Selection of promising non-conventional yeast strains for the production of added-value bioproducts from forest and agro-industrial residues

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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 September 2020 to October 2021, under the supervision of Prof. Dr. Isabel Maria de Sá Correia Leite de Almeida and Dr. Margarida Isabel Rosa Bento Palma.

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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I'd like to express my heartfelt appreciation and gratitude in this section and in the following paragraphs because all of the learning and work that I accomplished through my master's thesis would not have been possible without the people who taught me, guided me, and shared this journey with me. This segment is dedicated to them.

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Abstract:

Energy from fossil fuels is not sustainable due to environmental impact and limited supply. Consequently, there is a critical need for sustainable alternative energy sources. Biodiesel production based on oleaginous yeasts using agro-forest residues offers an eco-friendly alternative to petroleum-derived fuels and develops a greener circular bioeconomy. A total of 32 strains from the oleaginous species *Rhodotorula babjevae, R. mucilaginosa, R. glutinis, R. toruloides, Meyerozyma caribbica, M. guilliermondii, Debaryomyces hansenii, Cystobasidium slooffia, Candida boidinii, Kodamaea ohmeri, Cryptococcus flavescens, Lipomyces tetrasporus, L. starkeyi*, were tested to evaluate their potential in the referred context. Cells were grown in YNB with a single carbon source (20 g/L) of either D-glucose, D-xylose, L-arabinose, D-raffinose, D-galactose or D-galacturonic acid. *R. mucilaginosa* IST461 showed the highest potential for bioconversion of pectin rich hydrolysates due to the efficient metabolization of D-galacturonic acid and L-arabinose, the major challenging sugars present. Yeast strains were also cultivated in a mixed-substrate medium (YNB with 50 g/L D-glucose, 25 g/L D-xylose, 4.5 g/L acetic acid at pH 5.5), mimicking eucalyptus biomass hydrolysates For the *Lipomyces* genus, lipid accumulation appears promising. To evaluate the effect of acetic acid-supplemented medium at pH 5.0 and 5.5, *L. tetrasporus* IST531 was selected for being a less studied species compared to *L. starkeyi*, and was cultivated in the referred eucalyptus biomass-synthetic medium. Growth was significantly inhibited at pH = 5.0 but lipid accumulation was not affected. This study provides useful information for further studies to implement the exploitation of yeast biodiversity for the valorization of agro-forest-industrial residues.

Keywords: Oleaginous yeasts; Microbial lipids; Biofuels; Added-value bioproducts; Forest and agro-industrial residues; Advanced biorefineries

Resumo:

O uso de combustíveis fósseis não é sustentável devido ao impacto ambiental e fornecimento limitado. Consequentemente, existe uma necessidade de encontrar fontes de energia alternativas sustentáveis. A produção de biodiesel com base em leveduras oleaginosas utilizando resíduos agroflorestais oferece uma alternativa ambientalmente sustentável. Um total de 32 estirpes das espécies: *Rhodotorula babjevae, R. mucilaginosa, R. glutinis, R. toruloides, Meyerozyma caribbica, M. guilliermondii, Debaryomyces hansenii, Cystobasidium slooffia, Candida boidinii, Kodamaea ohmeri, Cryptococcus flavescens, Lipomyces tetrasporus, L. starkeyi*, foram testadas para avaliar o seu potencial no referido contexto. As células foram cultivadas em YNB com uma única fonte de carbono, (20 g/L) de D-glucose, D-xilose, L-arabinose, D-rafinose, D-galactose ou ácido D-galacturónico. *R. mucilaginosa* IST461 monstrou maior potencial de bioconversão de hidrolisados ricos em pectina devido à metabolização eficiente do ácido D-galacturónico e L-arabinose, os principais açúcares desafiantes presentes. As estirpes foram também cultivadas em YNB com 50 g/L D-glucose, 25 g/L D-xilose e 4.5 g/L ácido acético a pH 5.5, numa composição semelhante a hidrolisado de biomassa de eucalipto. Para o género *Lipomyces*, a acumulação de lípidos parece promissora. Foi avaliado o efeito do pH 5.0 e 5.5 do meio suplementado com ácido acético tendo *L tetrasporus* IST531, sido seleccionada por esta espécie ser menos estudada que *L. starkeyi*. O crescimento foi significativamente inibido a pH = 5.0, mas a acumulação de lípidos não foi afectada. Este estudo apresenta informação importante para estudos posteriores com vista a implementar a exploração da biodiversidade de leveduras na valorização de resíduos agro-florestais e industriais.

Palavras chave: Leveduras oleaginosas; Lípidos microbianos; Biocombustíveis; Bioprodutos de valor acrescentado; Resíduos florestais e agro-industriais; Biorrefinarias avançadas

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List of Abbreviations:

3PG - 3-phosphoglycerate

3PGDH - 3-phosphoglycerate dehydrogenase

Acetyl-CoA - Acetylcoenzyme A

ACL - ATP citrate lyase

Adh - Alcohol dehydrogenase

AMP - Adenosine monophosphate

AR - Arabinose reductase

ATP - Adenosine triphosphate

C. boidinii **-** *Candida boidinii*

C/N ratio - Carbon/Nitrogen ratio

CCR - Carbon catabolite repression

cdw - cell dry weight

Cr. flavescens - *Cryptococcus flavescens*

C-sources - Carbon sources

CW - Cheese whey

Cy. slooffiae - *Cystobasidium slooffiae*

D. hansenii - *Debaryomyces hansenii*

DAG - Diacylglycerol

D-GalA - D-galacturonic acid

FAME - Fatty acid methyl esters

FAS - Fatty acid synthase

FBP - Fructose-1,6-bisphosphate

FFA - Free fatty acid

GSH - Glutathione

GTP - Guanosine triphosphate

HG - Homogalacturonan,

HPLC - High Performance Liquid Chromatography

ICDH - Isocitrate dehydrogenase

IST – Instituto Superior Técnico

K. ohmeri - *Kodamaea ohmeri*

L. starkeyi - *Lipomyces starkeyi*

L. tetrasporus - *Lipomyces tetrasporus*

LAD - L-arabitol dehydrogenase

LPA - Lysophosphatidic acid

M. caribbica - *Meyerozyma caribbica*

M. guillermondii - *Meyerozyma guillermondii*

M. guilliermondii - *Meyerozyma guilliermondii*

NAD^+ - Nicotinamide adenine dinucleotide (oxidised form)

NADH - Nicotinamide adenine dinucleotide (reduced form)

NADPH - Nicotinamide adenine dinucleotide phosphate

NCY - Non-Conventional Yeast

nm - nanometer

OAA - Oxaloacetate

OD_{600nm} - Optical density at 600nm

PA - Phosphatidic acid

PAL - Phenylalanine ammonia lyase

PBS - Potassium phosphate buffer

Pdc - Pyruvate decarboxylase

Pdh - Pyruvate dehydrogenase

PEP - Phosphoenolpyruvate

pKa – $-\log(K_a)$, K_a being the acid dissociation constant

PPP - Pentose phosphate pathway

R. babjevae - *Rhodotorula babjevae*

R. glutinis - *Rhodotorula glutinis*

R. mucilaginosa - *Rhodotorula mucilaginosa*

R. toruloides - *Rhodotorula toruloides*

RFU - Relative Flurescence Unit

RG-I - Rhamnogalacturonan I

RG-II - Rhamnogalacturonan II

rpm – rotations per minute

S. cerevisiae - *Saccharomyces cerevisiae*

SBP – Sugar Beet Pulp

SCO - Single cell oil

Sp. – Specie

TAG - Triacylglycerides

TCA cycle - Tricarboxylic acid cycle

TGL - Triglyceride lipase

THF - Tetrahydrofolate

UDP-glucose - Uridine diphosphate glucose

UV - Ultraviolet

XDH - Xylitol dehydrogenase

XGA - Xylogalacturonan

XI - Xylose isomerase

XR - Xylose reductase

XR-XDH - Xylose reductase-xylitol dehydrogenase

YNB - Yeast Nitrogen Base

YPD - Yeast Extract Peptone Dextrose

1. Thesis main objectives and outline

This thesis project was, in part, performed in the framework of the ERANET-IB project *YEASTPEC* (Engineering of the yeast *Saccharomyces cerevisiae* for bioconversion of pectin-containing agro-industrial side-streams) (ERA-IB-2/0003/2015). The main focus of the project was to genetically engineer *Saccharomyces cerevisiae* to catabolize the sugars present in hydrolysates from pectin rich agro-industrial residues and to increase yeast tolerance to multiple stress factors occurring during the bioconversion of those residues into value-added products. In this work, the approach was to select and explore promising non-conventional yeast species for their valorization.

Climatic changes and the global energy demands are increasing year after year and demand realistic and sustainable alternatives to petrochemicals. The synthesis of lipids similar to palm oil lipids from oleaginous yeasts provides a realistic and sustainable alternative to fossil liquid fuels and biodiesel produced from vegetable oils. Due to the continued production of organic residues from agro-forest industrial operations, there is a need for novel approaches to treat and valorize those resources in an environmentally responsible manner in the context of a circular bio-economy (1,2).

Oleaginous yeasts have the capacity to accumulate more than 20% of their cell mass in lipids as reserve storage material, with some strains being capable under optimized conditions to accumulate up to 80% of cellular biomass (3). Some of these non-conventional yeast strains also have a high tolerance to stress factors and fermentation inhibitors and the ability to use complex substrates such as agro-forest residues hydrolysates as nutrients which are desirable characteristics for its industrial implementation (4). However, significant variations in lipid synthesis and accumulation capacity are found within the same oleaginous species, indicating that oleaginous potential should be evaluated at a strain level rather than at the species level, alone (5). This intraspecific diversity is caused by a variety of natural factors such as local adaptation, artificial selection, parental conditions, and phenotypic plasticity, affecting not just carbohydrate catabolization but also lipid synthesis (6). Hence it is important to assess the oleaginous potential of different species as well as different strains within the same species.

Based on the scientific literature, the following species were selected from the IST yeast culture collection for further studies due to their reported oleaginous phenotype: *Rhodotorula babjevae* (7), *Rhodotorula mucilaginousa* (8)*, Rhodotorula toruloides* (9), *Rhodotorula glutinis* (10)*, Meyerozyma caribbica* (11), *Meyerozyma guilliermondii* (12)*, Debaryomyces hansenii* (13) and *Cystobasidium slooffiae* (14,15).

To determine the capacity to grow and efficiently catabolise different relevant C-sources in the context of biomass feedstocks, yeast cells of the before mentioned species, were cultivated in Yeast Nitrogen Base (YNB) supplemented with the single carbon source (20 g/L) of either D-glucose, D-xylose, L-arabinose, D-raffinose, D-galactose or D-galacturonic acid. These C-sources were selected because agro-industrial feedstocks are a complex matrix that can be classified in three categories: lignocellulosic substrates (such as wood, straw and grasses), starch feedstocks (such as wheat, corn, rice, or potatoes), and sugar materials (such as sugar beet, sugarcane, or fruits). Each feedstock has a specific carbohydrates composition (16) and the yeast strains able to achieve high biomass concentrations by metabolizing efficiently the sugars examined, they can potentially be used to bioconvert these residues hydrolysates to produce lipids and other industrially interesting metabolites and bioproducts.

Furthermore, mixed-substrate media (composed of YNB supplemented with 50 g/L of D-glucose, 25 g/L of D-xylose and 4.5 g/L of acetic acid, at pH 5.5), mimicking eucalyptus biomass hydrolysates, an abundant lignocellulosic feedstock available in Portugal (17), and also the same media, at pH 5.5, without acetic acid, to serve as control condition to assess acetic acid effect on yeast cells, was used to evaluate the ability of yeasts strains to metabolize various carbon sources when present simultaneously, the production of added-value metabolites and their biolipid accumulation potential. Based on the results obtained in the first experiment as well as on the scientific literature, the following species were selected for this assay: *Candida boidinii* (18), *Kodamaea ohmeri* (19), *Cryptococcus flavescens* (20), *Cystobasidium slooffiae* (14,15), *Lipomyces tetrasporus* (21), *Lipomyces starkeyi* (22), *Meyerozyma guillermondii* (12).

Additionally, to evaluate the inhibitory effect of pH variation, *L. tetrasporus* IST531, selected due to its high lipid accumulation in previous experiment, was grown in culture media mimicking eucalyptus leaves hydrolysates (YNB supplemented with 50 g/L of D-glucose, 25 g/L of D-xylose and 4.5g /L of acetic acid), at pH=5.0 and 5.5, and also in the same medium without acetic acid, to act as a control condition for the presence of acetic acid.

The non-conventional yeasts strains investigated in this thesis work provided interesting and useful results that highlight the potential of non-conventional yeasts as suitable cell factories. Some strains were found to have the potential to be further investigated in the future by envisaging the optimization of growth conditions leading to higher lipid production, quantification and subsequent characterization of the lipids produced by these strains, as well as providing essential genetic information to, in future assays, engineer *S. cerevisiae* strains to develop superior strains for the bioconversion of agro-forest residues into value-added products.

2. Introduction

2.1. Agro-forest-industrial residues as feedstocks for the production of added value bioproducts

Agro-industrial activities have the potential to be one of the main sectors of a country's economy, especially those who have high productions yields in the agricultural sectors (23). Agricultural wastes are originated from farm animals, agricultural production and product processing, and industrial wastes are the materials that are discarded in the industrial production processes (24). Therefore, wastes from the agro-industrial sector can be referred as all the by-products and waste produced in forestry, agro-industries, crops and livestock. The origin of the agro-industrial wastes determines their classification in the following categories: monosaccharides and disaccharides, starch, structural polysaccharides and sources rich in proteins or lipids (25).

The increase in the amount of agro-industrial wastes became a serious problem for the agro-industry since the majority of agro-industrial wastes are left untreated and most of them are discarded through inadequate landfill or incineration (26). These discarded residues are rich in several nutrients according to their origin, for example: fiber, minerals, carbohydrates, and proteins (27,28). However, these agro-industrial residues can hold several pollutants, ranging from inorganic products such as heavy metals and organic such as polyphenolic constituents, making these residues seemingly unsuitable for future use (29). Agro-wastes are also attractive alternative sources of renewable energy since they do not compete with food sources (30). The use these wastes limit environmental pollution and other associated problems, encouraging economic benefits, such as the conversion of wastes to added value bioproducts (e.g. biofuel, enzyme production, among other applications) (31).

Residues from the agricultural industry are currently the focus of research and development activities around the world; they are raw materials for the biotechnology industry, as renewable sources of carbon, nitrogen and other nutrients for microbial growth and the production of metabolites (32,33). The use of organic residues as substrates for the production of value-added products is an environmentally friendly strategy through the conservation and reuse of resources. The implementation of a circular bioeconomy based on microorganisms, in particular non-conventional (non-*Saccharomyces*) yeast strains with metabolic diversity and tolerance to bioprocess-related stresses, is an important societal challenge (4,34). Agro-industrial residues are obtained from raw materials rich in simple sugars (such as fruit and vegetables, sugar beet, sugar cane), starch (such as rice, corn, wheat and potatoes), and lignocellulose (such as straw, wood or grass) (16). Agricultural residues rich in pectin and residues from the food industry are also potential raw materials for the production of biofuels and other bio-related products (35). Currently, many of the pectin-rich residues (like sugar citrus peel and beet pulp) are dried for later use as fodder or dumped in landfills for soil improvement, although it is desirable to find new ways to convert these residues into renewable chemicals using natural microbes or engineering (36).

Residues rich in pectin (apple cut, citrus peel, beet pulp, etc.) accumulate in large concentrations worldwide from the sugar industry and the process of the vegetable and fruit industry (37). This residue is partially processed in the production of sugar (from sugar beets) and juice (from fruits) and has a low lignin content (38). Because of their different geographical distribution and seasonal diversity, pectin-rich agro-industrial substrates are available cheaply despite the difficulties associated with the diversity of these raw materials (39). There are several uses for pectin-rich waste and some uses of apple pulp, citrus waste and beet pulp include: vinegar, apple wax, aromatics, aromas, oxalic acid, xyloglucan, activated carbon, antioxidants, heteropolysaccharides, and furfural (40–42). However, it is an interesting intermediate product in microbial fermentation and can economically increase the enzymatic hydrolysis of polysaccharide components to fermentable neutral sugars (pentoses and hexoses) and D-galacturonic acid (D-GalA) (43). Chemically, pectin is considered a family of covalently linked D-galacturonic acid-rich polysaccharides in which D-galacturonic acid comprises around 70% of pectin and all the pectic polysaccharides contain D-galacturonic acid linked at the O-1 and the O-4 position. Pectin also contains side chains are composed by homogalacturonan (HG), rhamnogalacturonan I (RG-I), and also the substituted galacturonans rhamnogalacturonan II (RG-II), and xylogalacturonan (XGA) (44).

The biochemical conversion of residues from the agricultural industry goes through several steps: pretreatment, hydrolysis, fermentation or saccharification and separation and recovery of the desired products (45). The process of saccharification can be carried out by acidic or enzymatic hydrolysis. Although acid saccharification is quick and easy, the enzymatic process offers advantages in terms of high selectivity and conversion yields to sugar monomers, reduced glycolysis and release of inhibitors, and is more environmentally friendly (38,46). An important aspect is the fact that pretreatment and hydrolysis methods and their conditions have a significant influence on the final chemical composition of the hydrolysate. These problems limit the repeatability of the biochemical conversion of agro-forest residues and the availability of carbohydrates (38,46,47). After hydrolysis, carbon sources are available that can be converted by oleaginous yeasts into value-added products. Unfortunately, hydrolysates of lignocellulosic biomass even if rich in several carbohydrates, also contain several toxic compounds that are responsible for reducing yeast metabolism and fermentation capacity. Compounds such as weak acids (such as acetic acid, methanol and ethanol), furan derivatives, and phenolic compounds, are produced during the pretreatment and hydrolysis of pectin and lignocellulosic-rich materials (46). Heavy metals and pesticides are other potential inhibitors present in pectic substances. The toxicity of these compounds may be relatively low, but the combined action of their synergistic effects allows higher toxicity values to be achieved (48).

2.2. Biofuels: The green alternative for petro derived diesel

The use of fossil fuels is largely responsible for the emission of carbon dioxide into the atmosphere, increasing the greenhouse effect and contributing to global warming. In addition, since the time it takes to produce fossil carbon sources is much higher than their consumption rate, their use is unsustainable (49). Biodiesel is the most sustainable and renewable substitute for fossil diesel fuel among biofuels that are focused on biomass and can be obtained from vegetable oils and animal fats (biolipids using transesterification) (50). Biodiesel is also an alternative to fossil derived diesel and it is usually formed chemically by transesterification, where triacylglycerides (TAG), regardless of their origin, interact with short-chain alcohols (generally ethanol/methanol) to form alkyl esters (methyl/ethyl esters) (51). The use of biodiesel as an energy source has many advantages, such as: its availability, better combustion efficiency, no aromatics, higher octane number and biodegradability. Biodiesel is produced worldwide using oils ranging from vegetable oils to animal fats or waste oils. In order to be able to compete with fossil fuels, however, biodiesel must be produced in reasonable concentrations in order to cover part of the energy demand and to achieve a positive return on the energy used to produce it (52). The lack of oil feedstocks limits the large-scale development of biodiesel to some extent and the development of microbial oils is currently being on the focus of intensive research. Many microorganisms, such as algae, yeast, bacteria, and fungi, have the ability to accumulate oils under some special cultivation conditions. Compared to other plant oils, microbial oils have many advantages, such as short life cycle, less labor required, less affection by venue, season and climate, and easier to scale up (53). For all these reasons, microbial oils might become one of potential oil feedstocks for biodiesel production in the future.

2.3. Oleaginous Yeasts

Only a few microorganisms can accumulate more than 20% of their cell mass in lipids as reserve storage material. These microorganisms include some fungi, yeasts, and algae being called oleaginous species and the microbial oils accumulated can reach levels of up to 80% of cellular biomass (3).

Saccharomyces cerevisiae is widely used as a eukaryotic model organism to study cell cycle metabolism and stress response, among other things, and is one of the most important microbial species in human history (54). It is the best understood genetic model organism as it was the first eukaryote to have its entire genome sequenced, and still is the most annotated genome and most compatible organism with genetic engineering and post-genomic approaches (55). Despite its many interesting properties, such as the expression of valuable technically engineered proteins, its main differentiation lies in the ability to produce alcohol from sugar even when oxygen is accessible for aerobic respiration. This key function is known as the Crabtree effect. Although *S. cerevisiae* is the most widely used yeast for biotechnological applications, it still faces some challenges in modern industrial settings, including varying environmental pressures and tolerances to cytotoxic inhibitors, as well as the use of mixed sugars as the result of catabolic repression regulation and its inability to catabolise a wide range of sugars. *S. cerevisiae* can ferment hexoses, like D-glucose, but cannot metabolize most pentose sugars (56–58).

Apart from *S. cerevisiae* species, about 1500 yeast species have been identified, and are collectively referred to as non-conventional yeast (NCY). These yeasts represent a tremendous resource of yeast biodiversity that are yet to be explored. Many of these non-conventional yeast species have industrially relevant characteristics that are specific mechanisms for survival in harsh environmental conditions (2,59). Non-conventional yeasts have a high tolerance to stress and fermentation inhibitors. In addition, its ability to use complex substrates as nutrients provides alternative metabolic pathways for substrate utilization and product synthesis. These beneficial and sometimes unusual metabolic traits can be of interest to biotechnology. For example, they can improve the profitability of bioethanol production from agricultural industrial residues given that *S. cerevisiae* natural strains cannot use all the sugars present in this complex matrix. Therefore, research on non-conventional yeast strains with a wider range of assimilable C-sources and higher resistance to cytotoxic compounds is currently very active (4). To date, most of the non-conventional yeasts in industry that have been examined were considered as spoiling yeasts due to recurrent isolation from contaminated foods and beverages. They are usually isolated from high-sugar environments and are specifically linked to the spoilage of acidic foods such as wine, colas, fruit juices and cider (60).

Microbial lipids, also called single cell oils (SCO), are very similar to vegetable oils. Hence, they have many uses in the biotechnology industry. These include diglycerols, triglycerides, free fatty acids, carotenoids, sterols, phospholipids, polyprinol, sphingolipids and glycolipids, which are available in different concentrations in the cytosol (61). The main forms of SCO's are sterol esters and triglycerides, which accumulate in the lipid bodies of yeast cells. In addition, the most common fatty acids in most oleaginous yeasts are linoleic acid (18:2ω6), oleic acid (18:1ω9), stearic acid (18:0) and palmitic acid (16:0) and the minor ones include lignophosphate (24:0), behenic acid (22: 0), arachidic acid (20: 0), α-linolenic acid (18:3ω3), palmitic acid (16:1ω7) and myristic acid (14:0) (59). Despite other lipid-storing microorganisms, oleaginous yeasts are the most promising in biodiesel production, not only because they can accumulate triglycerides, but also because they have a broader metabolism of feed sources and tolerance to larger pH ranges, inhibitors, and ionic strengths. In addition, oleaginous yeasts are usually non-pathogenic, most are suitable for large fermentation processes and for some species genetic manipulation is already possible (62).

