiles observed are mostly generated from glucose, galactose and acetic acid utilization, as observed in previous results.

Still, at both 30ºC (Figure 28) and 35ºC (Figure 29), it is possible to observe that all strains presenting a similar pattern of growth, preferring H11 at pH 5.0 > H11 at pH 4.5 > H13 at pH 5.0 > H13 at pH 5.0.

K. marxianus and *S. cerevisiae* strains presented similar growth curves at both temperatures. *S. cerevisiae* Ethanol Red (Figure 28 and 29 E) and CEN.PK 122 (Figure 28 and 28 F) do not show a latency phase, while *K. marxianus* IST389 and CBS712 show a small duration latency phase (Figure 28 and 29 C and D), that was superior in H11, pH 4.5. *K. marxianus* is a thermotolerant specie, despite being less tolerant to acetic acid than *S. cerevisiae,* which is known for having an inherent acetic acid tolerance [7,47]. Nevertheless, Ethanol Red optimal temperature is 34ºC [157]. Still, the conjugated effect of stress factors seems to affect these strains in a similar way, not presenting significative growth impairment.

M. guilliermondii IST369 (Figure 28 and 29 B) showed a longer latency phase, and the highest differences between conditions. The profiles in H13 seem to stabilize faster and more abruptly than in H11, with lower final biomass than at 30ºC (Figure 28B). However, the biomass produced by this strain was also slightly less than the obtained for the other strains. Still, at 30ºC, *M. guilliermondii* growth is favoured in H11 over H13, which may be related with hydrolysates composition (higher glucose and less acetic acid). At 35ºC, pH 5.0 is favoured over pH 4.5, independently of the hydrolysates, consistent with the higher susceptibility to the conjugated effect of temperature, pH and acetic acid.

R. mucilaginosa IST390 (Figure 28 and 29 A) presented the more extended latency phases. At 35ºC, IST390 exhibited a marked decrease in biomass production, with reduced duration of exponential phases, due to a much faster growth stabilization, and in H13, pH 4.5, no growth occurred. The increased temperature only affected especially *R. mucilaginosa*. *R. mucilaginosa* is reported to present good growth from 25ºC to 30ºC, and variable growth ability at 35ºC [134], with this temperature impairing growth of some strains [137]. IST390 seems to be a temperature sensible strain. Still, *R. mucilaginosa* seems to be the most affected by the conjugated effect of temperature, pH and acetic acid, even more than *M. guilliermondii*.

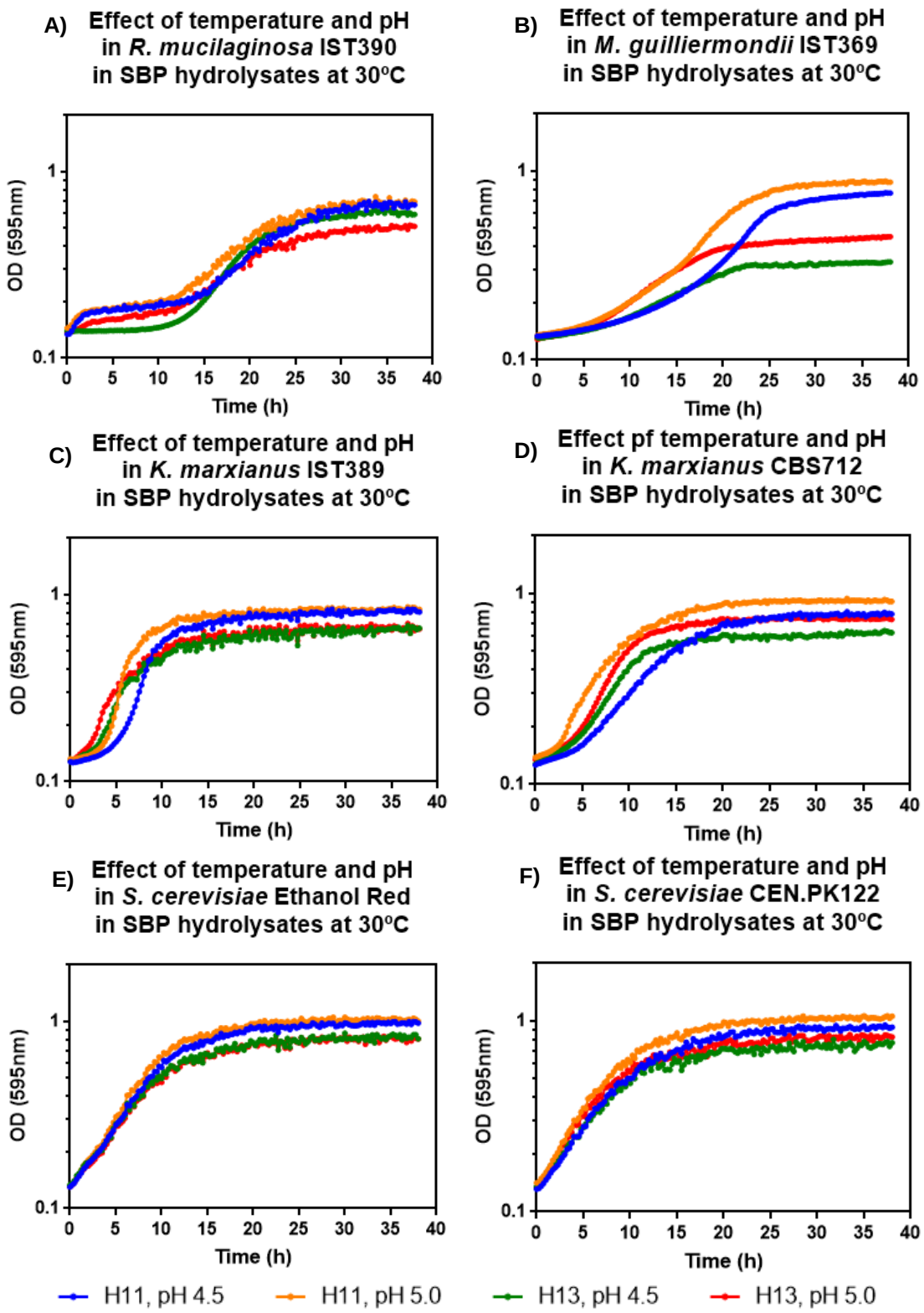

Figure 28 – Growth profiles of the selected yeasts in SBP hydrolysates H11 and H13 at 30ºC. Growth curves of *R. mucilaginosa* IST390 (A), *M. guilliermondii* IST369 (B), *K. marxianus* IST389 (C), *K. marxianus* CBS712 (D), (orange triangle), *S. cerevisiae* Ethanol Red (E) and *S. cerevisiae* CEN.PK122 (F) were performed in hydrolysate H11, at pH 4.5 (blue line), and pH 5.0 (orange line) and in H13, at pH 4.5 (green line) and pH 5.0 (red line), in a 96-well microplate, and curves were bases in OD measurements at 595nm in a fluorescence microplate reader, in kinetics mode.

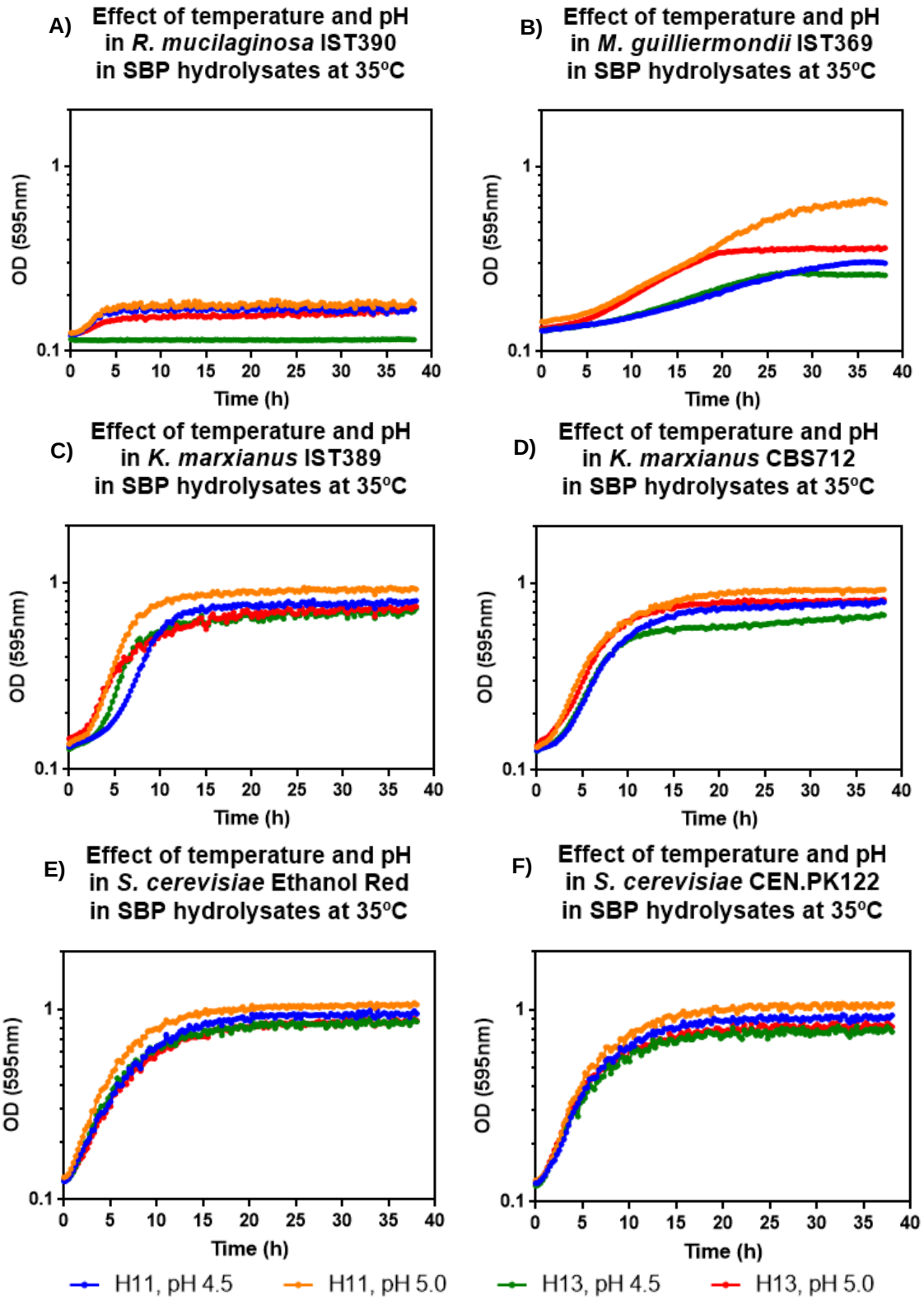

Figure 29 – Growth profiles of the selected yeasts in SBP hydrolysates H11 and H13 at 35ºC. Growths of *R. mucilaginosa* IST390 (A), *M. guilliermondii* IST369 (B), *K. marxianus* IST389 (C), *K. marxianus* CBS712 (D), (orange triangle), *S. cerevisiae* Ethanol Red (E) and *S. cerevisiae* CEN.PK122 (F) were performed in hydrolysate H11, at pH 4.5 (blue line), and pH 5.0 (orange line) and in H13, at pH 4.5 (green line) and pH 5.0 (red line), in a 96-well microplate, and curves were bases in OD measurements at 595nm in a fluorescence microplate reader, in kinetics mode.

5. Discussion

The exploitation of non-conventional yeasts (NCY) is gaining relevance due to their potential for the bioconversion of different biomasses constituted by distinct carbon sources of difficult metabolization and the production of diverse high added-value bioproducts with applications in Biotechnology and in the Food and Pharmaceutical Industries [1,2,16,42]. A number of these yeasts have recently been proposed as potentially interesting cell factories for pectin-rich agro-industrial wastes valorisation, given the carbon sources present and their catabolic abilities. [9,29]. Evidences reinforcing this idea were obtained during the present study.