Oleaginous yeasts synthesize lipids, mainly in the form of triglycerides (TAGs). When there is an excess of carbon and when essential nutrients (usually nitrogen) in the environment have been depleted, yeast diverts the flow of carbon from energy production to lipid synthesis (TAG) (3). Nitrogen deficiency reduces intracellular levels of adenosine monophosphate (AMP) and thus inhibits isocitrate dehydrogenase (ICDH), which leads to citrate accumulation in mitochondria, which is a unique property of oleaginous yeasts. Citrate is transported into the cytoplasm where it is cleaved by ATP citrate lyase (ACL), a key enzyme for lipogenesis in all oleaginous yeasts. Acetyl-CoA is then directed to the synthesis of new fatty acids in the form of a fatty acid synthase (FAS) complex (63). The products of the FAS complex are palmitoyl-CoA and stearoyl-CoA, which are transported to the endoplasmic reticulum, where they are used for TAG production or for NADPH-dependent desaturation and/or two-carbon prior to TAG synthesis. TAG synthesis follows the Kennedy pathway leading to the production of lysophosphatidic acid (LPA), phosphatidic acid (PA), diacylglycerol (DAG) and finally TAG, which is stored in lipid droplets (64). A schematic diagram of lipogenesis in the oleaginous yeast *Lipomyces starkeyi* is represented in **Figure 1**.

Free fatty acid (FFA) can be released from TAG through the action of intracellular lipase (TGL) and further activated by acyl-CoA synthetases (FAA1) to produce acyl-CoA. Under starvation conditions, both acyl-CoA and FFA can enter the peroxisome, where it is broken down via the beta-oxidation pathway. NADPH is required for lipid synthesis in oleaginous microorganisms by a variety of sources, including NADPH-dependent isocitrate dehydrogenase, oxidized pentose phosphate pathway (PPP), or malate enzyme (ME), which appears to be the main source of NADPH for many oleaginous yeasts (3).

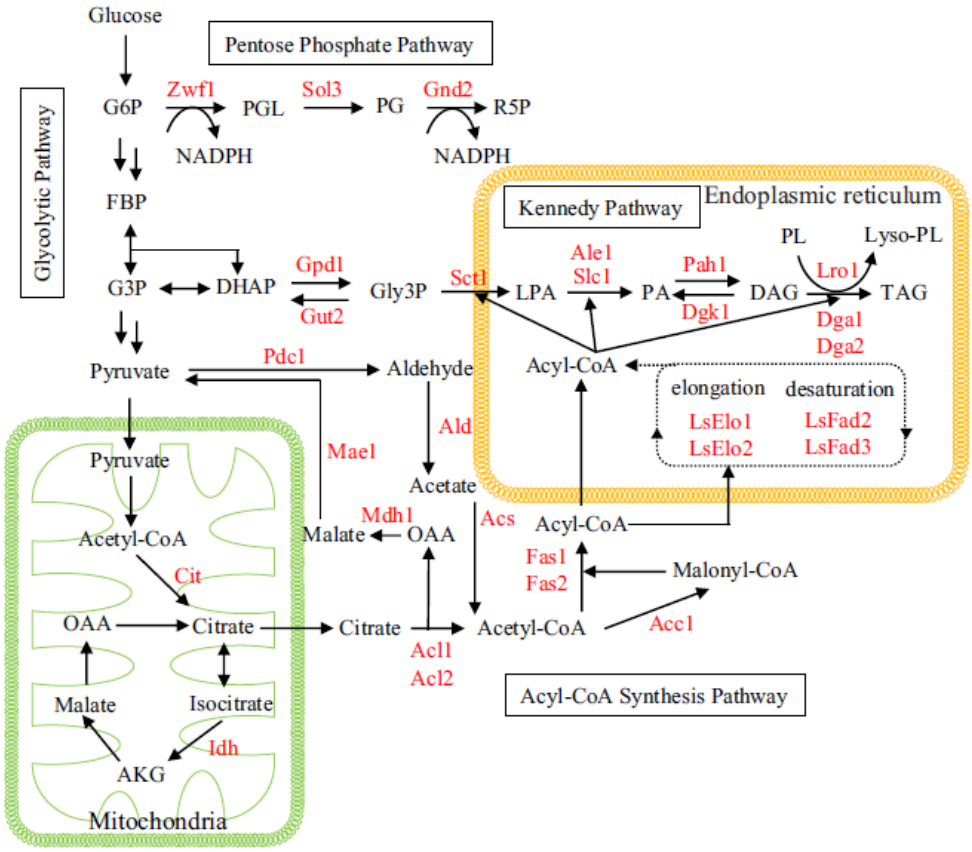

Figure 1 - **Lipogenesis in the oleaginous yeast *Lipomyces starkey*i, with an emphasis on acyl-CoA and TAG synthesis**. Figure from Takaku et al. (2020) (65).

The growth of oleaginous yeasts and the fatty acid composition of lipids are strain-specific, depends on their stage of growth and on the type and concentration of carbon and nitrogen sources, C/N ratio, temperature, pH, aeration rate and growth inhibitors. The incubation time is also an important factor because the lipid concentrations are higher during the stationary phase compared with exponential growth phase (66).

The ratio of C/N needed to induce lipid production depends on the yeast, composition of the culture medium, and carbon and nitrogen sources. However, reducing one or more nutrients when there is an excess of carbon in the medium can result in the production of triglycerides in yeasts (67). The pH also has a strong influence on lipid yield and biomass. Despite the fact that yeasts can accumulate lipids from basic to acidic pH levels and lower pH levels are considered less harmful, the appropriate pH range for lipid production is between 5 and 6.5, this is also because it is essential to decrease potential for bacterial contamination (68). In addition, the aeration rate is an important factor in the synthesis of triacylglycerols, even though it also varies with the species of yeast used. Temperature is also an important parameter in lipid production. While different species have different optimal temperatures, triglyceride production occurs at temperatures between 25°C and 30°C, and extreme temperatures can affect lipid yield (66). In addition, choosing a carbon source that allows growth and lipid accumulation is crucial to the success of the bioprocess. Hence, there are a number of different carbon sources that have been reported to enable the growth of oleaginous yeasts and the synthesis of triglycerides from sugars to organic acids, lipids, and alcohols (69).

2.4. Carbohydrate metabolism

Adenosine triphosphate (ATP) is a complex organic molecule that is required for several cellular metabolic activities, including motility, biosynthesis, growth, and transport. Yeast may produce ATP via two distinct mechanisms: respiration and fermentation. Fermentation does not require oxygen but the output of ATP produced is lower when compared to respiration (70).

In general, non-conventional yeasts prefer D-glucose and D-fructose over alternative carbon sources since these may enter directly the glycolytic pathway. When D-glucose is unavailable, alternative carbon sources can be used depending on natural metabolic activities of the different yeast species. These alternative C-sources include D-xylose, L-arabinose, maltose, ethanol, glycerol, and acetic acid which may be utilized to generate metabolic energy and cellular biomass. In order to adapt to different oxygen conditions and fermentable carbohydrates, yeast cells may rapidly switch metabolism between respiration and fermentation (71). A diauxic shift occurs when the preferred carbon source is depleted, followed by a considerable drop in growth, resulting in adaptation to the usage of an alternative carbon source. There is extensive reprogramming of gene expression in several signaling pathways such as protein synthesis, carbon metabolism, and carbohydrates storage during this transition (72).

2.4.1. D-Glucose

Glycolysis, a metabolic process with variations in most species, converts D-glucose to pyruvate. The conversion of D-glucose to fructose-1,6-bisphosphate (FBP) requires the consumption of two molecules of ATP in the first reactions of glycolysis, and the subsequent reaction results in the formation of two 3-carbon sugars, which are then broken down into pyruvate, yielding a total of four ATP and two NADH (2 ATP and 1 NADH for every 3 carbon sugar). Pyruvate can be broken down further by respiration or the fermentation process (70).

Pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (Adh) are responsible for turning pyruvate into ethanol during alcoholic fermentation, with a net gain of 2 ATP molecules per D-glucose. Adh performs the re-oxidation of NADH, which is produced during lower glycolysis, to NAD+, which offers an oxygen-independent means of generating ATP (73). Pyruvate undergoes oxidative decarboxylation in the respiratory system, mediated by pyruvate dehydrogenase (Pdh), and is transformed to acetyl coenzyme A (Acetyl-CoA), which is subsequently oxidized to carbon dioxide in the tricarboxylic acid (TCA) cycle. As a result, guanosine triphosphate (GTP), NADH, and other reduced coenzymes are formed, which are then oxidized to ATP in the mitochondria. The resultant respiratory output for *S. cerevisiae* is around 18 ATP per D-glucose (70,74).

Glucose in the medium inhibits the uptake and dissociation of other carbon sources in the yeast *S. cerevisiae* via three signalling pathways: inhibition of AMPK/Snf1, activation of PKA, and regulation of transporter expression and cytoplasmic membrane stability by yeast casein kinases Yck1 and Yck2 (75).

In *S. cerevisiae*, when oxygen is present, D-glucose is responsible for inhibiting gene expression associated to various cellular processes such as respiration, general stress response mechanisms and gluconeogenesis, in addition to suppressing the metabolism of other carbon sources (76). This phenomenon is called the Crabtree effect, with *S. cerevisiae* being considered Crabtree-positive (77). Despite significant research into D-glucose-mediated catabolite repression, it is still unknown whether various carbon sources have comparable priorities and what are the sensing mechanisms of these carbohydrates (78).The impact of Crabtree effect in the cell is perplexing at first look, as the output of ATP per unit of D-glucose is significantly larger during respiration than during fermentation. Fermentation, on the other hand, is thought to allow for quicker ATP generation and hence faster development (79). Furthermore, the buildup of ethanol generated during fermentation might inhibit the development of competing microorganisms (80). The Crabtree-positive species may employ fermentation and respiration simultaneously if there is an appropriate quantity of oxygen and D-glucose. Once the D-glucose is depleted, ethanol produced by fermentation may be recycled in yeast respiratory metabolism to create ATP. However, as compared to direct pyruvate oxidation, this method produces less ATP since the synthesis of Acetyl-CoA from ethanol needs ATP (70,79).

2.4.2. D-Xylose

The catabolic mechanisms of the original D-xylose strains may be divided into oxidoreductase (fungal) and isomerase (bacterial). The D-xylose isomerase (XI) route as well as the D-xylose reductase-xylitol dehydrogenase (XR-XDH) pathway are the two primary metabolic processes for the breakdown of D-xylose. NADH- or NADPH-dependent D-xylose reductase (XR) converts D-xylose to xylitol. NAD- or NADP-dependent xylitol dehydrogenase (XDH) then oxidizes xylitol to D-xylulose, although it can be expelled from the cell. The XR-XDH cofactor's dual affinity for NADPH and NADH produces an unbalance within those two cofactors and is a key bottleneck in this pathway (81,82).

2.4.3. L-Arabinose

L-arabinose is a five-carbon carbohydrate that is a component of pectin and is more common in nature than D-arabinose. Despite extensive study into D-xylose metabolism, little attention has been paid to L-arabinose metabolism. The catabolic mechanisms of the original L-arabinose strains, similar to D-xylose, may be divided into oxidoreductase (fungal) and isomerase (bacterial). In both cases, L-arabinose is transformed to D-xylulose-5-phosphate, which will then be degraded via the non-oxidative pentose phosphate route (83).

L-arabinose reductase (AR) favours NADPH as a cofactor in the fungal route, while sugar alcohol dehydrogenases (such as L-arabitol dehydrogenase (LAD) and XDH) are NAD^+ dependent. Under low oxygen circumstances, the cellular ability to replenish NAD+ is limited, which may result in the synthesis of arabitol. Furthermore, L-arabinose can be converted to xylitol, the common thread of the L-arabinose and D-xylose degradation pathways. There is a significant link between the usage of these pentoses in yeasts due to their largely overlapping routes (83,84).

2.4.4. D-Raffinose

Raffinose is a trisaccharide consisting of D-galactose, D-glucose, and fructose linked together by both α- and β-glycosidic bonds. Raffinose is progressively degraded extracellularly by invertase (SUC2) to monosaccharides, which can enter the cell through SNF3 and other hexose transporters, and these monosaccharides eventually enter glycolysis (85,86).

2.4.5. D-Galactose

Galactose is an aldohexose and a D-glucose C-4 epimer, and the enzymes of the Leloir pathway, which convert D-galactose to D-glucose-6-phosphate for subsequent entrance into glycolysis, start the breakdown of D-galactose in yeast. Galactokinase (Gal1p) phosphorylates D-galactose to D-galactose-1-phosphate in the yeast Leloir pathway. Galactose is switched with a D-glucose moiety from UDP-D-glucose via Gal7p after being converted to D-galactose-1-phosphate, resulting in UDP-D-galactose and D-glucose-1-phosphate. Phosphoglucomutase (Pgm2p or Pgm1p) can then convert the D-glucose-1-phosphate to D-glucose-6-phosphate, a glycolysis substrate. By using Gal10p's epimerase activity, the UDP-D-galactose product, which contains carbon from the original D-galactose substrate, may be transformed to UDP-D-glucose. During a second round of the Leloir pathway, the D-glucose unit of this UDP-D-glucose molecule may be transformed to D-glucose-6-phosphate (87).

2.4.6. D-Galacturonic Acid

There are four stages in the fungal D-galacturonic acid pathway. GAR1, a NADPH-utilizing reductase, transforms D-galacturonic acid to L-galactonic acid in the first step (88). The second enzyme is responsible for removing a water molecule from L-galactonic acid to create 3-Deoxy-L-threo-hex-2-ulosonate, also known as 2-keto-3-deoxy-L-galactonic acid (89).

In the D-galacturonic acid pathway, the 2-keto-3-deoxy-L-galactonate aldolase enzyme breaks down the preceding substrate into pyruvate and L-glyceraldehyde. While pyruvate may be metabolized in a variety of ways, L-glyceraldehyde is not a metabolite of investigated pathway and is converted into glycerol by the pathway's last enzyme, a NADPH-dependent reductase (90).

S. cerevisiae is unable to catabolize D-galacturonic acid (D-GalA), particularly for this uronic acid sugar absorption, necessitating the introduction of a heterologous D-GalA transporter (73). One disadvantage of fermenting D-GalA to synthesize bioethanol is that D-GalA is more oxidized than hexoses such as D-glucose. As a result, NADPH cofactors are needed in greater concentrations than in hexose fermentation (73). D-GalA fermentation would require two more NADPH than D-glucose fermentation and hence would not be redox-neutral. A significant amount of the carbon is redirected to the synthesis of glycerol to compensate for the generation of NADH during anaerobic hexose fermentation (90).

2.4.7. Acetic acid

S. cerevisiae and other species may use sub-lethal concentrations of acetic acid as a carbon and energy source during aerobic development. Acetyl-CoA is an essential intermediate metabolite in yeast and is involved in metabolism as activated form of acetate (by acetyl-CoA synthetase) in the cytosol, mitochondrion, peroxisome, and cell nucleus. Acetyl-CoA is a substrate for the TCA cycle, which generates ATP as well as the major molecules of amino acids, nucleotide bases, and porphyrins; fatty acid synthesis and the end product of fatty acid breakdown; and protein acetylation, which regulates DNA transcription and enzyme function, and finally, the glyoxylate cycle, a variant of the TCA cycle employed for carbohydrate synthesis (91,92).

Acetic acid appears to be a toxic or beneficial compound for lipid accumulation, depending on the concentration of acetic acid present and the yeast strain, and many species that can be grown in high concentrations of acetic acid are oleaginous yeasts because acetate enters the cell and is converted to acetyl-CoA, a lipid biosynthesis precursor (93,94). Carbon catabolite repression in yeast is not limited to D-glucose; acetate also inhibits ethanol use as a C-source, even when other carbohydrates are present (78).

The toxic effect of acetic acid comes from the fact that undissociated weak acids are liposoluble and may distribute over the plasma membrane. The growth-inhibiting effect on microorganisms has been proposed to be due to the inflow of undissociated acid into the cytosol (95). In the cytosol, dissociation of the acid occurs due to the neutral intra cellular pH, therefore decreasing the cytosolic pH (96). According to the uncoupling theory, the decrease in intracellular pH is caused by the inflow of weak acids that are neutralized by the ATPase function of the plasma membrane, which removes protons from the cell at the expense of ATP hydrolysis (96). To maintain intracellular pH, additional ATP must be produced, and in anaerobic conditions this is achieved by increasing ethanol production at the expense of biomass formation. At high acid concentrations, the cell's ability to pump protons is reduced, which results in a decrease in ATP content, loss of proton motive force and acidification of the cytoplasm (97).

2.5. Added-value compounds synthesized by non-conventional yeasts

In the literature, a vast range of products generated from various yeast species have been documented. Antibiotics, enzyme inhibitors, nutraceuticals biofuels, anticancer drugs, enzyme inhibitors, dyes are only a few examples (98).

2.5.1. Bioethanol

On the global market today, ethanol is the most important and widely manufactured biofuel. Bioethanol can be derived from renewable agricultural resources rather than non-renewable petroleum products. It is less harmful than other fuel alcohols and the by-products (acetic acid and acetaldehyde) are also less toxic (99). Most yeast can produce ethanol from D-glucose. The process of intracellular conversion of hexoses (D-glucose and fructose) to pyruvate is known as glycolysis and involves the formation of ATP and NADH. NAD^+ must be recreated from NADH to restore the cell's redox potential. The reducing power of NADH is transmitted to acetaldehyde and regenerates NAD^+ consumed by glycolysis. This process is known as alcoholic fermentation and involves two additional enzymatic reactions after glycolysis: the first being the decarboxylation of pyruvate to acetaldehyde by pyruvate decarboxylase, and the second being the reduction of acetaldehyde to ethanol by the enzyme alcohol dehydrogenase using zinc as a co-factor. The final products of alcoholic fermentation, carbon dioxide and ethanol, diffuse freely out of the cell (100,101).

At sublethal concentrations, ethanol acts as a yeast growth inhibitor (inhibits cell division decreasing maximum specific growth rate and metabolic activity) by affecting protein activity and increasing the fluidity and permeability of the plasma membrane and dissipating transmebrane proton motive force (102). At sublethal concentrations, ethanol reduces cell viability leading to cell death and the presence of other stress factors, such as supraoptimal temperatures, acetic acid, among other factors, increases the deleterious effects of ethanol (103).

2.5.2. Acetic Acid

Acetic acid, also known as ethanoic acid, is a volatile organic acid composed by two carbons that is formed during the alcoholic fermentation. The biochemical pathways involved in acetic acid production in yeasts include: the reversible synthesis of acetyl Co-A and acetyl adenylate by acetyl Co-A synthetase; citrate lyase converting citrate to acetyl Co-A; acetyl Co-A hydrolysis by acetyl hydrolase; reversible formation of acetyl-phosphate by acetyl kinase and finally, aldehyde dehydrogenase oxidizing acetaldehyde (104). Acetate, in addition to being a substrate for acetyl Co-A synthetase, acetate metabolism has a physiological function by lowering NADH and NADPH and preserving the redox equilibrium when aldehyde dehydrogenases oxidize acetaldehyde to acetic acid (105).

2.5.3. Sugar alcohols

Sugar alcohols are polyols that is commonly derived from sugar. They are water-soluble solids that can arise naturally or be synthesized. These polyols are extensively utilized as thickeners and sweeteners in the food sector due to their beneficial qualities. A few examples being: lower calorie content while keeping equivalent or superior sensory characteristics, sugar alcohols have a lower glycemic index, cause less change in blood D-glucose levels than "natural" sugars, and are non-carcinogenic because oral bacteria cannot digest them (81,106).

Xylitol is one of those sugary alcohols, being a crystalline substance that is colorless or white and soluble in water. It is classed as a polyalcohol and a sugar alcohol, and it has a wide range of applications as a sweetener in the creation of diabetic-friendly baking items. It also enhances the shelf life of food by preventing saprophytes like *Salmonella typhi* and *Clostridium butyrican* from growing, as a result, xylitol is found in a variety of consumer goods, including chewing gum, soft beverages, ice cream, and toothpaste (81,107). In yeasts, a xylose reductase (XR) reduces D-xylose to xylitol, which is then oxidized to xylulose by a xylitol dehydrogenase (XDH). Depending on yeast species, these two enzymes require the cofactors NAD^+/NADH or NADP/NADPH. XR is dual-dependent (NADPH and NADH) in some yeasts, with NADPH activity being the greatest and XDH being mostly NAD+ dependent (108). These two stages are regarded as limiting factors since the regeneration of cofactors is extremely dependent on oxygen levels and oxygen transfer rates, and yeasts lack transhydrogenase enzymes (which allow for the oxidation of NADH). The respiratory chain converts NADH to NAD^+ under aerobic circumstances, and the high NAD^+/NADH ratio promotes xylulose production. The electron transport system is unable to fully oxidize intracellular NADH when oxygen levels are low, resulting in a rise in NADH concentration and xylitol excretion from the cell. However, due to a redox imbalance between NAD^+ and NADH, yeasts are unable to metabolize D-xylose in anaerobic circumstances (108,109).

2.5.4. Malate

Malic acid, often referred as the dissociated form, malate, is a four-carbon dicarboxylic acid that is primarily utilized in the beverage and food industries as an acidulant and flavor enhancer (110). For the synthesis of L-malic acid from glucose, there are four metabolic pathways. The first is pyruvate carboxylation to oxaloacetate (*S. cerevisiae* lacks phosphoenolpyruvate carboxylase), followed by oxaloacetate reduction to malate. The second one being the condensation of oxaloacetate and acetyl-coenzyme A to citric acid, procceded by its oxidation to malate via the tricarboxylic acid (TCA) cycle. The third process is the glyoxylate cycle, which produces malate from two molecules of acetyl-CoA. Due to the oxidative decarboxylation process necessary for acetyl-CoA generation from pyruvate in this alternate oxidative pathway for malate formation, the maximal malate yield on glucose is restricted. The fourth mechanism is a noncyclic one that uses glyoxylate cycle enzymes but replanish oxaloacetate with pyruvate carboxylation (111).