The isolation during this work of the yeast species *Pichia kudriavzevii*, *Clavispora lusitaniae*, *Rhodotorula mucilaginosa, Metschnikowia pulcherrima* and *Hanseniaspora opuntiae* from sugar beet pulp and fruits is consistent with their presence in vegetal and fruit environments. The first three species, found in multiple environments, including fresh and rotten fruits and trees, fermented food and beverages and pectin-containing residues [15,134,158], were isolated from sugar beet pulp (SBP). These species were reported to catabolize glucose and as having a variable metabolization of galactose. However, only *R. mucilaginosa* is capable to metabolize xylose, arabinose and galacturonic acid, while *C. lusitaniae* metabolizes xylose. Together with acetic acid, these are the main carbon sources present in pectin-rich residues hydrolysates, namely SBP, and their catabolization ability a desired trait. The SBP received from a Belgium sugar refinery was stored at 4ºC during several weeks, this may have impaired yeast growth and affected their vitality. For this reason, it is probable that the strains isolated do not represent the SPB associated cultivable microbiome. *Metschnikowia pulcherrima* was isolated from cherries, and *Hanseniaspora opuntiae* was found in grapes, which are, respectively, common environments for these species [159,160]. Despite the low pectin content of cherries and grapes [161], these are interesting sources for the isolation of strains with different ability to catabolize pectin-related sugar monomers. However, *H. opuntiae* was only reported to metabolize glucose, while *M. pulcherrima* also catabolizes galactose [159,160]. The yeasts isolated from pectin-containing residues may also be interesting for pectinases production, useful for pectin hydrolysis. However, since this thesis work was carried out using SBP hydrolysates supplied by German partner of the YEASTPEC EraNet project, these pectinolytic activities were not considered relevant in this context.

The strains *M. guilliermondii* IST369 (isolated from oak beer barrel), *K. marxianus* IST389 and *R. mucilaginosa* IST390 (both isolated from SBP) present in IST Yeast Collection were selected after an initial screening to search for the capacity to growth in pectin sugar monomers and the ability to reach high culture OD values were the selection criteria. The type-strain *K. marxianus* CBS712 and *R. mucilaginosa* IST423 (isolated from lupine processing effluent and chosen from IST collection due to the interesting isolation source) were also studied for comparison, and *S. cerevisiae* Ethanol Red was used as control. These yeasts were grown, and the metabolites produced assessed in SBP hydrolysates in aerobic conditions. SBP hydrolysates H8 and H11 differ in the carbon sources composition, with H11 containing higher glucose, galactose, arabinose and galacturonic acid concentrations. These hydrolysates are also composed by acetic acid at similar concentrations (33.5 -

35.3 mM), which was initially thought to be a potential yeast growth inhibitor, and low concentrations of methanol (0.04 and 0.3 %(v/v)) and ethanol (0.08 %(v/v)).

The analysis of the profiles of consumption of the carbon sources present in SBP hydrolysates, the sequential consumption of carbon sources was observed, but those profiles varied among the studied species. Glucose was the preferred carbon source by all the studied yeasts over galactose, xylose and arabinose, these sugars being metabolized in this order, as stated in the literature [81,109,135]. Interestingly, some carbon sources were found to be co-consumed. For example, *M. guilliermondii* IST369 and *R. mucilaginosa* strains IST390 and IST423 appeared to co-consume acetic acid, another carbon source present in the hydrolysates, as the result of no inhibitory effect by this weak acid, together with glucose, showing absence of glucose catabolite repression in these species. A previous study also reported the co-consumption of acetic acid with glucose, xylose and arabinose by *M. guilliermondii*, not suffering glucose catabolite repression as occurs in *S. cerevisiae*. Also, the acetic acid consumption did not interfere with the uptake of other carbon sources [154].

Acetic acid was found to be an important carbon source present in these hydrolysates and the concentrations present are not significantly inhibitory at pH 5.0 and 30ºC, being metabolized by all yeast species. The effect that increasing concentrations of acetic acid have in the metabolization of 10 g/L of glucose or of arabinose (similar to the maximum concentrations present in SBP hydrolysates) was assessed. Concentrations up to 35 mM, close to those present in SBP hydrolysates, do not exert toxic effects nor inhibit the growth of any of the yeast strains examined at pH 5.0, and were used as sole carbon source (for S cerevisiae in arabinose medium) or additional carbon source (for all the strains in any media), increasing the final biomass concentration attained. The maximum specific growth rates obtained in media supplemented with acetic acid concentrations in the range from 10 mM to 35 mM slightly increased in arabinose medium, while in glucose media the specific growth rates maintained similar values. This indicates that in glucose media, yeasts' growth in the exponential phase was mostly due to glucose consumption, while in arabinose, acetic acid was also actively metabolized and yeast growth was improved. A previous report on *M. guilliermondii* cultivation in glucose, xylose and arabinose at pH 5.5, also refer that the addition of 5 g/L acetic acid slightly improved the global specific growth rate compared with an un-supplemented medium and it was proved that acetic acid was co-consumed with all the other carbons sources [154].

Concentrations of 50 mM of acetic acid at pH 5.0 showed to be toxic for all the yeast species examined but the toxicity was higher for *M. guilliermondii* IST369 and *R. mucilaginosa* IST390 grown in glucose or arabinose media. In fact, a more extended lag phases and more decreased specific growth rates were found, compared with *K. marxianus* and *S. cerevisiae,* but *M. guilliermondii* and *R. mucilaginosa* produced a higher final biomass, and after an adaptation period, they were able to resume growth. This hypothesis is proven in a study performed with *K. marxianus*, in which increasing acetic acid concentrations led to higher latency phases where cells were acquiring tolerance, and the newly resistant cells reinoculated in fresh acetic acid medium immediately resume growth [162]. *S. cerevisiae* Ethanol Red was the most tolerant strain to acetic acid. This is a robust industrial strain used for bioethanol production, adapted to resist to stresses present in lignocellulosic hydrolysates

and molasses fermentations [14,163]. Since this species does not metabolize arabinose, the biomass production in the corresponding medium occurred due to acetic acid consumption only.