2.5.5. Succinate

Succinate or succinic acid is a dicarboxylic acid with a wide range of uses, including as a surfactant, detergent extender, foaming agent, ion chelator, and food additive, as well as a precursor for compounds like tetrahydrofuran and 1,4-butanediol (112). When sugar or glycerol is utilized as a carbon source, succinate can be a fermentation end-product in addition to being an intermediary in the tricarboxylic acid (TCA) cycle. The reductive branch of the TCA cycle, the glyoxylate route, and the oxidative TCA cycle are the three pathways for succinate production. Because succinate is employed as the electron-acceptor instead of oxygen under anaerobic circumstances, the reductive branch of the TCA cycle is utilised. Besides being an intermediate of the tricarboxylic acid (TCA) cycle, succinate can also be a fermentation end-product when sugar or glycerol is used as a carbon source. There are three pathways for succinate formation including the reductive branch of the TCA cycle, the glyoxylate pathway, and the oxidative TCA cycle. Under anaerobic conditions, succinate is the electron-acceptor instead of oxygen, and therefore the reductive branch of the TCA cycle is used. Succinate is formed from phosphoenolpyruvate (PEP) and various TCA reductive branch intermediates, such as oxaloacetate (OAA), malate, and fumarate (113).

Under aerobic conditions, succinate can also be made from acetyl-CoA produced from pyruvate via the oxidative TCA cycle. Acetyl-CoA is metabolized to citrate, isocitrate, and succinate in this pathway, which is then transformed to fumarate by succinate dehydrogenase. Because succinate is merely an intermediary of the TCA cycle, it cannot be produced naturally under aerobic conditions. Inactivation of the SDHA gene, which prevents succinate from being converted to fumarate in the TCA cycle, is required to achieve succinate buildup under aerobic conditions (113).

2.5.6. Formate

Formate, formic acid or methanoic acid, has the chemical formula H_2CO_2 and is one of the simplest carboxylic acid. It's frequently utilized in a variety of industries such as: leather manufacturing, pharmaceuticals, and in chemical labs. Formic acid is a key intracellular metabolite that has developed to fulfill distinct functionalities in various organisms. In yeasts, tetrahydrofolate (THF)-mediated one-carbon (C1) reactions in the mitochondria are the principal source of formic acid (114,115). The branch point enzyme 3-phosphoglycerate dehydrogenase (PGDH), which is encoded by the yeast isozymes SER3 and SER33, catalyzes the first step in this pathway. In conditions where high levels of serine and formic acid are synthesized from the glycolytic intermediate 3-phosphoglycerate (3PG), the NAD-dependent oxidation reaction catalyzed by PGDH is nonfermentative: oxygen, rather than organic substrate, serves as the final electron acceptor to maintain redox homeostasis (115,116).

2.5.7. Carotenoids

Carotenoids are lipid-soluble pigments that have pink, orange, and red hues. They are tetraterpenoids made up of highly unsaturated isoprene derivatives, and have a variety of applications, including: as coloring agents for margarine, soft drinks, and baked goods in the food industry; as a precursor to vitamin A, which is the main source in human and animal feed; in feed ingredients for poultry, fish, and mollusks; as antioxidants in food additives, nutritional supplements, and vitamin preparations; and in cosmetics and pharmaceuticals, such as tinctures as well as antioxidants with photoprotective qualities that help to improve the immune system, accelerate wound healing, battle eye illnesses and age-related wear, decrease aging and cell and tissue damage, as a way of protecting the skin from harmful UV rays (117–119). Carotenoids can be found in a variety of foods, including eggs, fruits, vegetables, fish, and oil. Carotenoids are produced by a variety of plant species, yeast, filamentous fungi, protozoan bacteria, algae, and lichens (120). These compounds protect non-photosynthetic organisms from radiation-induced (strong light) photooxidative stress, therefore yeast makes carotenoids to flourish in both light and atmospheric environments (118,120). Carotenoids are classified into two types: carotenoids, which contain beta-carotene and torulene; and xanthophylls such as astaxanthin, canthaxanthin, and torularhodin (119,120). Because of the increased demand for carotenoids in the global market, interest in their synthesis by microbes has developed, albeit they are still mostly chemically produced (119).

2.6. Exploring non-conventional yeast biodiversity for the production of added-value compounds from agro-forest-residues

Intraspecific variation can be generated by a number of factors in nature, including local adaptation, artificial selection, and phenotypic plasticity (6,121). Local adaptation occurs when a population acquires features that enable greater fitness in its native environment compared to populations from other habitats (122). In response to external conditions, phenotypic plasticity refers to a genotype's ability to create several alternative forms of morphology, physiological state, and/or behaviour (123). Artificial selection is a cultivation method in which a population of organisms is screened for a quantitative characteristic or traits, with the highest-rated individuals serving as parents for the following generation (124).

Intraspecific trait variation can reflect microgeographic adaptation, and even divergent selection when it is caused by evolutionary forces (122). This intraspecific variation highlights the importantce to isolate and examine different strains even if belonging to the same species, since their phenotypic characteristics and industrial potential may differ greatly. For instance, significant differences in lipid accumulation capacity were identified for some species in a study conducted by Miranda et al. (2020), in which it was possible to detect differences in oleaginous potential at the strain level rather than the species level (5). The lipid accumulation capacity, for example, of *Candida tropicalis* isolates varied from 2 to 78 Relative Flurescence Units (RFU), with isolate V311 having the greatest lipid accumulation capacity (RFU = 78) and isolate V286 having the lowest (RFU = 2) (5).

In the present thesis work, a total of 32 strains belonging to 13 different species were examined. The species are summarized in **Table 1** and briefly described thereafter.

Table 1 - Selected yeast strains from IST private collection that are considered oleaginous in the lliterature and promising for further studies in this thesis project context. The species, strain and respective origin of isolation are indicated.

Species	Strain (IST ID Collection)	Origin of isolation
Rhodotorula babjevae or *R. diobovatum*	IST323	Aragonese Grape, Azambuja Beer
Rhodotorula babjevae	IST537	PYCC 5168
	IST549	Walnut outer shell
	IST550	Walnut outer shell
Rhodotorula mucilaginosa	IST390	Hydrated SBP (Pulp+Water)
	IST423	Effluent from lupine production
	IST461	Beer Wort
Rhodotorula glutinis	IST535	PYCC 4177
Rhodotorula toruloides	IST536	PYCC 5615
Meyerozyma caribbica	IST494	Oak soil
	IST541	Arrábida Sea, grown in YPD
	IST542	Arrábida Sea, enriched in D-xylose
	IST601	Fuel contaminated soil
Meyerozyma caribbica or *M. guilliermondii*	IST595	Fuel contaminated soil
Meyerozyma guilliermondii	IST369	Beer Barrel
	IST502	Plum
	IST587	Fuel contaminated soil
	IST591	Fuel contaminated soil
Debaryomyces hansenii	IST334	Beer wort
	IST339	Beer wort
	IST340	Beer wort
	IST375	Chorizo from Alentejo
	IST586	Fuel contaminated soil
Cystobasidium slooffiae	IST544	Walnut outer shell
	IST547	Walnut outer shell
Candida boidinii	IST509	Olive Water
	IST599	Fuel contaminated soil
Kodamaea ohmeri	IST538	PYCC 7192
Cryptococcus flavescens	IST325	Branch and fruit by the vine in Colares
Lipomyces tetrasporus	IST531	PYCC 4033
Lipomyces starkeyi	IST532	PYCC 4045
	IST533	PYCC 4046

Relevant characteristics based on which strains of the different species were selected for a preliminar screening follow. The streak culture of *Rhodotorula mucilaginosa* strains is pink, ranging from saffron-orange to deep coral, sparkling (sometimes dull), mucoid, typically smooth but rarely rugoid, with an entire border. It metabolizes D-glucose, D-xylose, L-arabinose while D-Galactose and D-raffinose is variable. In patients with severe underlying diseases and impaired host defenses, opportunistic fungal infections can emerge as a leading cause of morbidity and death (125). *Rhodotorula glutinis* colonies are red to salmon or slightly orange, smooth to wrinkled, glossy, mucous to butyrous, and smooth to wrinkled, frequently with small transverse striations when grown on agar. The cross-section is flat to wide convex, with a complete border. This species is able to metabolize: D-glucose, D-xylose, L-arabinose and D-Galactose while D-raffinose is variable (125). The biomass of this yeast can provide a source of microbiological oils, with oleic, linoleic, and palmitic acid dominating the fatty acid pool. The lipids may be beneficial as a source for the synthesis of so-called third-generation biodiesel due to their composition. Carotenoids like β-carotene, torulene, and torularhodin can also be synthesized by these yeasts and are the reason for their colonies´ colours. Carotenoids are widely employed in the cosmetic, pharmaceutical, and food sectors due to their health-promoting properties. They're also utilized as ingredients in animal, fish, and crustacean feed. The capacity of *R. glutinis* to synthesize a variety of enzymes, including phenylalanine ammonia lyase (PAL), is one of its most notable features. This enzyme is utilized in the food industry to make L-phenylalanine, which is the starting material for the manufacturing of aspartame, a sweetener widely used in the food industry (126).

The streak culture of *Meyerozyma caribbica* is tannish-white in hue, smooth and glossy. This species ability to metabolize: D-glucose, D-xylose and D-galactose while D-raffinose and D-galactose is variable (127). *Meyerozyma guilliermondii* streak culture is similar to *Meyerozyma caribbica.* It metabolizes D-glucose, D-xylose, L-arabinose and D-galactose while D-rafinnose metabolization is variable. *Meyerozyma guilliermondii* is a microorganism that generates riboflavin and xylitol. Other yeasts now generate both chemicals, but biotechnological advances may offer *M. guilliermondii* an advantage in commercial manufacturing. Riboflavin is a vitamin that is utilized in human nutrition and as a supplement in animal feed all over the world. Xylitol is used as a sweetener that may be used in place of sugar in goods such as chewing gum (127).

The streak colour of *Debaryomyces hansenii* is grayish-white to yellowish and can be sparkling or dull, smooth or wrinkled. This species is able to metabolize: D-glucose, D-xylose, D-raffinose and D-galactose while L-arabinose metabolization is variable (128). Due to its osmo and xerotolerance, as well as its halotolerance, *D. hansenii* is an extremeophile. As evidenced by its participation in cheese ripening and meat production, *D. hansenii* may synthesize biotechnologically significant products. *D. hansenii* is an osmotolerant yeast that generates solutes such D-arabinitol, which might be an industrially appealing characteristic. D-arabinitol is created in batch culture following the development phase, at the same time that riboflavin is excreted (129).

Candida boidinii is cream colored or beige, glossy to dull, butyrous to membraneous, and have an uneven or fringed edge when plated on agar. This species is able to metabolize: D-glucose and D-xylose, while L-arabinose metabolization is variable and is unable to metabolize D-raffinose and D-galactose. *Candida boidinii* is a yeast species that has garnered prominence as a non-conventional yeast. The use of methanol endows the species with a variety of characteristics that are beneficial in heterologous protein production (130). Only a few microbes, such as the yeasts *Candida boidinii and Candida famata* can naturally produce sorbitol (131).

The streak colony of *Cryptococcus flavescens* is dull, yellowish-cream in color, and has an uneven, poorly pustulate surface. This species is able to metabolize: D-glucose, D-xylose, D-raffinose and D-galactose while L-arabinose is variable. A strain of this species was found to be able to produce salt-tolerant and thermostable glutaminase. *Cr. flavescens* is also capable to produce an acidic extracellular polysaccharide, which contains D-xylose, D-mannose, D-glucuronic acid (132).

Lipomyces tetrasporus is mucoid, shiny, smooth, and white to light-cream in color when plated on agar. Its able to metabolize: D-glucose, D-xylose, L-arabinose, D-raffinose and D-galactose (133). The streak colonies of *Lipomyces starkeyi* are alike *L. tetrasporus* and metabolize the same carbohydrates with the exception of L-arabinose, whose metabolization is variable for *L. starkeyi*. *Lipomyces starkeyi* has several important biotechnological capabilities. The species produces dextranases, which have been extensively characterized. *L. starkeyi* also produces delta 12-desaturase, an enzyme that converts oleic acid into linoleic acid. This species is also reported to convert components of sewage sludge into lipids for biodiesel production (133).

3. Materials and Methods

3.1. Yeast strains and growth conditions

The yeast strains used in this study were obtained from the yeast culture collection of Instituto Superior Técnico. These include strains isolated from different samples and molecularly identified in the Biological Sciences Research Group of iBB/IST and strains obtained from other culture collections. The complete list of species and strains, as well as their origin is summarized in **Table 1**.

All strains were recovered from glycerol stocks stored at -80ºC and plated on YPD agar (1% (w/v) of yeast extract (Difco), 2% (w/v) of bactopeptone (Difco), 2% (w/v) of D-glucose (Sigma-Aldrich) and 2% (w/v) of agar (Merck)) and grown at 30°C for 2 to 3 days, depending on strains' growth kinetics.

3.2. Assessment of the ability of different yeast strains to grow on different sugars

Yeast cells were pre-grown aerobically overnight, or longer if necessary, at 30°C in liquid YPD medium with orbital agitation (New Brunswick Innova 2100, platform shaker) at 250 rpm. Those cells were collected by centrifugation and washed twice with sterile distilled water. A second centrifugation process was performed, and cells were re-suspended in the different media used with an initial optical density at 600nm (OD(600nm)) of 0.5 ± 0.05 in 50 mL Erlenmeyer flasks containing 25 mL of Yeast Nitrogen Base (YNB) (Difco) medium supplemented with 20 g/L of either one of the following sugars: D-xylose (≥99%, Sigma-Aldrich), D-galactose (≥99%, Sigma-Aldrich), L-arabinose (≥99%, Sigma-Aldrich), D-raffinose (≥98%, Sigma-Aldrich), D-glucose (≥99%, Sigma-Aldrich) or D-galacturonic acid (≥97%, Sigma-Aldrich).

Cell cultures were cultivated at 30℃, in orbital incubators (New Brunswick Innova 2100, platform shaker) at 250 rpm. Cell growth was monitored daily by collecting a sample of 1 mL of culture and measuring the optical density at 600 nm (following dilution of the cell culture, if necessary, to OD(600nm) values between 0.1 ± 0.05 and 0.7 ± 0.05) until the cells reached stationary phase. By the end of yeast cultivation, a sample of the culture was collected, centrifuged at 9700 X *g* for 3 min, and culture supernatant was used for the determination of the concentration of sugar and other metabolites in the growth medium by High Performance Liquid Chromatography (HPLC), as described below.

3.3. Assessment of the ability of selected yeast strains to grow and accumulate lipids and produce other metabolites in a growth medium mimicking lignocellulosic biomass hydrolysates

Cells from different yeast strains were pre-grown aerobically at 30°C in liquid YPD, with orbital agitation (New Brunswick Innova 2100, platform shaker) at 250 rpm, during 2 or 3 days. Cells were centrifuged and washed twice with sterile distilled water. Cells were resuspended in YNB medium (Difco) supplemented with 50 g/L of D-glucose (≥99%, Sigma-Aldrich), 25 g/L of D-xylose (≥99%, Sigma-Aldrich) and containing, or not, 75 mM (corresponding to 4.5 g/L) of acetic acid, at pH 5.5. Whenever stated, the growth of specific strains was also tested in the same medium, but at pH 5.0. Yeast growth started with an optical density (600nm) of 4 ± 0.5 and was performed in 250 mL Erlenmeyer's flasks containing 50 mL of medium. Yeast cells were cultured at 30℃ with orbital agitation (New Brunswick Innova 2100, platform shaker) at 250 rpm. Cell growth was monitored periodically by collecting 1 mL the culture and measuring the optical density at 600 nm (following dilution of the cell culture, if necessary, to OD(600nm) values between 0.1 ± 0.05 and 0.7 ± 0.05). At the same time, a sample was collected, centrifuged at 9700 X *g* for 3 min, and culture supernatant was used for the determination of the concentration of sugars, acetic acid, and other metabolites in the growth medium by HPLC, as described below.

3.4. Determination of the concentration of sugars, acetic acid and other organic acids by High Performance Liquid Chromatography (HPLC)

The methodology for determination of the concentration of sugars, acetic acid and other organic acids is the same as described by Martins et al. (2021) (134). The samples were collected during cultivation of the different yeast strains tested (1 mL of each) were centrifuged at 9700 X *g* for 3 min in a microcentrifuge MiniSpin Plus (Eppendorf) and 100 µL of the supernatant were diluted with 900 µL of 50 mM H_2SO_4. The concentration of carbon sources present in each sample was determined by HPLC (Hitachi LaChrom Elite) using a column Aminex HPX-87H (Bio-Rad) coupled with UV/visible detector (for organic acids D-galacturonic acid, acetic acid, malate, formate, oxalate, citrate, and succinate detection) or a refractive index detector (for sugars (D-glucose, D-xylose, L-arabinose, L-raffinose and D-galactose), xylitol, glycerol, and ethanol detection). Ten microliters of each sample were run and compounds were eluted with 5 mM H_2SO_4 as mobile phase at a flow rate of 0.6 mL/min for 30 minutes. The column and refractive index detector temperatures were set at 65ºC and 40ºC, respectively.

The concentrations of D-glucose, D-xylose, L-arabinose, L-raffinose, D-galactose, acetic acid, D-galacturonic acid, malate, formate, oxalate, citrate, succinate, xylitol, ethanol, and glycerol were calculated using the respective calibration curves prepared for each compound.

3.5. Preliminary assessment of the accumulated lipids in yeast cells using Nile Red fluorescence staining

During cultivation of oleaginous yeast strains in medium mimicking lignocellulosic biomass hydrolysates, cells were collected, centrifuged at 9700 X *g* for 3 min, and the supernatant discarded. The pellet was resuspended and washed twice with 10 mM of potassium phosphate buffer (PBS) (NaCl (1.37 mM), KCl (2.7 mM), Na_2HPO_4 (10 mM), KH_2PO_4 (1.8 mM), pH 7) adjusting the OD(600nm) to 1. A total of 200 µL of each cell suspension was transferred to a black 96-well optical bottom plate (Thermo Fisher Scientific, NY, USA) and then mixed with 20 µL of Nile Red solution (Merck) (2.5 µg/mL from a Nile Red stock solution of 25 µg/mL dissolved in acetone). Three technical replicates were performed for each cell sample, and relative fluorescence units (RFU) were measured using a FilterMax F5 Multi-Mode Microplate Reader (Molecular Devices) using the excitation and emission wavelengths of 535 and 625 nm, respectively. Relative neutral lipid content was represented as RFU.

3.6. Microscopic observation of yeasts' lipid droplets

For microscopic observation of lipid droplets inside the cells of oleaginous yeasts, 800 µL of the growth culture were collected, centrifuged at 9700 X *g* for 3 min and the supernatant discarded. The pellet was resuspended and washed twice with 10 mM of PBS (pH 7) adjusting the OD(600nm) to 10 and stained with Nile Red solution (final concentration 2.5 µg/mL). A total of 4 µL of this cell suspension were examined with a Zeiss Axioplan microscope equipped with adequate epifluorescence interface filters (Ex/Em of 495/520 nm). Fluorescence emission was collected with a coupled device camera (Axiocam 503 color; Zeiss, Jena, Germany), and the images were analysed with ZEN 2 Microscope Software (Zeiss).

4. Results

4.1. Growth profile of selected yeasts strains on different carbon sources

In order to select the most promising species and strains from the IST yeast culture collection reported to be oleaginous in the scientific literature, the strains listed in **Table 2** were chosen for subsequent phenotypic screenings.

According to the literature, *Rhodotorula babjevae* and *R. diobovatum* produce lipids when cultivated with glycerol as a carbon source under nitrogen-limiting conditions. After 120 hours of cultivation, biomass concentrations of *R. babjevae* reached 9.4 ± 0.8 g/L with a final lipid accumulation of 34.9 ± 3.0 % cell dry weight (cdw) while for *R. diobovatum* the biomass concentration achieved was 12.0 ± 0.8 g/L with a final lipid accumulation of 63.7 ± 4.5 % cdw (7). *Rhodotorula mucilaginosa* when grown on pentose fraction of acid pre-treated sugarcane bagasse, its dried biomass, directly transesterified *in situ*, yielded 97.3 mg g^{-1} of fatty acid methyl esters (FAMEs) with a significant monounsaturated fatty acid content (8). *Rhodotorula glutinis* grown on glycerol-supplemented (5%) urban wastewater for 7 days at 28°C and 180 rpm, showed a 17.9 ± 0.1 % lipid content (10). *Meyerozyma caribbica,* using of glycerol as a carbon source, yielded 42.12 g L^{-1} of total dry weight (11). *Meyerozyma guilliermondii* when grown on D-glucose or pure glycerol accumulated a total lipids relative to dry weight of 52.38% and 34.97%, respectively (12). *Debaryomyces hansenii* has been described to accumulate up to 50% w/w of neutral lipids when cultivated on glycerol (13). Although *Cystobasidium slooffiae* was not reported to be an oleaginous yeast, this species phylogenetically related to oleaginous species such as *Cystobasidium iriomotense* (with lipid levels above 20% cdw when cultivated on D-glucose/D-xylose (GX) medium) and *Cystobasidium oligophagum* (with lipid levels of 29.42% cdw) (14,15). Therefore, it was decided to further test this species in this study.

Table 2 - Strains from yeast species available at the IST collection that have been reported in the literature as oleaginous species. The species, strain collection number and origin of isolation, are indicated.

Species	Strain (IST ID Collection)	Origin of isolation	Reference
Rhodotorula babjevae or *Rodothorula diobovatum*	IST323	Aragonese Grape	(7)
Rhodotorula babjevae	IST537	PYCC 5168	(7)
	IST549	Walnut outer shell	
	IST550	Walnut outer shell	
Rhodotorula mucilaginosa	IST390	Hydrated SBP (Pulp+Water)	(8)
	IST423	Effluent from lupine production	
	IST461	Beer Wort	
Rhodotorula glutinis	IST535	PYCC 4177	(10)
Rhodotorula toruloides	IST536	PYCC 5615	(9)
Meyerozyma caribbica	IST494	Oak soil	(11)
	IST541	Arrábida Sea, grown in YPD	
	IST542	Arrábida Sea, enriched in D-xylose	
	IST601	Fuel contaminated soil	
Meyerozyma caribbica or *M. guilliermondii*	IST595	Fuel contaminated soil	(11,12)
Meyerozyma guilliermondii	IST502	Plum	(12)
	IST587	Fuel contaminated soil	
	IST591	Fuel contaminated soil	
Debaryomyces hansenii	IST334	Beer wort	(13)
	IST339	Beer wort	
	IST340	Beer wort	
	IST375	Chorizo from Alentejo	
	IST586	Fuel contaminated soil	
Cystobasidium slooffiae	IST544	Walnut outer shell	(14,15)

The strains listed in **Table 2** were cultivated in YNB containing different single carbon sources, namely, 20 g/L of either D-glucose, D-xylose, L-arabinose, D-raffinose, D-galactose or D-galacturonic acid.