However, with 50 mM acetic acid, even *K. marxianus* IST389 and *S. cerevisiae* Ethanol Red showed a lower final biomass (OD decrease from 10 to 3, compared with glucose medium as sole carbon source). In case of *K. marxianus*, biomass reduction was observed in arabinose medium with only 20 mM and 35 mM of acetic acid supplementation. An interesting study on *K. marxianus* showed that acetic acid toxicity at 40 mM is highly pH and substrate dependent, being growth more affected in galactose and lactose medium than in glucose, inulin and fructose [162]. It was hypothesised the interference of acetic acid with lactose cellular uptake, which occurs only by symport, an energetically unfavoured process when cells are responding to acetic acid stress, trying to export protons to counteract cytosol acidification [162]. This is an highly energetically expensive adaptation mechanism, in which cytoplasmic ATP consumption by ATPases increases and inhibits growth and mitochondria function, as reported for *S. cerevisiae* [47]. It is also considered that enzymes of lactose and galactose metabolic pathways may be affected by acetic acid stress [162]. Similarly to galactose, arabinose is mostly transported by symport through high affinity transporters, as reported for several non-conventional yeasts as *M. guilliermondii* [164], while glucose can more easily enter by facilitated diffusion. Possibly, acetic acid affects more *K. marxianus* cells growing in arabinose than in glucose by impairing cellular uptake, and possibly by interfering with enzymes of arabinose metabolic pathway, while for glucose only at 50 mM acetic acid a detectable effect was observed.

During aerobic cultures in SBP hydrolysates H8 and H11, cells started to consume ethanol after glucose and/or galactose and acetic acid metabolization. This led to changes in acetic acid concentrations, especially observed in *S. cerevisiae* and *K. marxianus* strains in H8 cultures, in which 5 to 10 mM of acetic acid was produced. Ethanol metabolization starts with conversion of ethanol into acetaldehyde by alcohol dehydrogenase (*ADH2* gene), which is further converted into acetate by aldehyde dehydrogenase (*ALD4*, *ALD5* and *ALD6* genes) [52,53] (Figure 4, Introduction section), showing that ethanol catabolization leads to acetate generation, increasing the concentration of this compound in the medium.

Arabinose was only metabolized after the consumption of all the above referred carbon sources in both SBP hydrolysates by *M. guilliermondii* IST369 and co-consumed with acetic acid by *K. marxianus* strains IST389 and CBS712, while galacturonic acid was not metabolized by any of the strains of the species studied. *M. guilliermondii* IST369 was the only strain that fully metabolized arabinose during the time of the culture (148 h to 210 h). A study comparing one strain of each species *M. guilliermondii* and *Candida arabinofermentans* showed that *C. arabinofermentans* leads to a much lower accumulation of arabitol and to traces of ethanol production in arabinose medium, due to a more efficient arabinose catabolic pathway [125]. Arabinose metabolic pathway starts with arabinose conversion into arabitol by an NAD(P)H-dependent L-arabinose reductase (aldose reductase) (AR). It is posteriorly oxidized to L-xylulose by L-arabitol-4-dehydrogenase (LAD), which is converted into xylitol by L-xylulose reductase (LXR). Xylitol, a common metabolite of arabinose and xylose pathways, is then converted to D-xylulose by xylitol dehydrogenase (XDH) [89]. When arabitol is not a desired

product, it represents one of the major drawbacks to an efficient arabinose fermentation and metabolization, due to its accumulation resulting from a cofactor imbalance (AR prefers NADPH, while LAD prefers NAD^+) [89,125]. Subsequently, it was confirmed by analysis of the kinetics of arabinose pathway enzymes of each strain, that *C. arabinofermentans* enzymes showed much higher catalytic efficiency (higher V_{max} and lower K_m) compared with *M. guilliermondii*, especially the enzyme responsible for arabitol oxidation (LAD) [164]. Therefore, the studied *M. guilliermondii* IST369 seems to have a more efficient arabinose metabolism when compared to *K. marxianus* strains IST389 and CBS712, since these last strains accumulates higher arabitol concentrations and were unable to fully metabolize arabinose during culture duration.

Galacturonic acid (GalA) revealed to be a carbon source very difficult to be metabolized by most yeasts, with only few reports known so far. One study reports that the yeast *Rhodosporidium toruloides* possess an efficient D-GalA metabolism, similar to D-glucose and D-xylose metabolism in MM at 30ºC and pH 5.5 [165], and the same authors state that *R. mucilaginosa* also possesses an efficient D-GalA metabolic pathway, that it is not described yet (unpublished results) [136]. In our study, *R. mucilaginosa* strains IST390 and IST423 did not metabolize arabinose and galacturonic acid in SBP hydrolysates during the standardized culture time (168 h), but in H11 cultures, both strains showed a slight decrease of 1 g/L to 2 g/L in arabinose and GalA concentration in the last 24 hours of culture. Since both *R. mucilaginosa* strains exhibit low carbon source consumption rates (similar in both hydrolysates) compared with other strains (glucose consumption rates are approximately 3- and 8-times below the other tested species in H8 and H11, respectively) (Table 8, Results section). So, it is possible that if the cultures were maintained during a longer period of time, both GalA and arabinose would possibly be metabolized. When compared with MSc student Paula Semedo study, IST390 and IST423 strains showed arabinose and GalA consumption in MM with concentrations of carbon sources resembling the hydrolysates, with preference for arabinose over galacturonic acid, especially when acetic acid (a carbon source preferred over arabinose a GalA, was not supplemented to the medium. These cultures were maintained for 268 h, and only in the medium without acetic acid, the 10 g/L arabinose and GalA present were almost exhausted. This is consistent with the observation that *R. mucilaginosa* strains were capable to grow using GalA as sole concentration source, although only around 30 % of the initial concentration was used.

Considering arabinose metabolic pathway, reductases prefer NADPH as cofactor, whereas dehydrogenases are dependent on NADH, which generates a double cofactor redox imbalance [18,88,89]. Similarly, GalA metabolic pathway of *Rhodosporidium toruloides,* as the ones in filamentous fungi, generates a double redox imbalance. GalA is firstly reduced to L-galactonate by a NADPH-specific D-GalA reductase, being then transformed into 3-deoxy-L-threo-hex-2-ulosonate by a dehydratase, and posteriorly into L-glyceraldehyde and pyruvate by an aldolase. L-glyceraldehyde is reduced to glycerol, a central metabolite, by a NADPH-dependent glyceraldehyde [165]. Cofactor imbalance is another and most probable reason for the arrestment of GalA catabolism. Despite two NADPH being generated during ethanol and acetate catabolization, which should solve this problem, cells may utilize NADPH in other processes, since this is the preferred cofactor in biosynthetic reactions [166].

SPB hydrolysates are complex mixtures, not only of distinct carbon sources, but also of nitrogen sources. Despite proteins (approximately 10 % protein in dry SBP) [46] and other compounds that can be used as nitrogen sources being present, SPB hydrolysates H8 and H11 were supplemented in this work with urea as an additional nitrogen source, and in the case of H8 results, were compared with non-supplemented H8. In general, urea supplementation slightly improved final biomass concentrations, carbon sources consumption and ethanol and arabitol production rates, but had no effect in *K. marxianus* CBS712 growth (Table 8, Results section). Consistent with our results, urea supplementation improved carbon sources consumption and ethanol productivity by *Scheffersomyces stipitis* in sugarcane bagasse hydrolysate, compared with non-supplemented hydrolysate [167]. Urea supplementation may be interesting for improving industrial processes, especially for substrates with poor nitrogen sources but also for SBP hydrolysates, if used at sufficient low concentrations that allow increasing consumption rates, production rates and yields, namely of ethanol, but that also allows to maintain a cost-effective production [168]. However, the major improvements in maximum specific growth rates (approximately 1.3-times faster), consumption rates (especially glucose, with approximately 3-fold higher) and ethanol production rates (2- to 10-times higher) were observed in SBP hydrolysate H11 plus urea cultures when compared with H8 plus urea (Table 8, Results section). This indicates that yeasts' performances are mostly dependent on hydrolysates composition, with higher carbon sources concentrations improving yeasts' growth and metabolism [166].

Considering biorefinery processes, with the utilization of lignocellulosic and pectin-rich substrates, it can be advantageous to utilize different culture conditions, as increased temperature and lower pH, which may improve ethanol production and avoid undesirable contaminations by other microorganisms, in particular bacteria [15]. The study of the possible stress effects of increasing temperature (30ºC to 35ºC) and decreasing pH (5.0 to 4.5) allowed to observe that *K. marxianus* strains IST389 and CBS712, and *S. cerevisiae* Ethanol Red and CEN.PK122 are more tolerant strains to the combined effect of temperature and pH than *M. guilliermondii* IST389 and *R. mucilaginosa* IST390. *K. marxianus* is a thermotolerant specie, being capable of growing until maximum temperatures of 52ºC [107], so it as expected that a 5ºC increase did not exert negative effects on growth. *S. cerevisiae* is known for having an inherent acetic acid tolerance [7,47], higher than *K. marxianus*. The combined effect of high temperature and pH did not affect significantly their growth profiles. Contrarily, *M. guilliermondii* IST389 and *R. mucilaginosa* IST390 were the most affected strains by the conjugated effect of temperature and pH, with temperature effect being evident in the impairment of *R. mucilaginosa* growth. As mentioned above, acetic acid affects the uptake and metabolization of carbon sources, especially at lower pH (pH < pKa 4.7), by cytosol acidification [162], and the combined effect with temperature and pH increase exert higher stress and biomass inhibition.

Since the above referred assays were performed in 96-wells microplates, it is necessary to consider the limitations in culture conditions. Reports focus the insufficient aeration in 96-well microplate cultivations for microorganisms growth, being a difficult parameter to control, which generated differences in *K. marxianus* biomass yields under acetate stress between 96-plate and fermenter cultivations [162]. Also, it is not possible to assess the production of added-value products production. Nevertheless, these assays were useful to simulate different culture conditions, in which *S. cerevisiae*

and *K. marxianus* were found to be the more suitable species for the use of hydrolysates at higher temperature (35ºC), for ethanol or ethanol and arabitol production, respectively. However, if the desired product are the carotenoids, the use of 30ºC and pH not below 5.0 are necessary for *R. mucilaginosa*. Therefore, the selection of the adequate species and optimal conditions are required to obtain the most cost-effective and productive process. Additionally, cultures carried out in microaerophilic conditions were also performed. Despite the slight increase in final ethanol concentrations, the limitation of the oxygenation levels impaired the consumption of respirable carbon sources and arabitol production. Aerobic conditions are necessary for maximum carbon sources utilization and arabitol and carotenoids production, which is desirable in industrial applications.

Ethanol was considered to be produced from glucose and galactose by *M. guilliermondii* IST369, *K. marxianus* IST389 and *S. cerevisiae* Ethanol Red, and only from glucose by the other strains. Despite the reported *M. guilliermondii* ability to ferment arabinose [81], no ethanol was produced from this substrate by IST369. Ethanol yields obtained in this study varied between 0.32 $g_{ethanol}/g_{sugars}$ and 0.44 $g_{ethanol}/g_{sugars}$ for *K. marxianus* strains IST389 and CBS712, *M. guilliermondii* IST69 and *S. cerevisiae* Ethanol Red, showing similar maximum concentrations produced (0.2 to 0.3 %(v/v) in H8 and 0.7 to 0.8 %(v/v) in H11). The obtained yields are similar to those reported in studies with lignocellulosic and pectin-containing residues, in comparable conditions. *K. marxianus* yield was approximately 0.40 g/g in sugar beet molasses (100 g/L reducing sugars), at 30ºC, pH 5.0 with aeration [20]. *S. cerevisiae* Ethanol Red attained 0.40 g/g in soybean hull rich in glucose, at 32ºC, 100 rpm, during 48 h [169]. On the other hand, *R. mucilaginosa* IST390 only fermented glucose, showing lower ethanol concentrations in H8 (maximum of 0.1 %(v/v)), and almost no production occurred in H11, consistent with *R. mucilaginosa* classification as obligate aerobic and generally non-fermentative species [134]. Nevertheless, since SBP hydrolysates contain lower concentrations of fermentable sugars (e.g. glucose and galactose), this substrate is not a suitable for ethanol production, but it can be useful for obtaining other products, such as arabitol (mostly produced from arabinose) or carotenoids.