The growth profiles of different strains of the species *R. babjevae* (strains IST537, IST549 and IST550) and strain IST323, whose identification was inconclusive between *R. babjevae* and *R. diobovatum* through the nucleic acid sequencing of the D1/D2 region, when cultivated in media containing each one of the tested carbon sources are shown in **Figure 2**. The growth curves were followed based on the periodic measurement of the culture OD at 600nm.

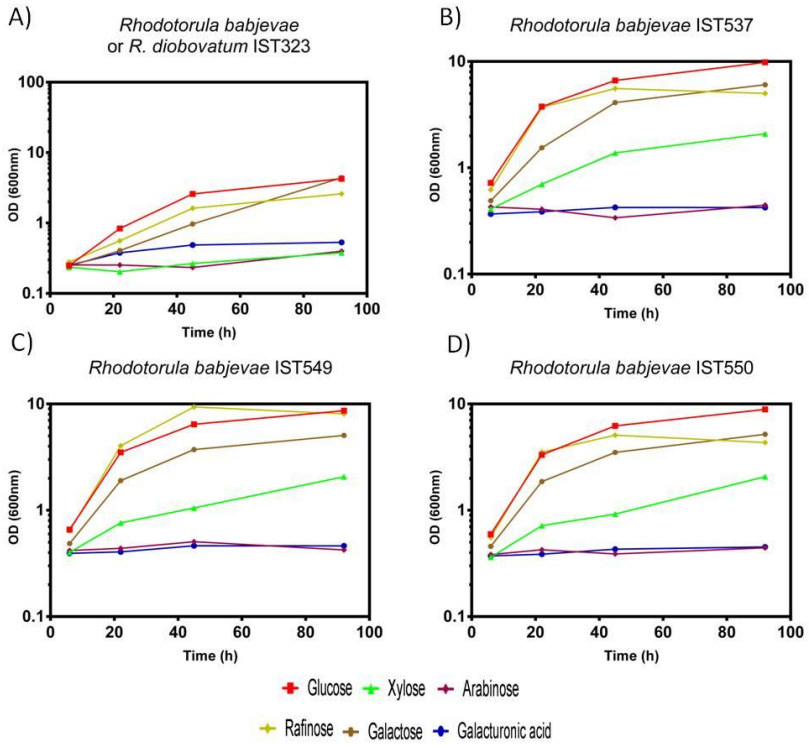

Figure 2 - Growth curves of strain IST323, whose identification was inconclusive between *Rhodotorula babjevae* and *Rhodotorula diobovatum,* and *Rhodotorula babjevae* strains IST537, IST549 and IST550 in media containing different individual carbon sources. Yeast strains were grown at 30ºC, pH 5.0 and 250 rpm. Growth profiles were obtained by periodically measuring the OD at 600nm.

Overall, this species grows better on D-glucose, D-raffinose and D-galactose. No growth was observed when *R. babjevae* strains were cultivated in medium containing either L-arabinose or D-galacturonic acid, with the exception of strain IST323, whose identification was inconclusive between *R. babjevae* and *R. diobovatum,* which showed a slight increase in the final OD when cultivated on these two carbon sources (**Fig.2A**). Among the four strains tested, strain IST323 showed a lower increase in the final biomass when cultivated in D-xylose, but was also apparently the only strain able to show some grow on D-galacturonic acid-medium (**Fig.2A**).

To know if the different strains tested can fully catabolise the different carbohydrates during cultivation, the final concentration of each sugar in the last time-point of every fermentation was assessed. *R. babjevae*/*R.diobovatum* IST323 and *R. babjevae* strains capability to consume these sugars is indicated in **Table 3**. Although the initial concentration of each sugar in the growth medium was not determined, it is evident that in all *R. babjevae* strains the residual concentration of D-glucose, D-galactose and D-raffinose in the culture medium (**Table 3**) is consistent with the growth curves obtained for each strain/sugar, reinforcing the notion that D-glucose, D-raffinose and D-galactose are the preferred carbohydrates. D-xylose is also used, although less efficiently, while D-galacturonic acid and, in general, L-arabinose are not. The only strain that shows slight growth in L-arabinose and D-galacturonic acid is IST323 but the residual concentration of these sugars in the culture medium are similar for all strains which does not indicate that IST323 can really exhibit some growth using these sugars.

Table 3 - **Residual concentration of the different sugars present in the growth medium after cultivation of *Rhodotorula babjevae/Rhodotorula diobovatum* IST323 and the different *Rhodotorula babjevae* strains IST537, IST549, and IST550.** The culture supernatant was collected after 92 h of cultivation.

Species	Strain	Carbon source	Final carbon source present in the growth medium (g/L)
Rhodotorula babjevae or *Rhodotorula diobovatum*	IST323	D-Glucose	6.8
		D-Xylose	18.2
		L-Arabinose	17.0
		D-Raffinose	10.5
		D-Galactose	14.6
		D-Galacturonic acid	17.0
Rhodotorula babjevae	IST537	D-Glucose	2.9
		D-Xylose	12.7
		L-Arabinose	17.5
		D-Raffinose	9.9
		D-Galactose	7.9
		D-Galacturonic acid	18.8
	IST549	D-Glucose	2.1
		D-Xylose	12.0
		L-Arabinose	17.4
		D-Raffinose	8,8
		D-Galactose	12.8
		D-Galacturonic acid	16.9
	IST550	D-Glucose	5.9
		D-Xylose	17.7
		L-Arabinose	17.1
		D-Raffinose	9.0
		D-Galactose	12.3
		D-Galacturonic acid	16.8

The growth profiles of the different strains tested of *R. mucilaginosa* (strains IST390, IST423 and IST461), *R. toruloides* IST536 and *R. glutinis* IST535 are depicted in **Figure 3**.

When comparing the *R. mucilaginosa* strains with each other, they showed the best growth parameters in the presence of D-glucose, D-xylose and D-galactose. These strains achieved a higher biomass concentration at the stationary phase, possibly due to a higher consumption of each carbon source (**Table 4**). However, strain IST423 does not grow in medium containing L-arabinose (**Fig.3B**), contrary to strain IST390 (**Fig.3A**) and strain IST461 (**Fig.2D**). *R. toruloides* IST536 was able to grow on all the carbon sources tested, but reached lower biomass concentrations when compared to *R. mucilaginosa* IST390 and IST461 when cultivated in D-galactose, D-raffinose, and D-galacturonic acid. Although D-galacturonic acid was efficiently used, the other two sugars, D-galactose, D-raffinose, were not fully consumed after 92 hours of cultivation (**Table 4**). *Rhodotorula glutinis* IST535 was able to grow on all carbon sources tested, although growth on D-raffinose was slower (**Fig.3E**). *R. glutinis* IST535 reached higher biomass concentration when cultivated in D-xylose, L-arabinose, and D-galactose when compared to *R. babjevae* strains IST537, IST549 and IST550, *R. mucilaginosa* strains IST390, IST423, IST461, and *R. toruloides* IST536, although it does not seem capable of metabolizing efficiently D-galactose (**Table 4**).

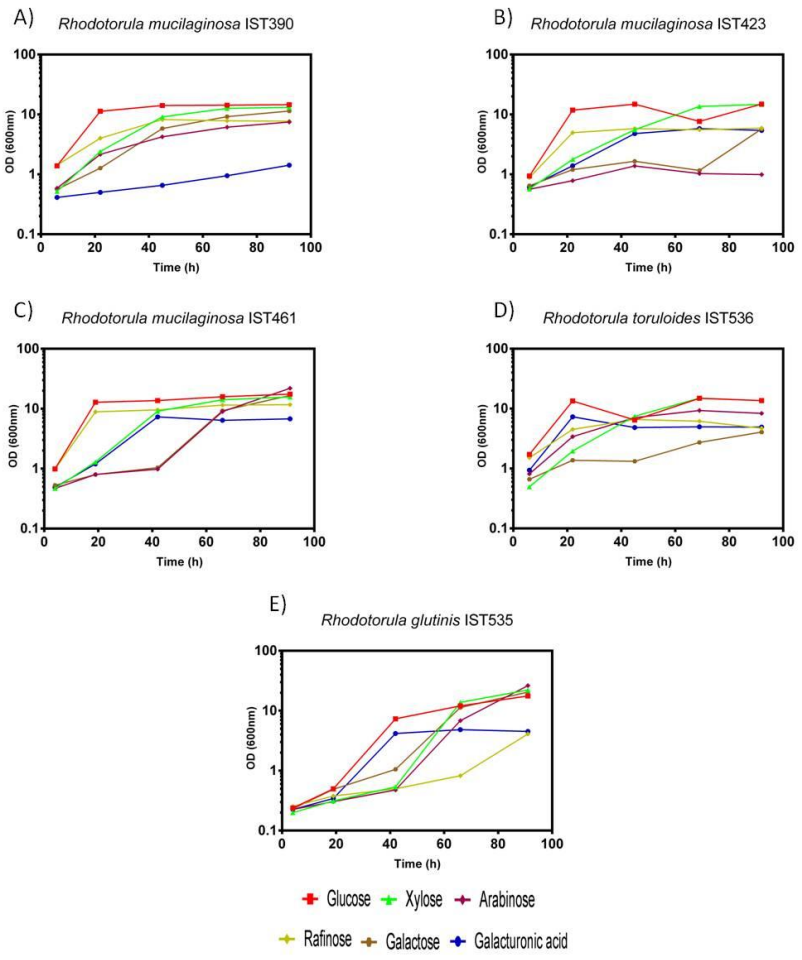

Figure 3 - Growth curves of *Rhodotorula mucilaginosa* strains IST390, IST423, IST461, *Rhodotorula toruloides* IST536 and *Rhodotorula glutinis* IST535 in media containing different individual carbon sources. Yeast strains were grown at 30ºC, pH 5.0 and 250 rpm. Growth profiles were obtained by periodically measuring the OD at 600nm.

To know if the different strains tested can fully catabolise the different carbohydrates during cultivation, the final concentration of each sugar in the last time-point of every fermentation was assessed. *R. mucilaginosa* strains IST290, IST423 and IST461, *R. toruloides* IST536 and *R. glutinis* IST535 capability to consume these sugars is indicated in **Table 4**. Analyzing the consumption of the different carbohydrates (**Table 4**), in general, *R. mucilaginosa* fully catabolizes D-glucose and D-xylose. It also seems capable of consuming D-galacturonic acid, with strain IST461 consuming the highest percentage, leaving 5.2 g/L of residual D-galacturonic acid in the growth medium at the end of the assay, in comparison with the remaining strains, which leave 11.8 g/L and 6.0 g/L for strains IST390 and IST423, respectively.

Regarding the growth profiles obtained for all the *Rhodotorula* species examined (**Figures 2 and 3**), the ones that showed, in general, a higher final biomass concentration are: *R. babjevae* IST537 (**Fig.2B**), *R. mucilaginosa* IST461 (**Fig.3C**), *R. toruloides* IST536 (**Fig.3D**) and *R. glutinis* IST535 (**Fig.3E**). *R. babjevae* IST537 (**Fig.2B**) had the least residual concentration present in the medium of D-glucose (2.9 g/L), D-galactose (7.9 g/L) and D-raffinose (9.9 g/L), indicating these carbohydrates are the best metabolized by this strain. *R. mucilaginosa* IST461 (**Fig.3C**) showed no residual concentration of D-glucose present in the media, indicating an efficient catabolization of this sugar, and had the least residual concentration of D-xylose (0.1 g/L), D-galacturonic acid (5.2 g/L) and L-arabinose (7.8 g/L) present in the medium, indicating that these carbohydrates are the best metabolized by this strain. Strain *R. toruloides* IST536 (**Fig.3D**) exhibited no residual concentration of D-glucose present in the media and residual concentrations of D-xylose (0.1 g/L) and D-galacturonic acid (0.6 g/L), indicating a better metabolization of these carbohydrates by this strain.

Table 4 - Residual concentration of the different sugars present in the growth medium after cultivation of *Rhodotorula mucilaginos*a strains IST290, IST423, and IST461*, Rhodotorula toruloides* IST536, and *Rhodotorula glutinis* IST535. The culture supernatant was collected after 92 h of cultivation.

Specie	Strain	Carbon source	Final carbon source present in the growth medium (g/L)
Rhodotorula mucilaginosa	IST390	D-Glucose	0.0
		D-Xylose	0.9
		L-Arabinose	9.9
		D-Raffinose	6.7
		D-Galactose	7.1
		D-Galacturonic acid	11.8
	IST423	D-Glucose	0.0
		D-Xylose	1.5
		L-Arabinose	12.5
		D-Raffinose	7.7
		D-Galactose	10.0
		D-Galacturonic acid	6.0
	IST461	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	7.8
		D-Raffinose	8.6
		D-Galactose	11.0
		D-Galacturonic acid	5.2
Rhodotorula toruloides	IST536	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	7.2
		D-Raffinose	11.3
		D-Galactose	12.5
		D-Galacturonic acid	0.6
Rhodotorula glutinis	IST535	D-Glucose	0.0
		D-Xylose	2.0
		L-Arabinose	1.7
		D-Raffinose	6.5
		D-Galactose	12.5
		D-Galacturonic acid	5.3

Another yeast genus examined was the genus *Meyerozyma,* namely the species: *Meyerozyma caribbica*, strains IST494, IST541, IST542 and IST601, *Meyerozyma guilliermondii,* strains IST502, IST587 and IST591, and strain IST595 (in this case, species identification was inconclusive between *M. caribbica* and *M. guilliermondii)*. The growth profiles of *M. caribbica* strains IST94, IST541, IST542, and IST601, in media containing each one of the tested carbon sources, are depicted in **Figure 4**. In general, the growth curve profiles obtained for the different strains are similar for all carbon sources with the exception of D-raffinose. In medium containing this carbohydrate, strain IST494 reached stationary phase faster (**Fig.4A**), after 45 h of cultivation close to the profiles for the other carbon sources. The growth of other strains in D-raffinose was slower, especially for strains IST541 and IST542. None of the *M. caribbica* strains tested was able to grow on D-galacturonic acid. All the growth profiles are consistent with the residual concentration of each sugar present after 98 h of cultivation, given that all *M. caribbica* strains can fully catabolise D-glucose, D-xylose, L-arabinose and D-galactose (**Table 5**). The residual concentration of D-raffinose present in the growth medium of strain IST601 was 0.5 g/L while strain IST494 left 0.8 g/L of D-raffinose, strain IST541 left 2.5 g/L of D-raffinose and strain IST542 left 2.2 g/L of D-raffinose, reinforcing the idea that *M. carribica* strains IST601 and IST494 catabolize better this carbohydrate when compared to strains IST541 and IST542.

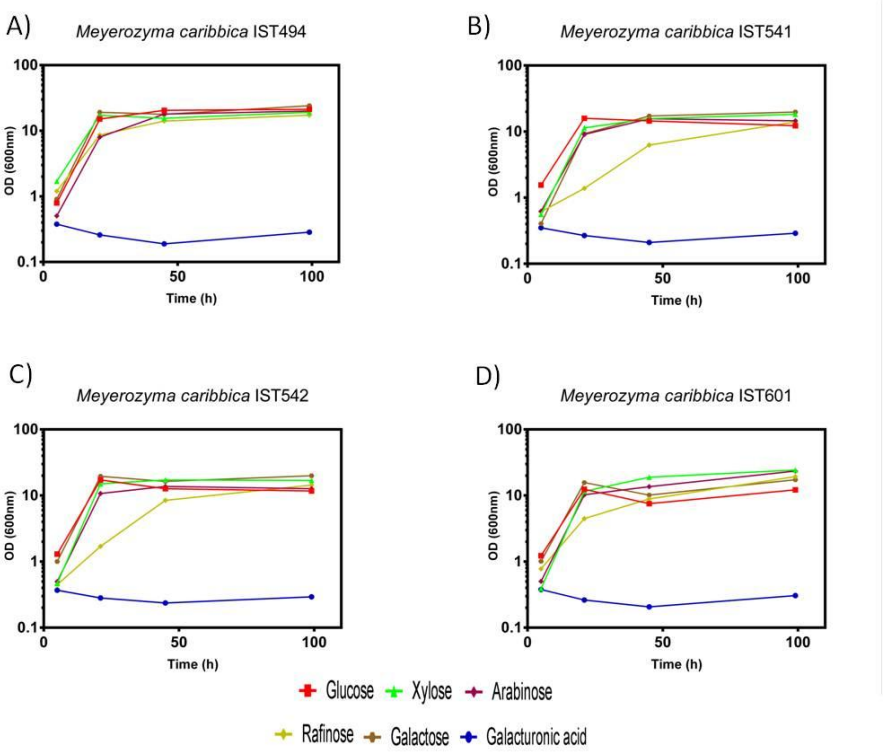

Figure 4 - Growth curves of *Meyerozyma caribbica* strains IST494, IST541, IST542 and IST601 in media containing different individual carbon sources. Yeast strains were grown at 30ºC, pH 5.0 and 250 rpm. Growth profiles were obtained by periodically measuring the OD at 600nm.

Table 5 - Residual concentration of the different sugars present in the growth medium after cultivation of *Meyerozyma caribbica* strains IST494, IST541, IST542 and IST601. The culture supernatant was collected after 99 h of cultivation.

Species	Strain	Carbon source	Final carbon source present in the growth medium (g/L)
Meyerozyma caribbica	IST494	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	0.8
		D-Galactose	0.0
		D-Galacturonic acid	18.5
	IST541	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	2.5
		D-Galactose	0.0
		D-Galacturonic acid	18.1
	IST542	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	2.2
		D-Galactose	0.0
		D-Galacturonic acid	18.9
	IST601	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	0.5
		D-Galactose	0.0
		D-Galacturonic acid	18.7

The growth profiles of *M. caribbica/M. guilliermondii* IST595 and *M. guilliermondii* strains IST502, IST587 and IST591 strains examined are depicted in **Figure 5**. As referred above, the identification of strain IST595 was inconclusive between *M. caribbica* and *M. guilliermondii*. These two species are included in *Saccharomycotina* CTG clade and are very closely related, being impossible to distinguish phenotypically and phylogenetically when comparing theD1/D2 domain sequences from ribosomal rDNA (135). *Meyerozyma guilliermondii* strains examined in this study showed a similar growth behaviour as the *M. caribicca* strains previously examined (**Figure 4**). When comparing *M. guilliermondii* strains with each other, a higher final biomass concentrations, as assessed based on culture OD (600nm), were observed in all strains when cultivated in D-glucose, D-xylose, L-arabinose and D-galactose while the final biomass concentration on D-raffinose, was lower when compared to the remaining sugars. *Meyerozyma guilliermondii* IST587 (**Fig.5C**) reached a highest final biomass concentration, in all sugars when compared with the other strains of the same species, with the exception of D-raffinose in which this strain, like IST502, attained lower final OD (600nm) values (7.3) compared to *M. caribbica/M. guilliermondii* IST595 and *M. guillermondii* IST591 (**Fig.5A** and **Fig.5D**, respectively) that reached a final OD (600 nm) of approximately 15.0.

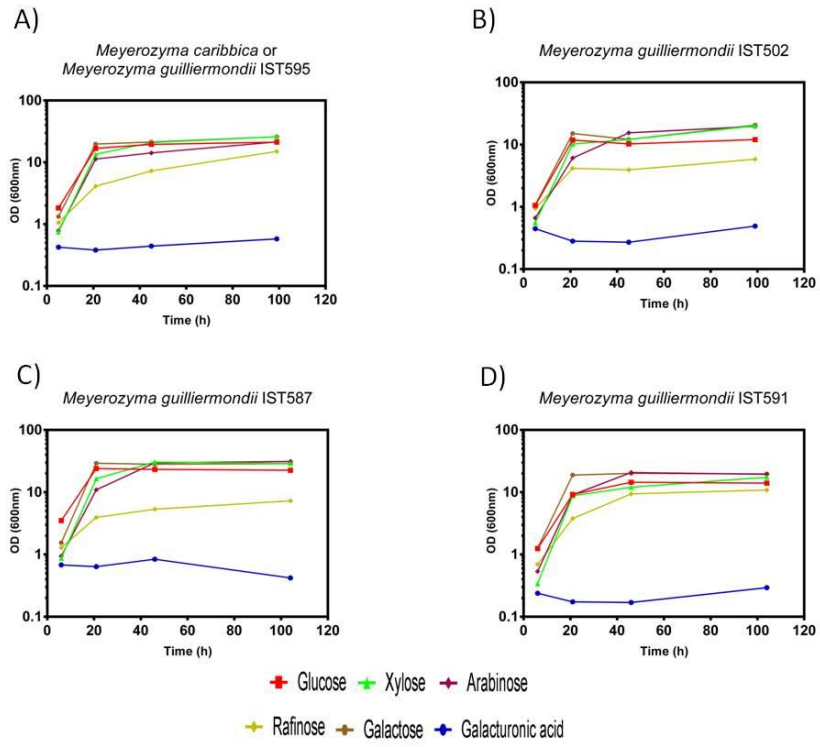

Figure 5 - Growth curves of strain IST595, whose identification was inconclusive between *Meyerozyma guilliermondii* and *Meyerozyma caribbica,* and *Meyerozyma guilliermondii* strains IST502, IST587 and IST591 in media containing different individual carbon sources. Yeast strains were grown at 30ºC, pH 5.0 and 250 rpm. Growth profiles were obtained by periodically measuring the OD at 600nm.

The residual concentration of each sugar in the last time-point of every fermentation was also determined (**Table 6**). *M. caribbica/M. guilliermondii* IST595 and *Meyerozyma guilliermondii* strains IST502, IST587 and IST591, depletion of the different sugars (**Table 6**) is consistent with the growth profiles obtained for each strain/sugar (**Figure 5**). The strains tested fully or almost fully catabolized all the sugar (even D-raffinose), except D-galacturonic acid.

Table 6 - **Residual concentration of the different sugars present in the growth medium after cultivation of *Meyerozyma caribbica/Meyerozyma guilliermondii* IST595 and *Meyerozyma guilliermondii* strains IST502, IST587 and IST59.** The culture supernatant was collected after 99 h of cultivation for strains IST595 and IST502 and after 104 h of cultivation for strains IST587 and IST591.

Species	Strain	Carbon source	Final carbon source present in the growth medium (g/L)
Meyerozyma caribbica/ Meyerozyma guilliermondii	IST595	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	0.1
		D-Galactose	0.0
		D-Galacturonic acid	18.7
Meyerozyma guilliermondii	IST502	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	0.8
		D-Galactose	0.0
		D-Galacturonic acid	19.0
	IST587	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	0.2
		D-Galactose	0.0
		D-Galacturonic acid	18.5
	IST591	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	0.1
		D-Galactose	0.0
		D-Galacturonic acid	18.8

The other species examined in this screening were the species *Debaryomyces hansenii* (strains IST586, IST334 and IST339) and *Cystobasidium slooffiae* IST544. Their growth profile in the different carbon source media examined is shown in **Figure 6**. In general, *D. hansenii* strains could grow on D-glucose, D-xylose, L-arabinose, D-raffinose, D-galactose, but not on D-galacturonic acid. Strain IST586 reached stationary phase after 22 h of cultivation on D-xylose, D-raffinose, D-glucose, L-arabinose and D-galactose. Strain IST334 also reached stationary phase after 22h of cultivation on D-glucose, D-raffinose, similar to IST586, but not on D-xylose, L-arabinose and D-galactose. Strain IST339 was the slowest to reach the stationary phase, however it is the one that, although later, showed the highest final biomass concentration on the different sugars. All *D. hansenii* strains tested were unable to grow on D-galacturonic acid. For *D. hansenii* IST339, the growth profile and the ability to catabolise D-galacturonic acid could not be fully assessed given that the Erlenmeyer flask broke during the course of the assay, having just biomass concentration values for the first 22h, however during this time course no growth was observed. *Cy. slooffiae* IST544 only showed growth on D-glucose, D-xylose and L-arabinose. However, this strain did not grow on the remaining carbohydrates and the final biomass concentration is one of the lowest out of all the strains examined.

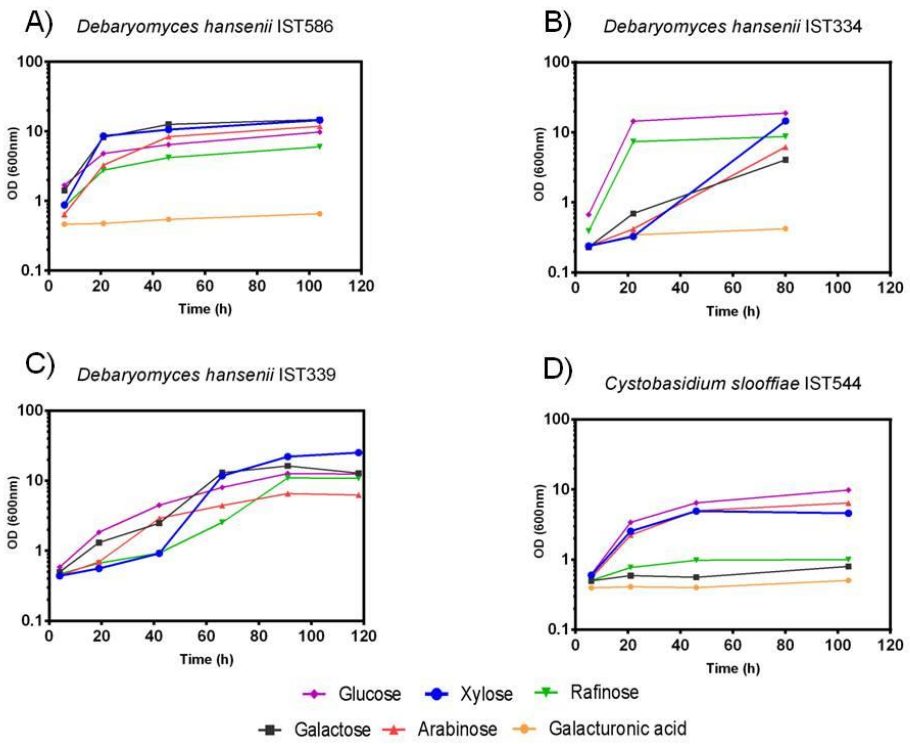

Figure 6 - Growth curves of *Debaryomyces hansenii* strains IST586, IST334, IST339, and *Cystobasidium slooffiae* IST544 in media containing different individual carbon sources. Yeast strains were grown at 30ºC, pH 5.0 and 250 rpm. Growth profiles were obtained by periodically measuring the OD at 600nm.

To determine if the different strains can fully catabolise the different carbohydrates, the final concentration of each sugar in the last time-point of every fermentation was assessed. *D. hansenii* strains IST586, IST334, and IST339, and *Cy. slooffiae* IST544 capability to consume these sugars is shown in **Table 7**. All *D. hansenii* strains completely depleted D-glucose and D-raffinose from the growth medium and with the exception of strain IST334 all other strains also fully catabolised D-xylose, L-arabinose and galactose. These sugars were still present in considerable amounts (10-13 g/L) after 80 hours of growth by strain IST334 indicating that for these carbohydrates, this strain was still in exponential growth phase when the assay ended, given that for the other strains they were fully catabolized. When comparing the *Debaryomyces* strains with each other, the one that showed the highest final biomass concentration, associated to culture OD, when cultivated in all sugars, except for D-galacturonic acid, was *D. hansenii* IST339 (**Fig.6C**). However, this strain was the slowest to reach stationary phase.

Table 7 - Residual concentration of the different sugars present in the growth medium after cultivation of *Debaryomyces hansenii,* strains IST586, IST334 and IST339, and *Cystobasidium slooffiae* IST544. The culture supernatant was collected after 80 h of cultivation for strain IST334, after 104 h of cultivation for strains IST586, IST544 and after 118 h for strain IST339.

Species	Strain	Carbon source	Final carbon source present in the growth medium (g/L)
Debaryomyces hansenii	IST586	D-Glucose	0.2
		D-Xylose	0.0
		L-Arabinose	0.1
		D-Raffinose	0.8
		D-Galactose	0.6
		D-Galacturonic acid	19.2
	IST334	D-Glucose	0.0
		D-Xylose	10.6
		L-Arabinose	12.1
		D-Raffinose	0.3
		D-Galactose	13.9
		D-Galacturonic acid	19.4
	IST339	D-Glucose	0.0
		D-Xylose	0.1
		L-Arabinose	0.0
		D-Raffinose	0.7
		D-Galactose	0.0
		D-Galacturonic acid	-
Cystobasidium slooffiae	IST544	D-Glucose	7.3
		D-Xylose	6.7
		L-Arabinose	5.5
		D-Raffinose	18.8
		D-Galactose	19.2
		D-Galacturonic acid	19.7

To synthesize all the information obtained, a heat map of the OD (600 nm) attained after different periods of cultivation, for the different species and strains studied are shown in **Table 8** and **Table 9**.

Table 8 - **Growth ability of the different strains selected from IST yeast culture collection in media containing an initial concentration of 20 g/L of either D-glucose, D-xylose or L-arabinose.** Growth profiles were based on OD measurements (at 600 nm) of the respective growth cultures attained after different periods of cultivation at 30ºC, pH 5.0 and 250 rpm. Values highlighted in dark green represent highly elevated OD (OD above 25), light green represent elevated OD (OD between 15 to 24.9), yellow represent medium growth (OD between 10 to 14.9), orange represents low growth (OD between 5 to 9.9) and red represent extremely low growth (OD between 0.5 to 4.9).

Species	Strain (IST ID Collection)	D-glucose					D-xylose					L-arabinose				
		0 h	22 h	45 h	69 h	Final OD	0 h	22 h	45 h	69 h	Final OD	0 h	22 h	45 h	69 h	Final OD
Rhodotorula babjevae or *Rhodotorula diobovatum*	IST323	0.5	0.8	2.6	-	4.3 [A]	0.5	0.2	0.3	-	0.4 [A]	0.5	0.3	0.2	-	0.4 [A]
Rhodotorula babjevae	IST537	0.5	3.8	6.6	-	9.8 [A]	0.5	0.7	1.4	-	2.1 [A]	0.5	0.4	0.3	-	0.4 [A]
	IST549	0.5	3.5	6.4	-	8.6 [A]	0.5	0.8	1.1	-	2.1 [A]	0.5	0.4	0.5	-	0.4 [A]
	IST550	0.5	3.3	6.2	-	8.9 [A]	0.5	0.7	0.9	-	2.1 [A]	0.5	0.4	0.4	-	0.4 [A]
Rhodotorula mucilaginosa	IST390	0.5	11.3	14.1	14.3	14.6 [A]	0.5	2.4	9.1	12.6	13.1 [A]	0.5	2.2	4.2	6.1	7.5 [A]
	IST423	0.5	11.8	14.8	13.9	14.9 [A]	0.5	1.8	5.6	13.6	14.6 [A]	0.5	0.8	1.4	1.0	1.0 [A]
	IST461	0.5	12.8	13.7	15.8	17.5 [A]	0.5	1.3	9.1	14.1	15.7 [A]	0.5	0.8	1.0	8.9	21.9 [A]
Rhodotorula glutinis	IST535	0.5	0.5	7.3	12.0	17.6 [A]	0.5	0.3	0.5	13.8	22.2 [A]	0.5	0.3	0.5	6.8	26.2 [A]
Rhodotorula toruloides	IST536	0.5	13.4	10.9	14.8	13.6 [A]	0.5	2.0	7.4	15.0	13.5 [A]	0.5	3.4	7.1	9.3	8.3 [A]
Meyerozyma caribbica	IST494	0.5	17.2	15.5	-	19.2 [B]	0.5	15.1	20.5	-	21.2 [B]	0.5	8.0	18.0	-	20.0 [B]
	IST541	0.5	15.9	14.5	-	12.3 [B]	0.5	11.4	15.7	-	18.3 [B]	0.5	9.0	15.6	-	14.5 [B]
	IST542	0.5	17.2	12.7	-	11.7 [B]	0.5	15.0	17.2	-	16.9 [B]	0.5	10.7	13.7	-	12.6 [B]
	IST601	0.5	12.4	9.5	-	12.2 [B]	0.5	11.5	18.9	-	24.2 [B]	0.5	10.3	13.6	-	23.4 [B]
Meyerozyma caribbica or *Meyerozyma guilliermondii*	IST595	0.5	16.8	19.5	-	21.2 [B]	0.5	13.5	20.9	-	26.0 [B]	0.5	11.3	14.2	-	21.4 [B]
Meyerozyma guilliermondii	IST502	0.5	11.9	10.3	-	12.0 [B]	0.5	10.2	12.1	-	20.1 [B]	0.5	6.1	15.4	-	19.6 [B]
	IST587	0.5	24.0	23.2	-	22.5 [C]	0.5	16.5	30.5	-	29.0 [C]	0.5	10.9	29.6	-	31.2 [C]
	IST591	0.5	9.2	14.5	-	14.0 [C]	0.5	8.7	12.0	-	17.4 [C]	0.5	9.1	20.8	-	19.4 [C]
Debaryomyces hansenii	IST334	0.5	14.4	-	-	18.8 [D]	0.5	0.2	-	-	14.5 [D]	0.5	0.4	-	-	6.2 [D]
	IST339	0.5	1.8	4.5	8.0	12.7 [E]	0.5	0.6	0.9	11.7	22.0 [E]	0.5	0.7	2.9	4.4	6.6 [E]
	IST375	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IST586	0.5	4.8	7.5	-	10.1 [C]	0.5	8.6	10.6	-	14.6 [C]	0.5	3.3	8.4	-	11.9 [C]
Cystobasidium slooffiae	IST544	0.5	3.4	6.5	-	9.8 [C]	0.5	2.5	4.9	-	4.6 [C]	0.5	2.2	5.0	-	6.4 [C]

Note: A - OD was measured after 92 h of cultivation; B - OD was measured after 99 h of cultivation; C - OD was measured after 104 h of cultivation; D - OD was measured after 80 h of cultivation; E - OD was measured after 118 h of cultivation.

Table 9 - **Growth ability of the different strains selected from IST yeast culture collection in media containing an initial concentration of 20 g/L of either D-raffinose, D-galactose or D-galacturonic acid.** Growth profiles were based on OD measurements (at 600 nm) of the respective growth cultures attained after different periods of cultivation at 30ºC, pH 5.0 and 250 rpm. Values highlighted in dark green represent highly elevated OD (OD above 25), light green represent elevated OD (OD between 15 to 24.9), yellow represent medium growth (OD between 10 to 14.9), orange represents low growth (OD between 5 to 9.9) and red represent extremely low growth (OD between 0.5 to 4.9).

Species	Strain (IST ID Collection)	D-raffinose					D-galactose					D-galacturonic acid				
		0 h	22 h	45 h	69 h	Final OD	0 h	22 h	45 h	69 h	Final OD	0 h	22 h	45 h	69 h	Final OD
Rhodotorula babjevae or *Rhodotorula diobovatum*	IST323	0.5	0.6	1.6	-	2.6 [A]	0.5	0.4	1.0	-	4.4 [A]	0.5	0.4	0.5	-	0.5 [A]
Rhodotorula babjevae	IST537	0.5	3.7	5.6	-	5.0 [A]	0.5	1.5	4.1	-	6.0 [A]	0.5	0.4	0.4	-	0.4 [A]
	IST549	0.5	4.0	9.4	-	8.1 [A]	0.5	1.9	3.7	-	5.1 [A]	0.5	0.4	0.5	-	0.5 [A]
	IST550	0.5	3.5	5.1	-	4.3 [A]	0.5	1.9	3.5	-	5.2 [A]	0.5	0.4	0.4	-	0.5 [A]
Rhodotorula mucilaginosa	IST390	0.5	4.0	8.2	7.8	7.7 [A]	0.5	1.3	5.8	9.2	11.4 [A]	0.5	0.5	0.7	0.9	1.4 [A]
	IST423	0.5	5.0	5.8	5.5	5.9 [A]	0.5	1.2	1.7	1.2	5.7 [A]	0.5	1.4	4.8	5.8	5.4 [A]
	IST461	0.5	8.9	9.6	11.5	11.6 [A]	0.5	0.8	1.0	9.3	16.7 [A]	0.5	1.2	7.3	6.4	6.8 [A]
Rhodotorula glutinis	IST535	0.5	0.4	0.5	0.8	4.1 [A]	0.5	0.5	1.1	11.3	20.5 [A]	0.5	0.3	4.2	4.8	4.5 [A]
Rhodotorula toruloides	IST536	0.5	4.5	6.6	6.2	4.7 [A]	0.5	1.4	1.3	2.7	4.1 [A]	0.5	7.3	4.8	5.0	4.9 [A]
Meyerozyma caribbica	IST494	0.5	8.6	14.0	-	17.3 [B]	0.5	19.1	17.8	-	24.1 [B]	0.5	0.3	0.2	-	0.3 [B]
	IST541	0.5	1.4	6.2	-	14.1 [B]	0.5	9.3	17.2	-	19.7 [B]	0.5	0.3	0.2	-	0.3 [B]
	IST542	0.5	1.7	8.4	-	14.4 [B]	0.5	19.5	16.4	-	19.9 [B]	0.5	0.3	0.2	-	0.3 [B]
	IST601	0.5	4.5	8.9	-	19.3 [B]	0.5	15.7	15.1	-	17.3 [B]	0.5	0.3	0.2	-	0.3 [B]
Meyerozyma caribbica or *Meyerozyma guilliermondii*	IST595	0.5	4.1	7.3	-	15.0 [B]	0.5	19.7	21.3	-	25.8 [B]	0.5	0.4	0.4	-	0.6 [B]
Meyerozyma guilliermondii	IST502	0.5	4.2	3.9	-	5.8 [B]	0.5	15.1	12.1	-	20.6 [B]	0.5	0.3	0.3	-	0.5 [B]
	IST587	0.5	3.9	5.3	-	7.3 [C]	0.5	29.2	28.1	-	28.8 [C]	0.5	0.6	0.8	-	0.4 [C]
	IST591	0.5	3.8	9.4	-	10.8 [C]	0.5	18.9	20.0	-	19.6 [C]	0.5	0.2	0.2	-	0.3 [C]
Debaryomyces hansenii	IST334	0.5	7.4	-	-	8.7 [D]	0.5	0.7	-	-	4.1 [D]	0.5	0.4	-	-	0.6 [D]
	IST339	0.5	0.7	0.9	2.5	11.0 [E]	0.5	1.3	2.5	13.0	16.2 [E]	0.5	0.5	-	-	-
	IST375	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IST586	0.5	2.8	4.2	-	6.0 [C]	0.5	8.2	12.7	-	14.8 [C]	0.5	0.5	0.5	-	0.7 [C]
Cystobasidium slooffiae	IST544	0.5	0.8	1.0	-	1.0 [C]	0.5	0.6	0.6	-	0.8 [C]	0.5	0.4	0.4	-	0.5 [C]

Note: A - OD was measured after 92 h of cultivation; B - OD was measured after 99 h of cultivation; C - OD was measured after 104 h of cultivation; D - OD was measured after 80 h of cultivation; E - OD was measured after 118 h of cultivation.

4.2. Selection of yeast isolates for growth and lipid production in culture medium mimicking lignocellulosic biomass hydrolysates

The objective of this part of the work was to evaluate the ability of selected yeast strains to use the different carbon sources (C-sources) present simultaneously in the culture medium and to determine their potential to produce lipids from these C-sources. For this purpose, the strains from different species considered in the literature of potential as oleaginous yeasts and listed in **Table 10** were tested.

Candida boidinii was selected based on former results obtained in our laboratory (18), since this species was previously selected when Rhodamine B was used to screen for preliminary identification oleaginous yeasts. The fluorescent dye Rhodamine B allows a preliminary assessment since its florescence is directly proportional to cells' lipid content (136). A *Kodamaea ohmeri* strain when cultivated for 5 days on a rotary shaker at 150 rpm and 28ºC in nitrogen-limited medium broth containing 70 g L^{-1} pure glycerol was also found to accumulate a total of 22.9 % of dry biomass (19). When *Cryptococcus flavescens* cells were placed in a 20% glycerol solution, they were buoyant, indicating that they may have oleaginous properties and are worth experimenting upon (20). As mentioned before, although *Cy. slooffiae* was not proven to be oleaginous but given the reasons previously mentioned, it was decided to further examine this species. *Lipomyces tetrasporus,* has been reported to achieve lipid titers between 16.3 and 20.8 g/L of cdw when cultivated in 10% w/v glucose media (21). *Lipomyces starkeyi* when cultivated on glucose and xylose as mixed carbon source media, achieved a lipid content of 84.9% (w/w) of cell dry weight (22). *Meyerozyma guilliermondii* when cultivated on glucose or pure glycerol was reported to accumulate a total lipids relative to dry weight of 52.38% and 34.97%, respectively (12).

Table 10- Strains from yeast species available at the IST collection that have been reported in the literature as oleaginous species. The species, strain collection number and origin of isolation, are indicated.

Species	Strain (IST ID Collection)	Origin of isolation	Reference
Candida boidinii	IST509	Olive Water	(18)
	IST599	Fuel contaminated soil	
Kodamaea ohmeri	IST538	PYCC 7192	(19)
Cryptococcus flavescens	IST325	Branch and fruit by the vine in Colares	(20)
Cystobasidium slooffiae	IST547	Walnut outer shell	(14,15)
Lipomyces tetrasporus	IST531	PYCC 4033	(21)
Lipomyces starkeyi	IST532	PYCC 4045	(22)
	IST533	PYCC 4046	
Meyerozyma guillermondii	IST369	Beer Barrel	(12)
	IST502	Plum	

The yeast strains listed in **Table 10** were screened in culture media mimicking lignocellulosic biomass hydrolysates, composed by YNB (Difco) supplemented with 50 g/L of D-glucose, 25 g/L of D-xylose and 75 mM (4.5 g/L) of acetic acid, at pH 5.5, and also in culture media composed by YNB (Difco) supplemented with 50 g/L of D-glucose, 25 g/L of D-xylose, to serve as the control for acetic acid effect on yeast. The growth profile was obtained through the measurement of the OD at 600 nm taken periodically and the C-sources´ consumption was assessed by HPLC analysis.

The growth, carbon sources consumption and synthesis of by-products of *C. boidinii,* strains IST509 and IST599 are shown in **Figures 7 and 8**. In the absence of acetic acid, *C. boidinii* IST509 has the capacity to metabolize all D-glucose present during the first 48 h of cultivation but not D-xylose (**Fig.7A**). Interestingly, acetic acid was produced during D-glucose metabolism, reaching 32.8 mM after 48 hours of cultivation. After this period, and after glucose exhaustion, acetic acid was consumed, being completely depleted.

When acetic acid at pH 5.5 was added to the growth medium used above, *C. boidinii* IST509 consumed all the available D-glucose (**Fig.7B**), similar to control conditions, and acetic acid concentration slightly increased during the first 24 h of cultivation, being entirely catabolized afterwards. Remarkably, D-xylose was also consumed, although slowly (**Fig.7B**). In these conditions, *C. boidinni* IST509 synthesized by-products such as formate, succinate and malate (**Fig.7C**). Malate reached a concentration peak of approximately 11.6 mM. Succinate had a concentration peak of 16.2 mM after 168 h of cultivation, and then was consumed, rapidly, reaching a stationary phase by 192 h of incubation with a concentration of 2.6 mM. Formate was produced steadily during 96 h of cultivation, reaching 4.4 mM, after this period, there was a remarkable increase in its production up to 192 h, reaching its maximum concentration of 35.6 mM, and being consumed after this period.

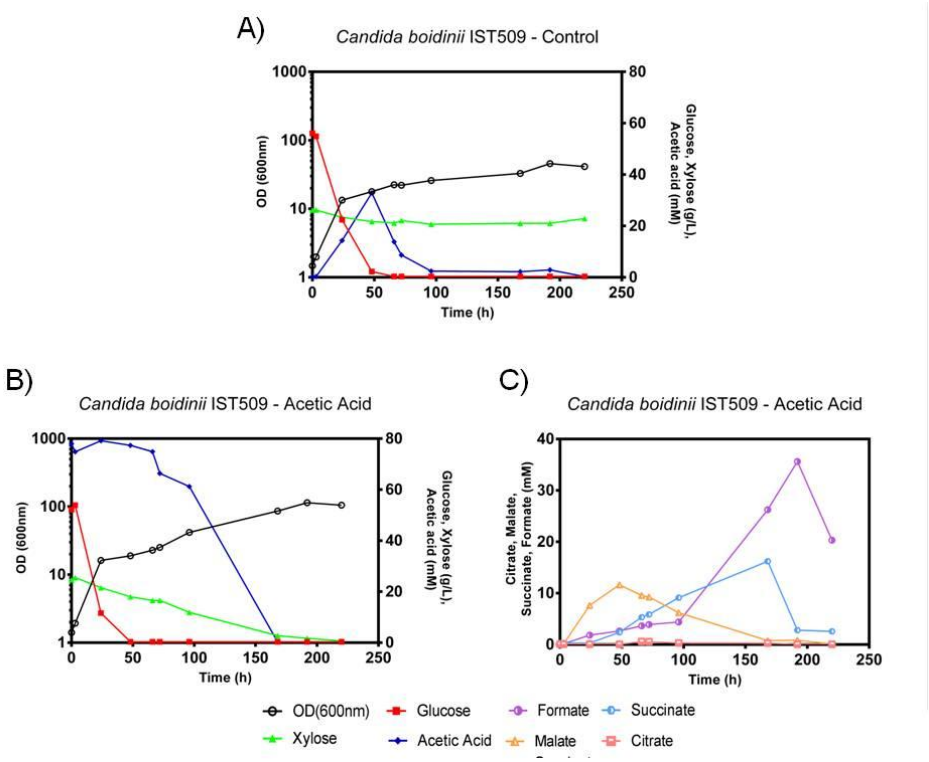

Figure 7 - Growth curves, carbon source utilization, and by-products' synthesis by *Candida boidinii* IST509. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (B and C) . Yeast growth (OD (600nm)), D-glucose, D-xylose and acetic acid are represented in A and B, and by-products synthesized by *C. boidinii* are represented in C.