K. marxianus strains IST389 and CBS712 showed a much higher accumulation of arabitol (2 g/L to 4 g/L in H8, and 6 g/L in H11) and the highest production yields (0.77 to 0.90 $g_{arabitol}/g_{arabinose}$ in both hydrolysates) in aerobiosis, indicating that SBP hydrolysates complex composition and arabinose metabolism of these strains favour arabitol production. Only one study showed that *K. marxianus* was able to produce arabitol in 20 g/L arabinose medium at 150 rpm, with yields approximately 0.27 g/g at 30ºC, and 0.48 g/g at 40ºC [110], much lower than those obtained in this study. On the other hand, *M. guilliermondii* IST369 presented low arabitol accumulation (maximum arabitol concentration of 3 g/L, and production yield of 0.22 $g_{arabitol}/g_{arabinose,}$ obtained in H11), which is related with the described efficient arabinose metabolic pathway that does not favour arabitol accumulation. However, there are other reports in which diverse *M. guilliermondii* strains were examined describing the species as a great arabitol producer [89,125,127].

An important production factor for arabitol accumulation is the level of medium oxygenation, as confirmed in studies in shake flasks, where 150 to 200 rpm were determined as suitable agitations [89,127]. In this study, 130 rpm was used as standard agitation, maintaining favourable dissolved

oxygen levels in liquid phase for arabitol accumulation by *K. marxianus* strains. Furthermore, as previously mentioned, the activity of the arabinose metabolic pathway enzymes influence both arabinose metabolization and arabitol accumulation [164].

Despite *R. mucilaginosa* strains IST390 and IST423 do have low fermentative ability, they show high pink pigmentation in aerobiosis, due to the production of carotenoids. Production assays in aerobiosis and extraction procedures were performed, since higher production had been observed in previous aerobic cultures in SBP hydrolysates. Carotenoids exerts a photooxidative protection to these cells, so in order to stimulate their production, higher aeration and illumination are favourable [94,95]. When cells were cultivated in minimal medium with glucose, IST423 produced much lower pink pigments, contrarily to IST390. This same behaviour was detected in MM assays performed by Paula Semedo in single sugar medium, with glucose, xylose and galacturonic acid. Accordingly, IST390 seems to be a better producer than IST423 by observation of the cultures, which was further confirmed after carotenoids extraction. Yet, both strains produced higher total carotenoids concentration in SBP hydrolysate H11 (312 μg $_{\text{total carotenoids}}$/g $_{\text{dry biomass}}$ extracted from IST390 culture, and 206 μg $_{\text{total carotenoids}}$/g $_{\text{dry biomass}}$ from IST423 culture) than in rich medium YPD (Table 12, Results section).

Several studies report the production of carotenoids by *R. mucilaginosa* strains and the most comparable studies to the one performed here present similar values to those obtained. The most similar study was performed using food wastes (4.3 to 5.9 g/L of reducing sugars) with 3 g/L yeast extract and mineral supplementation, reporting a maximum carotenoid production of 376.5 μg/g in ketchup, 268.6 μg/g in molasses and 245.0 μg/g in health drink. Carotenoids production varies among yeast strains, substrates consumed and culture conditions [170]. Metal ions addition (Fe^{2+} > Ca^{2+} > Mg^{2+}) was found to improve both carotenoid and biomass production, and, despite molasses having lower reducing sugar concentrations (4.3 g/L) compared with other substrates (5.9 g/L), they contains heavy metals, proteins, vitamins and fat, that also may favour this production [96], similarly to SBP hydrolysates [45,46]. Also, by observation of the cells growing in glucose and arabinose with acetic acid media in shake flasks, it was found that pink pigmentation production increased with increasing concentrations of acetic acid. Despite no carotenoid quantification was obtained, acetic acid concentrations usually present in SBP hydrolysates at pH 5.0 are metabolized as relevant carbon source for pigment generation. Tests using acetic acid as a sole carbon sources would be desirable to examine its potential for carotenoid production. Considering the carbon and nitrogen sources, and other trace elements available in SBP hydrolysates, these pectin-rich substrates are promising also for carotenoids production.

6. Conclusions and future perspectives

The economic valorisation of agro-industrial residues by yeasts requires the assessment of non-conventional yeasts potential, due to their metabolic diversity compared with *S. cerevisiae*. In this study, it was possible to successfully isolate and identify five distinct yeast species in pectin-rich (sugar beet pulp) or with pectin (macerated cherries and grapes) samples.

The non-conventional yeast species *M. guilliermondii*, *K. marxianus* and *R. mucilaginosa*, studied after selection for pectin-monomers catabolization ability, proved their capacity to metabolize most of the carbon sources present in the SBP hydrolysate H8 and H11 received from the YEASTPEC partner (galacturonic acid was the exception) and to produce interesting bioproducts in aerobiosis, but the better performances were obtained with H11 due to its higher concentrations of glucose, galactose and arabinose.

M. guilliermondii IST369 produced the highest final biomass concentration, being the only strain that efficiently catabolized arabinose. Several studies report the successful metabolic engineering of *S. cerevisiae* by expressing filamentous fungi pathways for arabinose (and xylose) utilization [171,172]. It would be interesting to express these metabolic pathways in *S. cerevisiae*, especially from other ascomycete yeast, as *M. guilliermondii*. *R. mucilaginosa*, despite being the only species examined that can grow using galacturonic acid as sole carbon source, GalA consumption in the hydrolysates was not possible during 168 h of incubation. This is not only due to the low specific growth rate and sugar utilization of this species strains, but also because GalA, as well as arabinose, are only utilized following the consumption of the more easily catabolized carbon sources. *S. cerevisiae* metabolic engineering with GalA yeast metabolic pathway, instead of using filamentous fungi pathways, as it was already reported [173,174], would also be desirable. For this, a more detailed study of *M. guilliermondii* IST369 and *R. mucilaginosa* strains IST390 and IST423 metabolic pathways of these sugars would be required, as well as the optimization of the cultivation conditions, especially for *R. mucilaginosa*, if to be directly used as cell factory.