In the absence of acetic acid (**Fig.8A**), *C. boidinii* IST599 was able to metabolize all D-glucose after 98 h of cultivation but the consumption rate of D-glucose was lower when compared to *C. boidinii* strain IST509 (**Fig.7A**). Strain IST599 also metabolized D-xylose, although at a slow rate, leaving nearly 9 g/L of residual concentration by 168 h of cultivation (**Fig.8A**). In comparison with strain IST509, acetic acid achieved a maximum peak later, consistent with a lower glucose metabolization rate by 62 h of cultivation, although a higher concentration of acetic acid was synthesized. This strain, in the absence of acetic acid, also produced small concentrations of succinate and citrate (**Fig.8B**). When acetic acid at pH 5.5 was added to the growth medium used above, *C. boidinii* IST599 (**Fig.8C**) completely metabolized D-glucose during the first 48 h of incubation, faster than in absence of acetic acid (**Fig.8A**). This behaviour has already been observed for strain IST509 in the same growth conditions (**Fig.7B**). D-xylose was also consumed and seemingly at a faster pace than in the absence of acetic acid (**Fig.8A**), leaving a residual concentration of 3.6 g/L by the end of the assay. Acetic acid was also produced from 24 h of cultivation up to 66 h, achieving a maximum peak of approximately 120 mM by 66 h of cultivation and thereafter it was consumed as C-source. Strain IST599 also produced by-products such as formate, succinate and malate (**Fig.8D**). Malate had a concentration peak of 11.34 mM, succinate and formate with concentration peak of 20.5 mM and 23.64 mM, respectively, by the end of the assay.

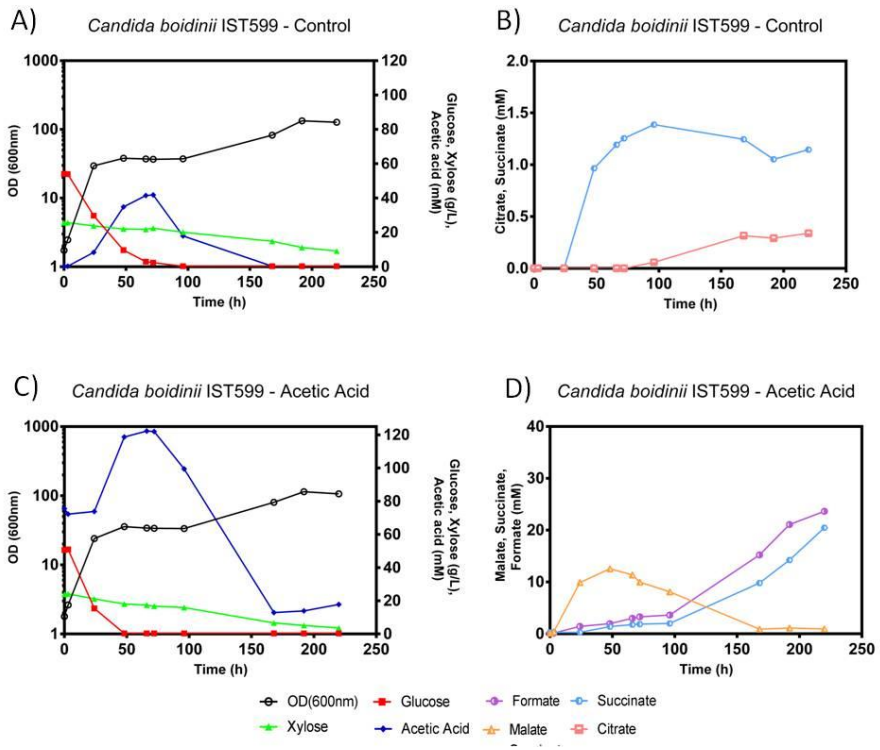

Figure 8 - Growth curves, carbon source utilization, and by-products' synthesis by *Candida boidinii* IST599. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect in yeast) (A and B) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (C and D) . Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid concentrations are represented in A and C, and by-products synthesized by *C. boidinii* are represented in B and D.

The growth curve, carbon sources´ consumption and synthesis of by-products by *K. ohmeri* IST538, is shown in **Figure 9**. In the absence of acetic acid (**Fig.9A**), *K. ohmeri* IST538 has the capacity to metabolize all the available D-glucose in the first 24 h of cultivation and also slowly metabolized D-xylose up to 48 h of cultivation, but then the consumption stopped prematurely leaving approximately 17.2 g/L of residual D-xylose concentration. This strain synthesized small concentrations of acetic acid in the first 24 h of incubation, reaching a peak of 5.7 mM. Biomass produced was maximal after 192 h of cultivation, with an OD600nm of 90.8. This strain also produced small concentrations of oxalate, citrate and formate (**Fig.9B**).

When acetic acid at pH 5.5 was added to the growth medium used above, *K. ohmeri* IST538 had the capacity to fully metabolize all the available D-glucose (**Fig.9C**), similar to when cultivated in media without of acetic acid (**Fig.9A**). The presence of acetic acid promoted the co-consumption of D-xylose. Acetic acid was synthesized in the first 24 h of cultivation, achieving a peak of 96.4 mM and was subsequently depleted. *K. ohmeri* IST538 achieved a higher biomass concentration, with an OD600nm of 192.3 at 192 h, in medium containing acetic acid (**Fig.9C**) than in the absence of acetic acid (**Fig.9A**), consistent with the catabolization of this additional C-source. In the presence of acetic acid, this strain also synthesized by-products such as succinate, citrate, malate, and formate (**Fig.9D**). Citrate synthesis started after 48h of cultivation and then achieved a peak at 72 h with a concentration of 7.1 mM. Contrary to control condition (**Fig.9B**), in the presence of acetic acid, citrate was synthesized later, but was not consumed after reaching a stationary phase. Formate concentration increased, being maximal at 192 h, with a concentration of 29 mM. In comparison with control condition (**Fig.9B**), the presence of acetic acid seems to stimulate the production of formate, reaching higher concentrations.

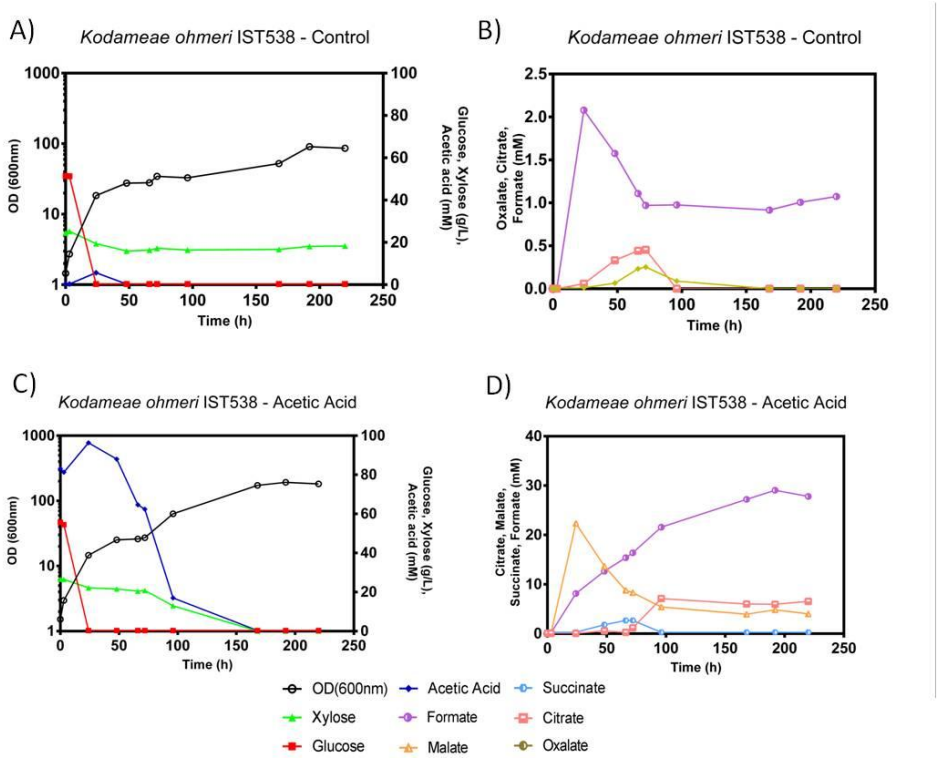

Figure 9 - Growth curves, carbon source utilization, and by-products' synthesis by *Kodameae ohmeri* IST538. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A and B) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (C and D) . Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid concentrations are represented in A and C, and by-products synthesized by *K. ohmeri* are represented in B and D.

The growth and carbon sources consumption of *Cr. flavescens* IST325, is shown in **Figure 10**. In the absence of acetic acid, *Cr. flavescens* IST325, (**Fig.10A**), did not metabolized D-xylose but has the capacity to metabolize D-glucose, leaving 11.8 g/L of this sugar after 144 h of cultivation (end of the assay). Strain IST325 reached stationary phase after 24 h of cultivation, with an OD600nm of 10.9. Biomass concentration increased slightly throughout cultivation time, reaching its maximum by 144 h, with an OD600nm of 17.2. When acetic acid at pH 5.5 was added to the growth medium used above (**Fig.10B**), *Cr. flavescens* IST325 also did not metabolize D-xylose and consumed slowly D-glucose, similarly to control conditions (**Fig.10A**). This strain was able to co-metabolize acetic acid and D-glucose, albeit at a slow rate.

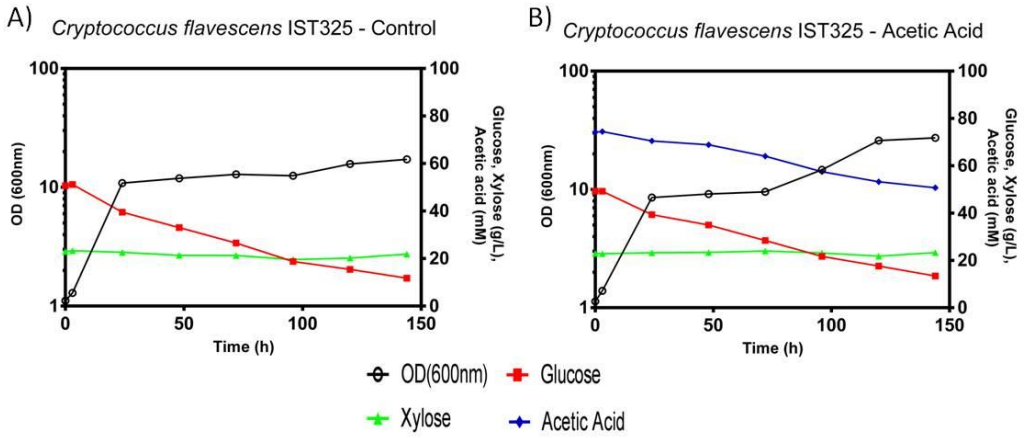

Figure 10 - Growth curves, carbon source utilization by *Cryptococcus flavescens* IST325. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (B) . Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid concentrations are represented in A and B.

The growth and sugar consumption by *Cy. slooffiae* IST547, is shown in **Figure 11**. In the absence of acetic acid, *Cy. slooffiae* IST547 (**Fig.11A**) has the capacity to metabolize slowly D-glucose and D-xylose, leaving 34.1 g/L of residual D-glucose and 10.4 g/L of residual D-xylose in the cultivation medium by the end of the assay. In the first 24 h of cultivation this strain reached stationary phase with an OD600nm of 17.9 and by 144 h it achieved a maximum growth peak with an OD600nm of 26.4. When acetic acid at pH 5.5 was added to the growth medium used above (**Fig.11B**), *Cy. slooffiae* IST547 metabolized faster D-glucose and D-xylose when compared to control condition (**Fig.11A**). This strain is capable of metabolizing acetic acid, having preference for this weak acid as carbon source than D-glucose and D-xylose. Remarkably, the presence of acetic acid in the growth medium had a positive effect on the co-consumption of D-glucose and D-xylose. Optical density at 600 nm after 144 h of cultivation had a maximal peak of 231.0, this OD600nm could be attributed to by-products not identified in the chromatograms that could possibly be influencing the respective OD600nm.

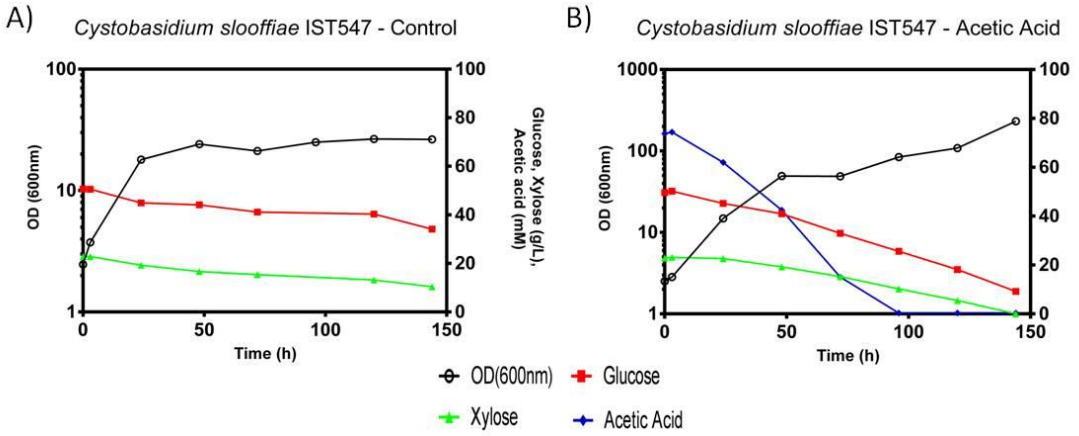

Figure 11 - Growth curves, carbon source utilization by *Cystobasidium slooffiae* IST547. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (B) . Yeast growth (OD (600nm)), D-glucose, D-xylose and acetic acid concentrations are represented in A and B.

The growth curve, sugar consumption and synthesis of by-products by *Meyerozyma guilliermondii* IST369 and IST502, is shown in **Figures 12** and **Figure 13**. In the absence of acetic acid (**Fig.12A**), *M. guilliermondii* IST369 metabolizes all the available D-glucose in 24 h of cultivation and metabolized D-xylose although at a slower rate when compared to D-glucose. This strain reached stationary phase after 24 h of cultivation and produced small concentrations of ethanol, succinate, xylitol, malate and glycerol (**Fig.12B**). Ethanol achieved a peak with a concentration of 2.2 % (v/v) by 24 h of cultivation. Succinate achieved a peak of 0.0164 mM at 24 h of cultivation. Xylitol achieved a peak by 98 h of cultivation with a concentration of 1.28 g/L. Glycerol is synthesized in the first 24 h of cultivation, achieves a maximal peak with a concentration of 0.269% p/v and afterwards is consumed slowly.

When acetic acid was added to the growth medium used above, *M. guilliermondii* IST369 fully metabolized all the available D-glucose in the first 24 h of inoculation (**Fig.12C**), similar to when cultivated in media without of acetic acid (**Fig.12A**), and also co-metabolized D-xylose with acetic acid and glucose, being completely depleted by 72 h of cultivation. Again, the presence of acetic acid at pH 5.5 promoted D-xylose consumption. Acetic acid is metabolized, showing a concentration of 9.9 mM by 48 h of cultivation. After this time of incubation, when all the initial sugars were exhausted acetic acid concentration suffered an increase synthesis, reaching a peak of 18.8 mM by 96 h of cultivation, being consumed afterwards. Regarding the growth curve the stationary phase was reached after 48 h of cultivation, with a final OD600nm of 79.8. As for the growth medium without acetic acid at pH 5.5, *M. guilliermondii* IST369 also produced small concentrations of ethanol, succinate, xylitol, malate and glycerol (**Fig.12D**). The profiles of production of these metabolites, followed by their consumption followed, in general, what was described before for the medium not supplemented with acetic acid (**Fig.12B**).

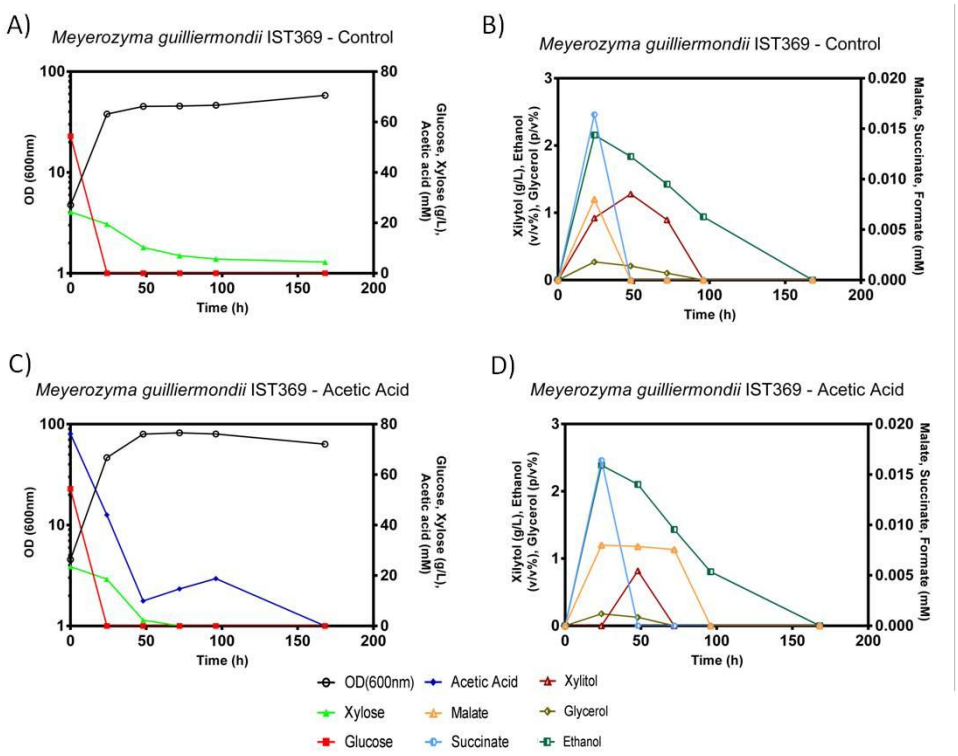

Figure 12 - Growth curves, carbon source utilization, and by-products' synthesis by *Meyerozyma guilliermondii* IST369. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A and B) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (C and D). Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid are represented in A and C, and by-products synthesized by *Meyerozyma guilliermondii* are represented in B and D.

In the case of the other stain tested of *M. guilliermondii* IST502 (**Fig.13A**), its capacity to use the sugars in the absence of acetic acid was less efficient than strain IST369. When acetic acid was present in the medium the co-metabolization of the three C-sources improved their catabolism and cell growth, as discussed above. This strain also produces small concentrations of ethanol and glycerol in the medium without acetic acid where the initial concentrations of sugars were not fully used (**Fig.13B**). When acetic acid was present, the utilization of the three C-sources by this strain was efficient and higher concentrations of ethanol and glycerol were produced compared with the medium without acetic acid (**Fig.13C** and **Fig.13D**). Moreover, succinate and malate were also synthesized as observed for strain IST369. The performance of strain IST502 in the medium with acetic acid mimicking lignocellulosic hydrolysates was close to strain IST369.

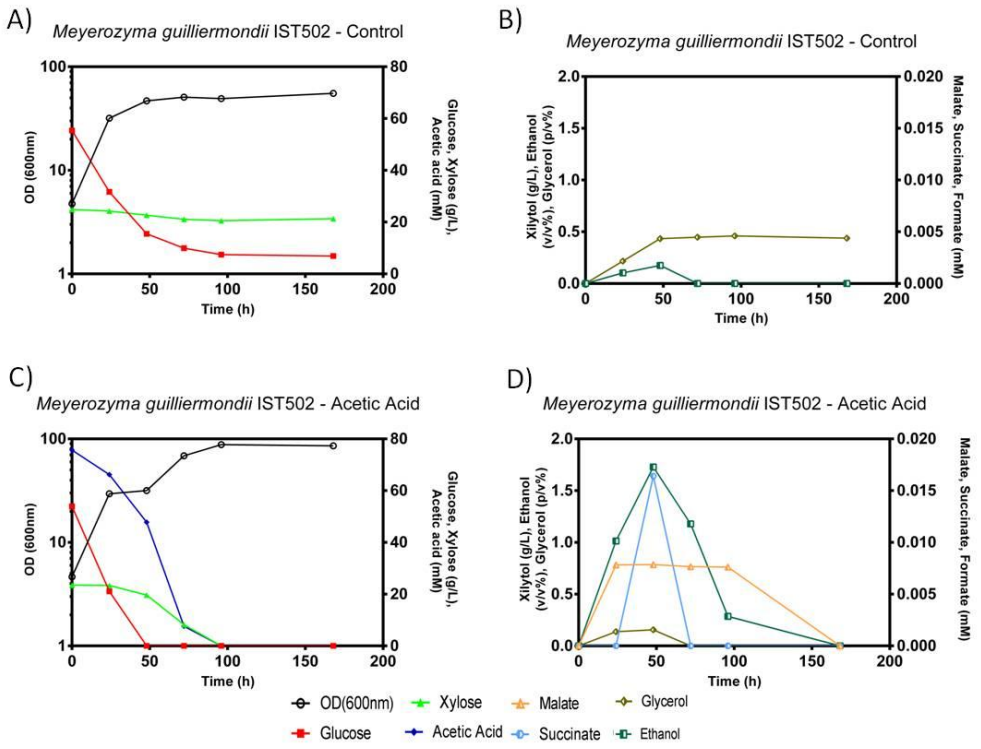

Figure 13 - Growth curves, carbon source utilization, and by-products' synthesis by *Meyerozyma guilliermondii* IST502. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A and B) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (C and D) . Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid concentrations are represented in A and C, and by-products synthesized by *Meyerozyma guilliermondii* are represented in B and D.

In the absence of acetic acid, *L. starkeyi* IST532 fully metabolized D-glucose and D-xylose (**Fig.14A**). Glucose was consumed first and by 96 h of cultivation, all D-glucose available in the medium was completely exhausted. D-xylose was metabolized slower when compared to D-glucose, having still a concentration of 4.2 g/L by the end of the assay, corresponding to 220 h of cultivation. When acetic acid at pH 5.5 was added to the growth medium used above, *L. starkeyi* IST532 showed the capacity of metabolizing acetic acid, consuming this carbon source first and then D-glucose and, lastly, D-xylose. *L. starkeyi* IST532 produced formate, malate and citrate (**Fig.14C**). Formate had a concentration peak of 3.9 mM and malate a maximal concentration of 1.6 mM. Citrate had a stationary phase at 72 h with a concentration of approximately 1.0 mM.

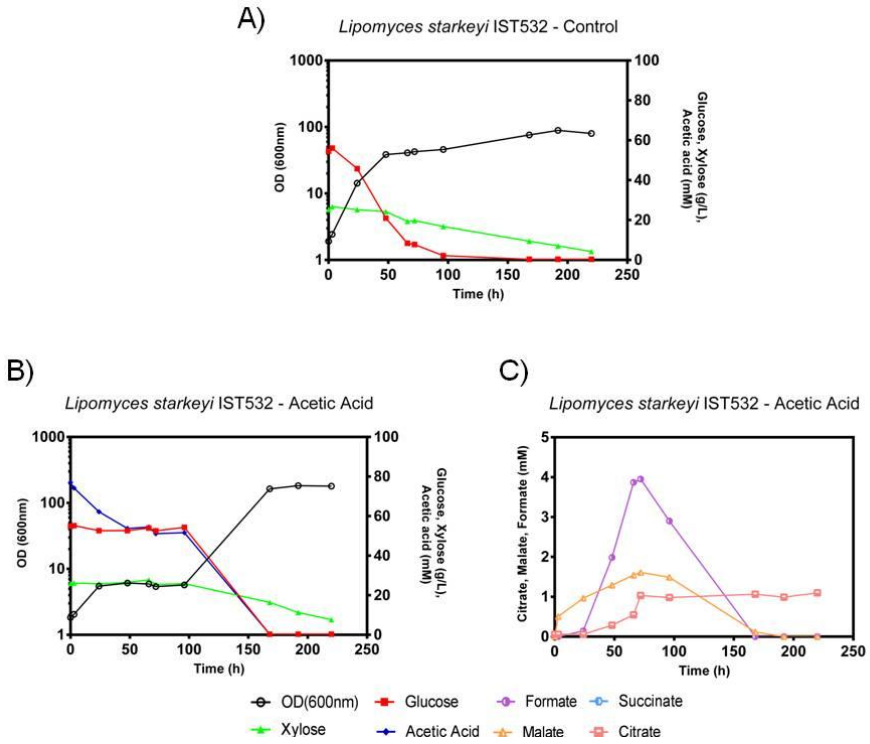

Figure 14 - Growth curves, carbon source utilization, and by-products' synthesis by *Lipomyces starkeyi* IST532. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (B and C) . Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid are represented in A and B, and by-products synthesized by *Lipomyces starkeyi* IST532 are represented in C.

In the absence of acetic acid, *L. starkeyi* IST533 shows a catabolized D-glucose slowly and was unable to fully consume all D-xylose available (**Fig.15A**). Glucose consumption profile reached a stationary phase after 168 h of cultivation and approximately 20.8 g/L of this sugar was still left in the growth medium. Contrary to strain IST532 (**Fig.14A**), strain *L. starkeyi* IST533 was unable to fully metabolize D-xylose and synthesized acetic acid after 98 h of cultivation. Interestingly, and contrary strain IST532 grown in control conditions (**Fig.14B**), strain IST533 synthesized by-products such as: citrate, malate, succinate and formate (**Fig.15B**). Citrate reached a concentration peak with 1.2 mM and malate had a maximal concentration of 4.8 mM. Succinate was synthesized later, compared to citrate and malate, and reached a concentration peak of 1.4 mM. Formate had a concentration peak of 1.6 mM, having similar concentration values afterwards.

When acetic acid at pH 5.5 was added to the growth medium used above, *L. starkeyi* IST533 was not capable to metabolize D-glucose and D-xylose, although it metabolized acetic acid, consuming it in the first 48 h of cultivation (**Fig.15C**). In this period, acetic acid concentration decreased to approximately 40 mM and remained constant from 48 h to 72 h. Thereafter, acetic acid was consumed again, albeit in a slower pace, reaching a second stationary phase by 192 h since inoculation with a concentration of 11.0 mM.

Strain IST533 was able to synthesize organic acids such as citrate, malate, succinate and formate (**Fig.15D**). Citrate reached a maximum concentration peak with 2.9 mM, and after 98 h of cultivation was completely depleted. Malate had a concentration of 5.3 mM, with similar concentration until 72h, where afterwards was metabolized again. Citrate reached a maximum concentration peak of 3.4 mM. Formate had a maximal concentration of 14.1 mM. Succinate reached a maximum concentration of 8.8 mM before being metabolized afterwards. The presence of acetic acid promotes higher synthesis of these organic acids (**Fig.15D**) when compared to control condition (**Fig.15C**).

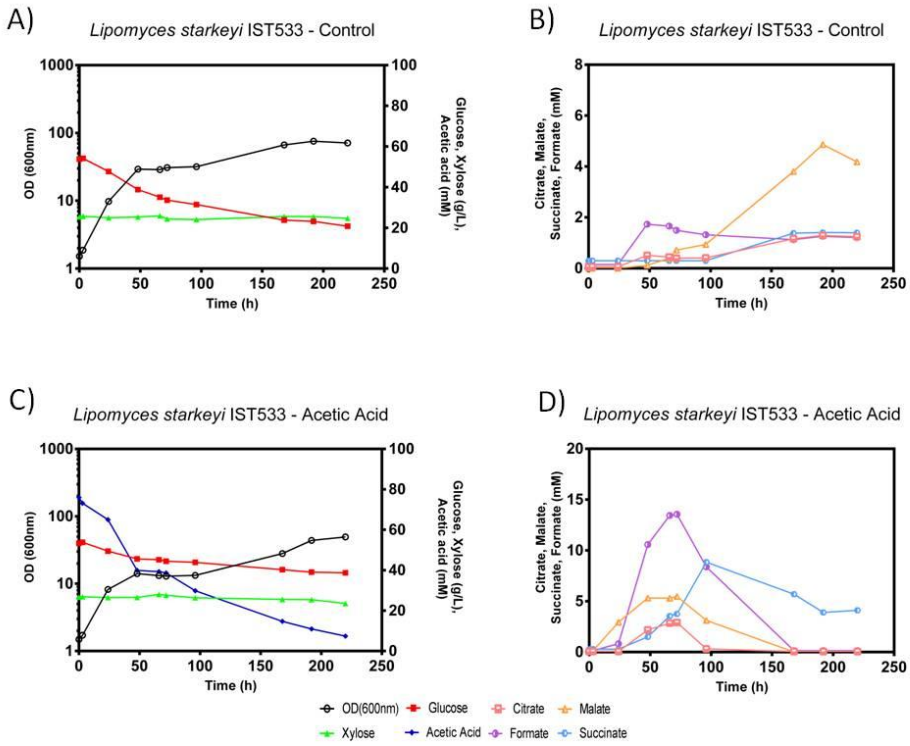

Figure 15 - Growth curves, carbon source utilization, and by-products' synthesis by *Lipomyces starkeyi* IST533. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A and B) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (C and D). Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid are represented in A and C, and by-products synthesized by *Lipomyces starkeyi* IST533 are represented in B and D.

The effect of decreasing the initial pH of the growth media was examined in *Lipomyces tetrasporus* IST531, using a pH 5.0 and pH 5.5 and an initial OD600nm of 4 (**Figure 16**). In the absence of acetic acid at pH 5.0, *L. tetrasporus* IST531 (**Fig.16A**) was capable of metabolizing D-glucose and D-xylose, although D-xylose consumption was slower than D-glucose consumption. In terms of growth, strain IST531 reached a maximum OD600nm of 58.0 by 96 h since inoculation. When acetic acid was added to the growth medium used above at pH 5.0 (**Fig.16B**) this strain did not metabolize D-glucose and D-xylose. It consumed approximately 10 mM of acetic acid and regarding growth, it reached a maximum OD600nm of 6.3 by the end of the assay, corresponding to 168 h of cultivation.

In the absence of acetic acid at pH 5.5 (**Fig.16C**), this strain showed a similar consumption profile compared to control condition at pH 5.0 (**Fig.16A**). Regarding growth, it reaches a maximum OD600nm of 63.8 by 96 h of cultivation. In the presence of acetic acid at pH 5.5 (**Fig.16D**), *L. tetrasporus* IST531 metabolizes acetic acid, consuming it first than D-glucose and D-xylose. Acetic acid, at 24 h of cultivation, has a concentration of 37.0 mM and reaches a stationary phase between 24 h up to 96 h, thereafter it begins to be consumed again being completely depleted by the end of the assay. D-glucose starts being metabolized at 96 h and by 168 h it still has a concentration of 25.7 g/L present in the growth medium. Regarding growth, it had a stationary 24 h to 96 h of cultivation with and OD approximately of 12 and by the end of the assay it had an OD of 54.4, being in exponential phase.

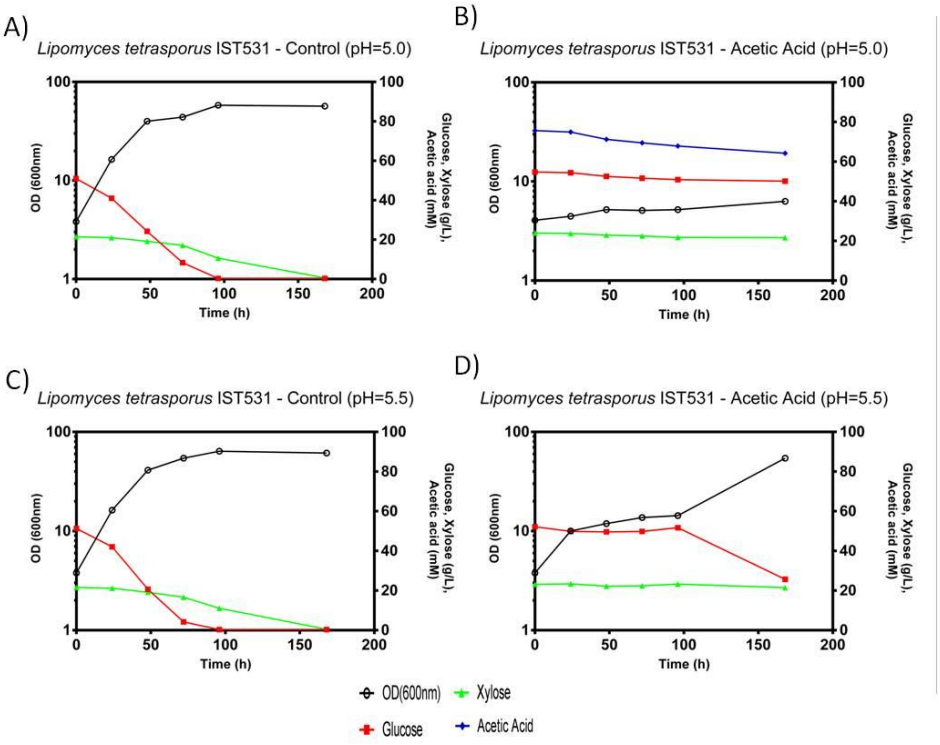

Figure 16 - Growth curves, carbon source utilization by *Lipomyces tetrasporus* IST531. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.0 (A and B) and at pH 5.5 (C and D), containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A and C) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (B and D). Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid are represented in A, B, C and D.

4.2.1. Preliminary screening of lipid production by selected yeasts in a culture medium mimicking lignocellulosic biomass hydrolysates

In order to determine whether *C. boidinii* strains IST509 and IST599, *K. ohmeri* IST538, *Cr. flavescens* IST325, *Cy. slooffiae* IST547, *L. tetrasporus* IST531 and *L. starkeyi* strains IST532 and IST533 are able to produce lipids during cultivation in a synthetic medium mimicking lignocellulosic biomass hydrolysates, the relative fluorescence unit (RFU) of strains cells stained with Nile Red was determined and shown in **Figure 17**.

Overall, *C. boidinii* strains IST509 and IST599, *K. ohmeri* IST538, *Cr. flavescens* IST325 and *Cy. slooffiae* IST547 showed low RFU when compared, in the same conditions, to *L. tetrasporus* IST531 and *L. starkeyi* strains IST532 and IST533. *C. boidinii* strains IST509 and IST599, *K. ohmeri* IST538 and *L. starkeyi* IST532 had higher RFU in control conditions (media containing 50 g/L of D-glucose and 25 g/L of D-xylose) compared to the same media supplemented with 4.5 g/L acetic acid, at pH 5.5, indicating that acetic acid negatively impacts lipid production for these species.

Regarding the RFU obtained for *L. tetrasporus* IST531, overall there seems to not be a significant difference between control condition (media containing 50 g/L of D-glucose and 25 g/L of D-xylose) and in the same medium supplemented with 4.5 g/L acetic acid, both for pH 5.0 and pH 5.5.

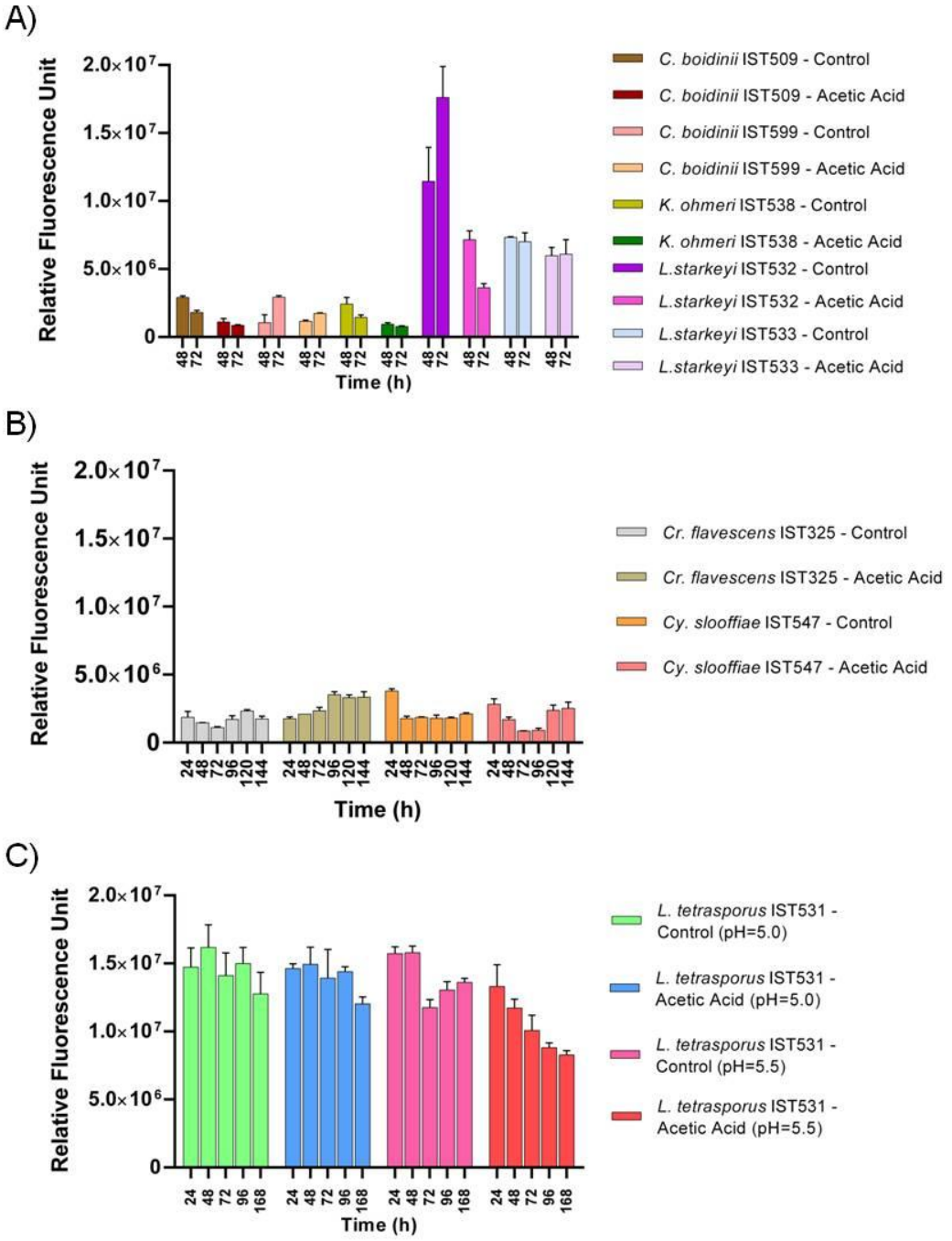

Figure 17 - **Relative Fluorescence Units (RFU) of *C. boidinii* (strains IST509 and IST599), *K. ohmeri* IST538, *Cr. flavescens* IST325, *Cy. slooffiae* IST547, *L. starkeyi* (strains IST532 and IST533) and *L. tetrasporus* IST531 cells**. *C. boidinii* strains IST509 and IST599, *K. ohmeri* IST538, *L. starkeyi* strains IST532 and IST533 cells after 48 h and 72 h of incubation in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (control condition) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid; B) RFU for *Cr. flavescens* IST325 and *Cy. slooffiae* IST547 cells, in the same media previously mentioned, after 24 h, 48 h, 72 h, 96 h, 120 h and 144 h of cultivation; C) RFUs values for *L. tetrasporus* IST531 cells after 24, 48, 72, 96 and 168 h of incubation, in the same media previously mentioned, at pH 5.5 or pH 5.0. Lipid production was assessed by Nile Red staining with a normalized cell suspension (OD600nm = 1) and based on RFU.

In order to assess the presence of lipid droplets inside *L. tetrasporus* IST531 cells when cultivated in media mimicking lignocellulosic biomass hydrolysates containing 50 g/L D-glucose and 25 g/L D-xylose (control condition) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid, at pH5.5, microscopic observations of cells stained with Nile Red were carried out (**Figure 18** and **Figure 19**). The fluorescence microscopy observations evidence the presence of a large single lipid droplet inside *L. tetrasporus* IST531 cells. At pH 5.5, in the presence of acetic acid, there seems to be a correlation between RFU and florescence intensity observed in the microscopy. Indeed, at 24 h of cultivation, florescence was higher and then gradually decreased through incubation time (**Fig.19**), similar to what has been previously observed (**Fig.17C**). At pH 5.5, in control conditions, it was not possible to correlate the apparent fluorescence intensity of lipid droplets (**Fig.18**) and RFU values previously obtained (**Fig.17C**), as lipid droplets showed a similar intense yellow color throughout incubation time, but RFU values were maximal after 24 and 48 h of incubation.

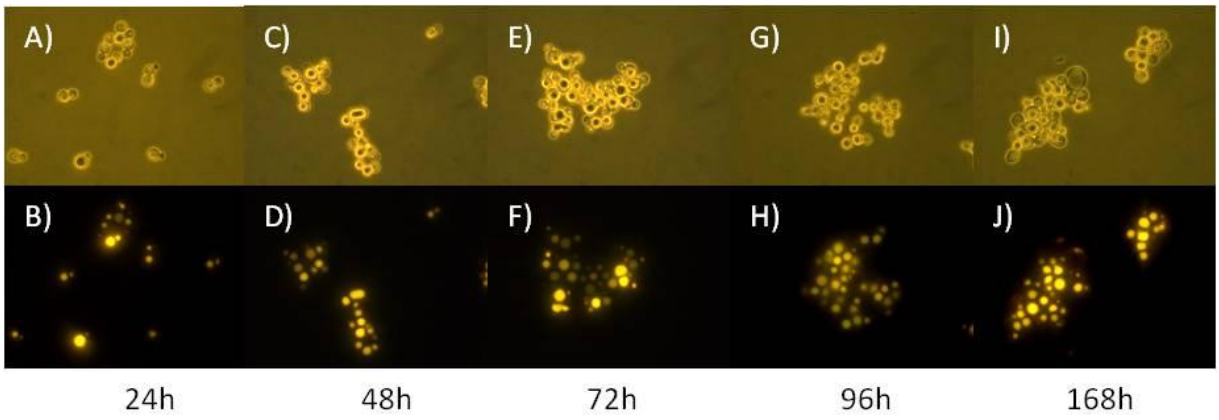

Figure 18 – Microscopic observations of *L. tetrasporus* IST531 cells stained with Nile Red fluorescence dye during cultivation in control media at pH 5.5. Cells were observed and photographed, with a total magnification of 1000x, at several time points along the growth curves depicted in **Fig.16C**. A, C, E, G, I were taken using bright-field microscopy and B, D, F, H and J were taken using epifluorescence microscopy.

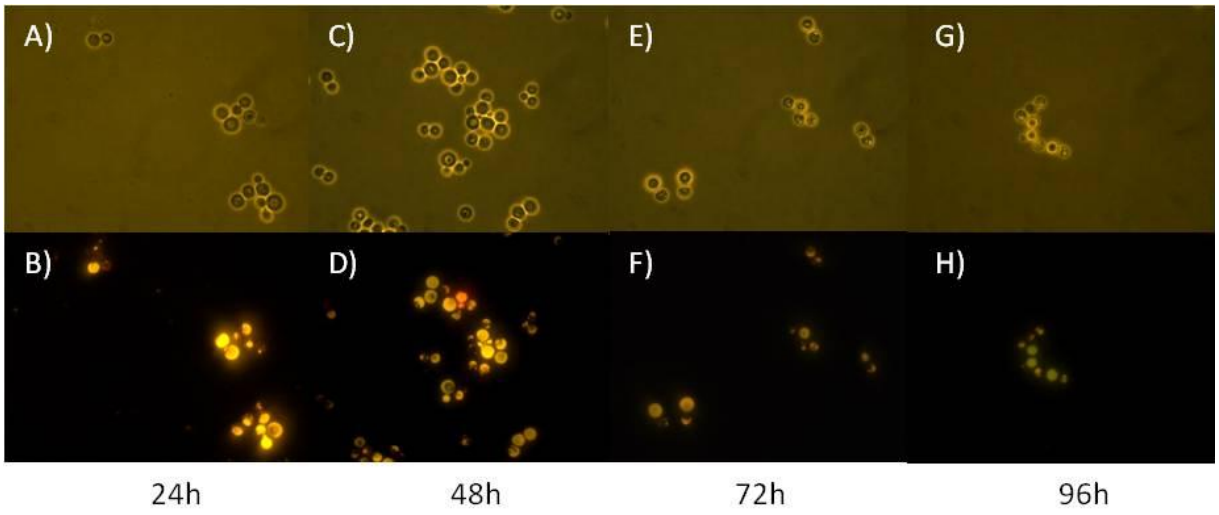

Figure 19 - Microscopic observations of *L. tetrasporus* IST531 cells stained with Nile Red fluorescence dye during cultivation in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5. Cells were observed and photographed, with a total magnification of 1000x, at several time points along the growth curves depicted in **Fig.16D**. A, C, E and G were taken using bright-field microscopy and B, D, F and H were taken using epifluorescence microscopy.