Acetic acid present in the hydrolysates, at concentrations of approximately 35 mM and pH 5.0, was found to be an important carbon source for all the yeast species tested. This concentration at pH 5.0 does not exert detectable toxicity, not altering maximum specific growth rates, independently of the carbon source (glucose or arabinose) in the culture medium. The increase of temperature and decrease of pH affect more significantly *M. guilliermondii* IST369 and *R. mucilaginosa* IST390 growth in SBP hydrolysates compared with *K. marxianus* and *S. cerevisiae* strains.

In terms of production potential, ethanol was produced by *K. marxianus* strains IST389 and CBS712 and *M. guilliermondii* IST389 at amounts and yields similar to *S. cerevisiae.* Both *K. marxianus* strains IST389 and CBS712 produce high arabitol concentrations, with high production yields. For these strains, SBP hydrolysates as substrate and culture conditions proved to be suitable for arabitol production. The thermotolerance of *K. marxianus* can be an interesting and advantageous feature to be explored in both ethanol and arabitol production. Despite its low fermentative ability and growth rate, *R. mucilaginosa* strains IST390 and IST423 possess high potential for carotenoid production, especially in SBP hydrolysates.

This thesis provides insights on the metabolization and production potential of new yeast isolates obtained in our laboratory to be explored for the bioconversion of pectin-rich residues, in particular of SBP hydrolysates. A general overview of this thesis' conclusions is displayed in Figure 30.

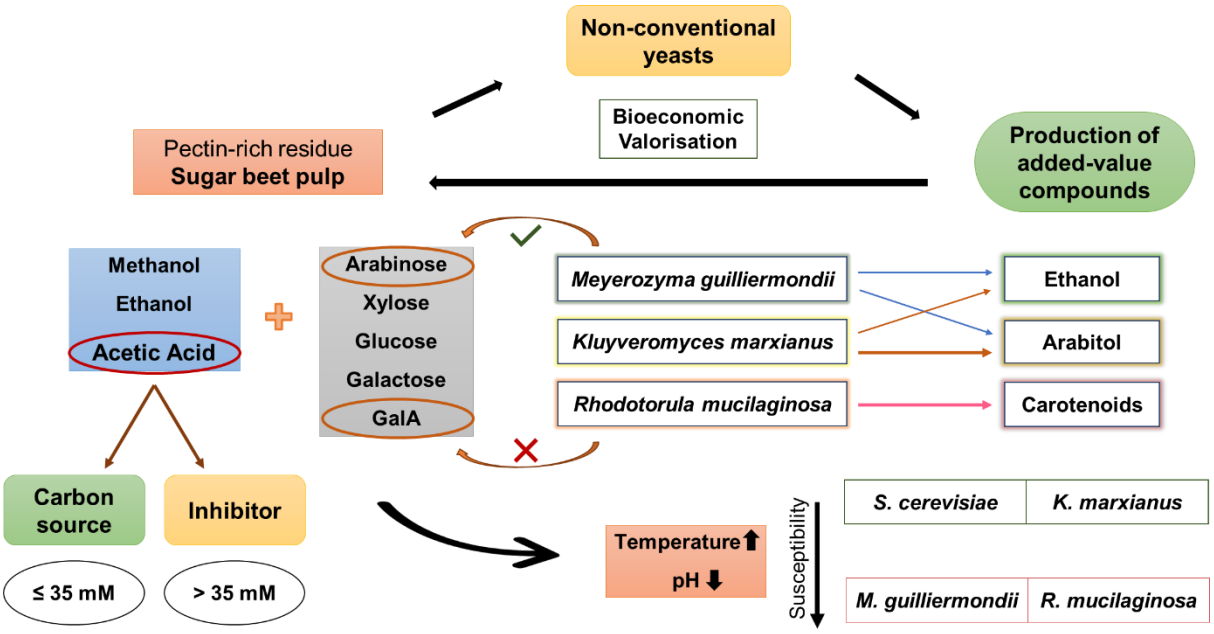

Figure 30 – Schematization of the most relevant conclusions obtained in the present study.

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8. Supplementary data

Table S1 – Isolated and identified yeast strains in this work, by BLASTN analysis, and respective sequences. The sequencing was performed by STABVIDA, Lda., and the results were subjected to analysis with BLASTN tool, being compared with the sequeces present in NCBI database.

Species and Strains	Sequences
Pichia kudriavzevii IST381	GTTTCTACTGATTTGAGGTCGAGCTTTTTGTTGTCTCGCAACACTCGCTCTCGGCCGCCAAGCGTCCCTGAAAAAA AGTCTAGTTCGCTCGGCCAGCTTCGCTCCCTTTCAGGCGAGTCGCAGCTCCGACGCTCTTTACACGTCGTCCGCT CCGCTCCCCCAACTCTGCGCACGCGCAAGATGGAAACGACGCTCAAACAGGCATGCCCCCCGGAATGCCGAGGG GCGCAATGTGCGTTCAAGAACTCGATGATTCACGATGGCTGCAATTCACACTAGGTATCGCATTTCGCTGCGCTCTT CATCGATGCGAGAACCAAGAGATCCGTTGTTGAAAGTTTTGTTTGTTTTTCGTAGATTTCTCTTGTCGACTATATGCTA TATTCCACATTTTAGGTGTTGTTGTTTTCGTTCCGCTCACGCAGTGTAGTACTAAATCACAGTAATGATCCTTCCGCA GGTTCACCTACGGAAGA
Metschnikowia pulcherrima IST383	GGAGTCTACGAGGGTGAGGAAAGACTGGGGCTAAAACTTATTCTAGCGCCGTTGATATTAGGCCGAAGCAGGACCA AACCGGAGGTTTGAGAGTAAATATCGCTCACCCACGCATGCCCTGGGGAATACCCCGGGGCGCAATGTGCGTTCA AAGATTCAATGATTCACGTCTGCAAGTCATATTACGTATCGCAATTCGCTGCGTTCTTCATCGATGCGAGAACCAAGA GATCCGTTGTTGAAAGTTTTTTAATTGTGTATTTGAAGAATAAGATGGAGAGTGTGTGCCTAAAAGGGTGTAAAAATG ATTATAATGAGCCTTCCGCACGTGCACCTACGGAGAGGTCCAATTTTAACGCTCACAACCACCTTTAATCACAAAAGT TTAGGTTTAACTT
Clavispora lusitaniae IST388	GGTCCTAAATCGCAGGCCTCGAAAGGGATGGAGGCGTCAACACGAGCTATAACACGCGCGCCCGAAGGTGCGCG CCACATTCTCGAGTTCTTGTTCCTCCCCCCTTTTCGACGCTGGCCCGGTAAAACCGTGTCTGCTTGCAAGCCCTTC CCTTTCAACAATTTCACGTGCTGTTTCACTCTCTTTTCAAAGTGCTTTTCATCTTTCCATCACTGTACTTGTTCGCTAT CGGTCTCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCCACTTAGAGCTGCATTCCCAAACAACTCGACTCGTC GGAGCCGCGGTGCAAAGAGTCGGCGTGCGCCATACGGGGCTCTCACCCTCCCAGGCGCCATGTTCCAATGGACT TGGGCGCGGCCGACTCAGACCACGAAACCTTCAAATTACAATTCCCGCAGGATTTCAAATTTGAGCTTTTGCCGCTT CACTCGCCGTTACTGGGGCAATCCCTGTTGGTTTCTTTTCCTCCGCTTATTTGATATGCAACG
Rhodotorula mucilaginosa IST390	CCCCGTCCTAAATGGGACGAGGTCCAAACCCCCGCCAAAAGGCACACTGCGTTCCTCAGTCCCCCAAGATGTATC CAGCAAAGAGCTATAACACAGCCGAAACTGCTACCTTCTCTCTACCATTATCCATCCCGGAAAACTGATGCTGGCCT GCAAACCGATTGCTCGGCAAGCAAGTCTGACTTCAAGCGTTTCCCTTCCAACAATTTCACGTACTGTTAACTCTCTT TCCAAAGTGCTTTTCATCTTTCCCTCACGGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATT TACCACCCAATTTGAGCTGCATTCCCAAACAACTCGACTCTTCGAAAATGTATCACAAAGCGCTGGGCGTCCGCACC ATATACGGGGGTCTCACCACTATGCCGCTGTATTCCAACAGACTTGTGTGCGGTCCAACGCGGAAAACATTTCTAGA GATTACAACTCGGACACCGAAGGTGCCAGATTATAAATTTGAGCTCTTCCCGCTTCGCTCGCCGCTACTAGGGGAAT CCTTGTTAGTTTCTTTTCCTCCGCTTATTGATATGCAACT
Hanseniaspora opuntiae IST408	ATGACCGGAGCGCAGGTCCTCAATCCCAGCTAGCAGTATTCCAATAAGCTATAACACTACCGAGGTAGCTACATTCTT AATGATTTATCCTACTGCCAGAATTGATGTTGGCCCAGTGAAATTTTTGAGAGGCCCAAGCCCACGAGAGGCGAGT GCATGCAAAAAACACCATGTCTGATCAAATGCCCTTCCCTTTCAACAATTTCACGTACTTTTTCACTCTCTTTTCAAAG TTCTTTTCATCTTTCCATCACTGTACTTGTTCGCTATCGGTCTCTCGCCAATATTTAGCTTTAGATGGAATTTACCACCC ACTTTGAGCTGCATTCCCAAACAACTCGACTCTTCGAAAAAGTCTTACAGAGAAAAGGTATCCTCGCCAAACGGGAT TCTCACCCTCTATGACGTCCTGTTCCAAGGAACATAGACAAGGACCTAATCAAAGACAAATTCTACAAATTACAACTC GGGCACTGAAAGTACCAGATTTCAAATTTGAGCTTTTACCGCTTCACTCGCCGTTACTAAGGTAATCCCAGTTGGTTT CTTTTCCTCCGCTTATTGATATGCAAGC

Table S2 – Yeasts strains subjected to the initial screening in single sugar media and SBP hydrolysate H6. Strains chosen were isolated from several biological origin and identified in our work and in other studies from YEASTPEC project, or from IST collection. *S. cerevisiae* industrial and engineered strains were also tested.

Source	Strains and Species	Origin of isolation
Type strains	*Saccharomyces cerevisiae* Ethanol Red	-
	Saccharomyces cerevisiae H4531	-
	Saccharomyces cerevisiae T18	-
	Saccharomyces cerevisiae T18:Haa1	-
IST collection	*Meyerozyma guilliermondii* IST335	Olives water AZTB1
	Meyerozyma guilliermondii IST369	Beer barrel 5 (B5)
	Pichia anomala IST317	Uva Aragonês, Mafra + moscas, Azambuja Cervejeira
	Pichia anomala IST358	Beer must 1 MC1Y2
	Candida intermedia IST385	White wine, Sardoal
Isolated in YEASTPEC project	*Kluyveromyces marxianus* IST382	Hydrated SBP + peptone
	Kluyveromyces marxianus IST389	Hydrated SBP + peptone
	Rhodotorula mucilaginosa IST392	Hydrated SBP + peptone
	Pichia kudriavzevii IST405	Wine must, Fundão
	Hanseniaspora opuntiae IST406	Wine must, Fundão
	Pichia kudriavzevii IST381	Hydrated SBP + peptone
	Metschnikowia pulcherrima IST383	Macerated cherries, Fundão
	Clavispora lusitaniae IST388	Hydrated SBP + peptone
	Rhodotorula mucilaginosa IST390	Hydrated SBP + peptone
	Hanseniaspora opuntiae IST408	Washed mature white grapes, Estremoz