5. Discussion

Oleaginous yeasts are gaining popularity for the bioconversion of agro-industrial biomass residues, which contain mixtures of different carbon sources, into a wide variety of added value bioproducts with applications in the biotechnological, food, and pharmaceutical industries (2,58). Due to their catabolic and unusual biosynthetic abilities, a number of yeast species has been proposed as promising cell factories for the recycling of agro-forest industrial residues (39).

In this study, a total of 23 yeast strains from 8 different promising oleaginous species, *Rhodotorula babjevae, R. mucilaginosa, R. glutinis, R. toruloides, Meyerozyma caribbica, M. guilliermondii, Debaryomyces hansenii and Cystobasidium slooffiae* from the IST yeast culture collection were tested to evaluate their potential for the catabolization of different carbon sources present in hydrolysates of different biomass residues. Out of all the strains tested, the one that showed more potential for biotransformation of hydrolysates from pectin-rich residues was *R. mucilaginosa* IST461 due to its effective catabolization of D-galacturonic acid and L-arabinose, the two major challenging carbon sources present in these hydrolyzates (137). The catabolization of D-galacturonic acid and L-arabinose present in sugar-beet pulp hydrolysates was reported before for *R. toruloides* PYCC 5615^{T} and *R. mucilaginosa* IST 390 (134), confirming the potential of *Rhodotorula* species for the bioconversion of these hydrolysates (39,134).

Rhodotorula sp. strain IST323, whose previous identification was inconclusive between *R. babjevae* and *R. diobovatum,* showed a different profile of growth and carbohydrate metabolization when compared to the other *R. babjevae* strains examined (IST537, IST549, IST550), being unable to consume D-xylose and catabolizing D-galactose much slower. This may indicate that strain IST323 is not from the *R. babjevae* species, but from *R. diobovatum.* Comparison of strain IST323 with other *R. diobovatum* strains regarding assimilation of different carbon sources, as well as the amplification and sequencing of ITS and/or other regions usually used for yeast identification should be conducted to identify this species (138,139). If in fact this strain is *R. diobovatum,* further experiments could be conducted to investigate its potential in the production of added-value products. For instance, *R. diobovatum* species has been investigated for a variety of new applications (140) that include, for example, the production of glutathione (GSH), a vitamin supplement with antioxidant properties that may also be used as an anti-toxicant, a cell metabolism regulator, and a neuromediator (141,142).

The results regarding *R. mucilaginosa* (strains IST390, IST423 and IST461) also showed the importance of screening different strains within the same species due do intraspecific variability, a characteristic already reported in literature for this species due to its genetic and phenotypic diversity (143). The growth profiles obtained when these strains were grown on D-galacturonic acid were different and the metabolization of D-galactose by IST390 and IST423 was also distinct. This intraspecific variation can be generated by a number of factors in nature, including local adaptation, artificial selection, parental conditions, and phenotypic plasticity (6) and has impact not just on carbohydrates catabolization, but also on lipid production. Since lipid accumulation differs amongst strains of the same species, yeasts' oleaginous potential should not be assessed at the species level but rather at the strain level, given *Rhodotorula* genus high intraespecific variability (5,143). For example, two distinct strains of *R. mucilaginosa* were investigated by Garay et al. 2016, and their lipid accumulation ranged from 20 to 45 % of cdw (144). To fully assess the potential for fermentation of agro-forest residues hydrolysates, it is important to isolate different strains from different ecosystems, in order to select the best strains and not to select only based on the species. Considering the results previously obtained for *R. mucilaginosa* IST390 (134) and the results obtained herein for *R. mucilaginosa* IST461, it would be interesting, in future assays, to analyze also how this later strain catabolizes mixture of carbohydrates present in sugar-beet pulp hydrolysates.

Meyerozyma caribbica (strains IST494, IST541, IST542, IST601) and *Meyerozyma guilliermondii* (IST502, IST587 and IST591), in general, showed higher final biomass concentrations when grown on D-galactose. However, these strains did not catabolize galacturonic acid, indicating that they are not suitable for the conversion of pectin-rich hydrolysates, since D-galacturonic acid is one of the main sugars present in these hydrolysates. Based on the results obtained in this assay, it would be interesting to further cultivate these *Meyerozyma* strains in media whose single carbon source is lactose, to evaluate if they are capable of growing and metabolize this carbohydrate. If they are capable of metabolizing lactose, these strains could be used for other industrial residues such dairy residues like cheese whey (CW), a far less studied substrate when compared to pectin-rich agro-industrial residues. CW is a liquid waste produced by the cheese-making process in the dairy industry. Despite the fact that CW is nutrient-dense and has a high usage potential, half of it is discarded without treatment (145). Because only a few yeasts have the lactose permease and β -galactosidase enzymes essential for lactose digestion, oleaginous yeast growth on CW medium is constrained (146). Nevertheless, a strain of *M. guilliermondii* was reported in literature as able to grow in cheese whey substrate as the only source of nutrients, supporting further experiments using those strains (147). Other yeast species like *R. glutinis* and *R. mucilaginosa* (148), *D. hansenii* (149) have been found to store lipids when cultivated in CW media.

Cystobasidium slooffiae, among all species and strains tested, showed the lowest final biomass concentration and was able to grow on D-glucose, D-xylose and L-arabinose, but not on D-galacturonic acid. This finding is in agreement with literature (14). This species was also reported to grow on cellobiose, thus being more suitable for biotransformation of other substrates where cellobiose is expected to be present in hydrolysates obtained from cellulose-rich residues (150). Nevertheless, *Cy. slooffiae* is poorly studied in literature, so further investigation is required to determine its full potential and to assess the formation of other value-added byproducts that could increase its potential for industrial applications.

In the present study, 10 strains from 7 different species, *Candida boidinii, Kodamaea ohmeri, Cryptococcus flavescens, Lipomyces tetrasporus and L. starkeyi,* were also grown in a synthetic medium mimicking lignocellulosic biomass hydrolysates to evaluate their ability to use the various carbon sources when present simultaneously in the culture medium and to determine their potential to produce lipids. Due to carbon catabolite repression (CCR) regulation, the sequential use of multiple sugars in biomass hydrolysates is a limitation (151). This regulatory mechanism restricts the effective simultaneous use of numerous carbon substrates in biotechnological processes such as those designed to valorize pectin-rich or lignocellulose-based agro-industrial residues. In fact, in the presence of the preferred substrate (D-glucose), the metabolization of secondary carbon sources (e.g. L-arabinose, D-galacturonic acid, D-xylose) is repressed, lengthening fermentation duration as a result of sequential, rather than simultaneous, usage of the carbon sources (152,153). This phenomenon is well described in *S. cerevisiae* (154), but, in general, non-conventional yeasts also have a preference for D-glucose over other carbon sources. The ability to metabolize all sugars present in a mixture is sometimes referred to as sugar co-consumption and can occur in three situations. Firstly, yeast species may consume sugars in a sequential manner, with a preference for glucose due to carbon catabolite repression (155), or they may co-consume glucose and pentoses because initial glucose concentrations are low, this sugar is immediately consumed and thus does not exert carbon catabolite repression (156), or, lastly, yeasts can co-consume freely glucose and other carbon sources like pentoses (157). In this study, glucose repression was observed in *L. starkeyi* IST532 and IST 533 in the absence of acetic acid: D-glucose repressed D-xylose catabolization, which prolonged the fermentation period owing to the sequential, rather than simultaneous, utilization of these two sugars. On the other hand, when in the presence of acetic acid, the acid had a negative impact in the consumption of D-glucose that never reached derepressing concentrations to allow D-xylose consumption by the two *L. starkeyi* strains. Remarkably, consumption of acetic acid by strain *L. starkeyi* IST532 was not repressed by D-glucose, contrary to what has been observed in *S. cerevisiae*. The lack of carbon catabolite repression was also observed in *Cy. slooffiae* IST547, where acetic acid was rapidly consumed first and then there was co-consumption of D-glucose and D-xylose. In *Meyerozyma guilliermondii* IST369 where there was also co-consumption of acetic acid, D-glucose and D-xylose and in *Cr. flavescens* IST325 there was co-consumption both of acetic acid and D-glucose and higher final biomass concentrations were achieved.

Remarkably, the presence of acetic acid seemed to promote D- xylose consumption by *C. boidinii* IST509 and IST599, *Cy. slooffiae* IST547, *K. ohmeri* IST538, *M. guilliermodii* IST369 and IST502. The molecular mechanisms behind such extraordinary and desired characteristic should be further explored. Acetate and xylose were co-consumed by an engineered *S. cerevisiae* strain (158). Acetate was assimilated as a secondary carbon, via the acetate reduction pathway, which consists of acetyl-CoA synthase and acetaldehyde dehydrogenase with NADH oxidation and acetate conversion to ethanol (159). On the other hand, D-xylose was metabolized via the XR/XDH route, producing D-xylulose and a surplus NADH, which serves as a reducing equivalent for acetate reduction (158,160). This process allows for the co-utilization of xylose and acetate, as well as acting as a redox sink for excess NADH (151). It is likely that *C. boidinii* IST509 and IST599, *Cystobasidium slooffiae* IST547, *K. ohmeri* IST538, *Meyerozyma guilliermodii* IST369 and IST502 have an intrinsically similar mechanism where the presence of acetic acid could promote D-xylose consumption by counter-acting the effects of excess of NADH by the XR/XDH pathway.

Citrate synthesis and accumulation was observed in *L. starkeyi* IST532 and *L. starkeyi* IST533. This citrate accumulation could be due to an increased activity of AMP deaminase that causes a reduction in adenosine monophosphate (AMP) concentration, the AMP-regulated mitochondrial isocitrate dehydrogenase ICDH is deactivated, resulting in an excess of mitochondrial citrate that is exported to the cytosol and used as a substrate for ATP-citrate lyase (ACL) to produce cytosolic acetyl-CoA, a lipogenesis precursor (161).

When in the presence of acetic acid at pH 5.5, *C. boidinii* IST599 and *L. starkeyi* IST533 produced higher succinate concentrations compared with their cultivation in the absence of acetic acid. Similar findings have been reported when a co-culture of *Aspergillus niger*, *Phanerochaete chrysosporium*, and *Trichoderma reesei* in a slurry fermentation set-up revealed that a low starting concentration of acetic acid, succinic acid production decreased, while higher starting acetic acid concentrations resulted in increased succinic acid synthesis (162). However, no reports, as of the time of the writing of this thesis, have been found regarding this effect on *C. boidinii and L. starkeyi.*

Lipomyces genus strains examined showed higher Relative Flurescence Unit (RFU) values, associated to lipid accumulation, when compared to the remaining strains studied: *Candida boidinii* (strains IST509 and IST599), *Kodamaea ohmeri* IST538, *Cryptococcus flavescens* IST325 and *Cystobasidium slooffiae* IST547. This indicates that these last strains mentioned are less suitable for lipid production under the conditions tested. However, it is important to note that not all RFU measurements were performed at the same stage of the growth curve across the different strains, since storage lipids accumulate in yeast during stationary phase, mostly of the assays were performed during this stage (163–165).

It is possible that assimilation and conversion of acetate to acetyl-CoA,improve, theoretically, lipid production since acetyl-CoA is a precursor to lipid biosynthesis (93,166). This effect is observed in *Cr. flavescens* IST325 where the presence of acetic acid at pH 5.5 led to an increase in RFU when compared to control condition. It has been reported that *Cr. curvatus, a* species close to *Cryptococcus flavescens,* over-produced lipids and achieved 73.4 % of lipid content when acetate was used as a sole carbon source at pH 5.5 (93). In future work, testing the production of lipids by *Cryptococcus flavescens* IST325 when cultivated in a medium containing acetate as the only carbon source could be performed to asses lipid production.

Interestingly, *L. tetrasporus* IST531, showed higher RFU than *L. starkeyi* IST533, indicating that is a species worth to further study and experimenting upon since this species is far less studied compared to *L. starkeyi.* In future assays, in order to obtain more relevant information regarding lipid biosynthesis from *L. tetrasporus* IST531, gravimetric determination of biomass and of total lipids could be perform in order to determine lipid yield, lipid content expressed as a proportion of lipid weight as a percentage of dry biomass weight (w/w %) and lipid productivity. Furthermore, lipid profile could be determined by GC-FID in order to assess linoleic acid, oleic acid, stearic acid and palmitic acid concentration as performed by other studies in this field (12).

The inhibitory effect of lower pH is observed in *L. tetrasporus* IST531 when cultivated in the presence of acetic acid at pH 5.0, where lower biomass concentration was achieved and D-glucose just slightly consumed when compared in the same medium at pH 5.5 where growth was not affected and neither the consumption of D-glucose. Since acetic acid has a pKa of 4.75, at pH 5.0 there is a higher percentage of undissociated acid than at pH 5.5. Weak acids, such as acetic acid, in its undissociated form can freely permeate the plasma membrane. When in the neutral pH of the cytosol, the acid dissociates and leads to a decrease in the internal pH and causes the growth-inhibiting impact on microorganisms (95,96,167).

In the process of accumulating storage lipid, the amount of biomass produced is frequently determined by the concentration of the limiting nutrient (nitrogen), whereas the amount of accumulated lipid is largely determined by the concentration of the carbon source (e.g., D-glucose) found in excess in the growth environment. As a result, the C/N molar ratio is critical in determining the oil content and biomass density of oleaginous microbes (168). Therefore in future assays, it is important to examine different growth conditions such as carbon source, C/N ratio, medium components, and culture conditions such as temperature and pH, in order to determine the ideal conditions for lipid synthesis (169).

6. Concluding Remarks and Future Perspectives

The non-conventional yeasts species/strains investigated in this thesis work provided interesting and useful results that highlight the potential of non-conventional yeasts as suitable cell factories. Some strains, such as *L. tetrasporus* IST531, were found to have the potential to be further investigated in the future to optimize the growth conditions leading to higher lipid production, quantification and subsequent characterization of the lipids produced by these strains,. It would also be interesting to provide essential genetic material to allow the engineering of promising yeast cell factories strains to develop superior strains for the bioconversion of agro-forest residues into value-added products.

In *M. guilliermondii* IST369, and *Cr. flavescens* IST325, there was no evidence of carbon catabolite repression in which co-consumption of acetic acid and D-glucose was observed and in *C. boidinii* (strains IST509 and IST599), *Cy. slooffiae* IST547, *K. ohmeri* IST538, *M. guilliermodii* (strains IST369 and IST502), the presence of acetic acid at pH 5.5 seemed to promote D-xylose consumption. In future assays, these industrially desirable methabollic characterisctics should be further studied to understand the genetic and biochemical pathways that underpin such unique and desirable traits.

This thesis work further confirmed the importance of screening different strains within the same species for specific purposes due do intraspecific variability. Namely in *R. mucilaginosa* and *D. hansenii* were a high variability in terms of growth and metabolization of sugars was observed in different strains. *R. mucilaginosa* IST461 exhibited the higher potential for biotransformation of pectin rich hydrolysates out of all the strains studied due to its' efficient catabolization of D-galacturonic acid and L-arabinose. In future experiments, it would be interesting to see how this strain catabolizes a mixture of carbohydrates present in sugar-beet pulp hydrolysates. *Meyerozyma caribbica* and *Meyerozyma guilliermondii* metabolized efficiently D-glucose and D-galactose and in future experiments, it would be interesting to test if they metabolize lactose; if they are capable of metabolizing this carbohydrate, these strains could be further tested for other industrial residues such as dairy residues like cheese whey (CW), a substrate that is less studied in literature than pectin-rich agro-industrial residues.

7. References

1. Cristobal-Sarramian A, Atzmüller D. Yeast as a production platform in biorefineries: conversion of agricultural residues into value-added products. 2018;476.4Kb.
2. Kręgiel D, Pawlikowska E, Antolak H. Non-Conventional Yeasts in Fermentation Processes: Potentialities and Limitations. In: Lucas C, Pais C, editors. Old Yeasts - New Questions [Internet]. InTech; 2017 [cited 2021 Oct 6]. Available from: http://www.intechopen.com/books/old-yeasts-new-questions/non-conventional-yeasts-in-fermentation-processes-potentialities-and-limitations
3. Ratledge C. Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie. 2004 Nov;86(11):807–15.
4. Rebello S, Abraham A, Madhavan A, Sindhu R, Binod P, Babu AK, et al. Non-conventional Yeast cell factories for sustainable bioprocesses. FEMS Microbiol Lett [Internet]. 2018 Sep 13 [cited 2021 Oct 18]; Available from: https://academic.oup.com/femsle/advance-article/doi/10.1093/femsle/fny222/5096020
5. Miranda C, Bettencourt S, Pozdniakova T, Pereira J, Sampaio P, Franco-Duarte R, et al. Modified high-throughput Nile red fluorescence assay for the rapid screening of oleaginous yeasts using acetic acid as carbon source. BMC Microbiol. 2020 Dec;20(1):60.
6. Des Roches S, Post DM, Turley NE, Bailey JK, Hendry AP, Kinnison MT, et al. The ecological importance of intraspecific variation. Nat Ecol Evol. 2018 Jan;2(1):57–64.
7. Munch G, Sestric R, Sparling R, Levin DB, Cicek N. Lipid production in the under-characterized oleaginous yeasts, *Rhodosporidium babjevae* and *Rhodosporidium diobovatum*, from biodiesel-derived waste glycerol. Bioresour Technol. 2015 Jun;185:49–55.
8. Khot M, Ghosh D. Lipids of *Rhodotorula mucilaginosa* IIPL32 with biodiesel potential: Oil yield, fatty acid profile, fuel properties. J Basic Microbiol. 2017 Apr;57(4):345–52.
9. Lopes HJS, Bonturi N, Miranda EA. *Rhodotorula toruloides* Single Cell Oil Production Using *Eucalyptus urograndis* Hemicellulose Hydrolysate as a Carbon Source. Energies. 2020 Feb 12;13(4):795.
10. Vasconcelos B, Teixeira JC, Dragone G, Teixeira JA. Optimization of lipid extraction from the oleaginous yeasts *Rhodotorula glutinis* and *Lipomyces kononenkoae*. AMB Express. 2018 Dec;8(1):126.
11. Chebbi H, Leiva-Candia D, Carmona-Cabello M, Jaouani A, Dorado MP. Biodiesel production from microbial oil provided by oleaginous yeasts from olive oil mill wastewater growing on industrial glycerol. Ind Crops Prod. 2019 Nov;139:111535.
12. Ramírez-Castrillón M, Jaramillo-Garcia VP, Rosa PD, Landell MF, Vu D, Fabricio MF, et al. The Oleaginous Yeast *Meyerozyma guilliermondii* BI281A as a New Potential Biodiesel Feedstock: Selection and Lipid Production Optimization. Front Microbiol. 2017 Sep 22;8:1776.
13. Yaguchi A, Rives D, Blenner M, Department of Chemical and Biomolecular Engineering, Clemson University, Clemson SC 29634, USA. New kids on the block: emerging oleaginous yeast of biotechnological importance. AIMS Microbiol. 2017;3(2):227–47.
14. Tanimura A, Sugita T, Endoh R, Ohkuma M, Kishino S, Ogawa J, et al. Lipid production via simultaneous conversion of glucose and xylose by a novel yeast, *Cystobasidium iriomotense*. Virolle M-J, editor. PLOS ONE. 2018 Sep 12;13(9):e0202164.
15. Vyas S, Chhabra M. Assessing oil accumulation in the oleaginous yeast *Cystobasidium oligophagum* JRC1 using dairy waste cheese whey as a substrate. 3 Biotech. 2019 May;9(5):173.
16. Balat M. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Convers Manag. 2011 Feb;52(2):858–75.
17. *Eucalyptus globulus* Coppices in Portugal: Influence of Site and Percentage of Residues Collected for Energy [Internet]. Available from: https://www.mdpi.com/2071-1050/13/11/5775
18. Pataco M. Isolation and Identification of oleaginous yeasts [Master Thesis]. [Lisboa]: Instituto Superior Técnico; 2020.
19. Polburee P, Yongmanitchai W, Lertwattanasakul N, Ohashi T, Fujiyama K, Limtong S. Characterization of oleaginous yeasts accumulating high levels of lipid when cultivated in glycerol and their potential for lipid production from biodiesel-derived crude glycerol. Fungal Biol. 2015 Dec;119(12):1194–204.
20. Sitepu IR, Jin M, Fernandez JE, da Costa Sousa L, Balan V, Boundy-Mills KL. Identification of oleaginous yeast strains able to accumulate high intracellular lipids when cultivated in alkaline pretreated corn stover. Appl Microbiol Biotechnol. 2014 Sep;98(17):7645–57.

21. S Dien B, J. Slininger P, P. Kurtzman C, R. Moser B, J. O'Bryan P, 1 Bioenergy Research Unit, National Center for Agricultural Utilization Research, USDA-ARS, Peoria, IL, USA. Identification of superior lipid producing *Lipomyces* and *Myxozyma* yeasts. AIMS Environ Sci. 2016;3(1):1–20.

22. Juanssilfero AB, Kahar P, Amza RL, Miyamoto N, Otsuka H, Matsumoto H, et al. Effect of inoculum size on single-cell oil production from glucose and xylose using oleaginous yeast *Lipomyces starkeyi*. J Biosci Bioeng. 2018 Jun;125(6):695–702.

23. Pellera F-M, Gidarakos E. Anaerobic digestion of solid agroindustrial waste in semi-continuous mode: Evaluation of mono-digestion and co-digestion systems. Waste Manag. 2017 Oct;68:103–19.

24. Mo J, Yang Q, Zhang N, Zhang W, Zheng Y, Zhang Z. A review on agro-industrial waste (AIW) derived adsorbents for water and wastewater treatment. J Environ Manage. 2018 Dec;227:395–405.

25. Zakaria ZA, Boopathy R, Dib JR, editors. Valorisation of Agro-industrial Residues – Volume I: Biological Approaches [Internet]. Cham: Springer International Publishing; 2020 [cited 2021 Nov 15]. (Applied Environmental Science and Engineering for a Sustainable Future). Available from: http://link.springer.com/10.1007/978-3-030-39137-9

26. Sadh PK, Duhan S, Duhan JS. Agro-industrial wastes and their utilization using solid state fermentation: a review. Bioresour Bioprocess. 2018 Dec;5(1):1.

27. Zihare L, Spalvins K, Blumberga D. Multi criteria analysis for products derived from agro-industrial by-products. Energy Procedia. 2018 Aug;147:452–7.

28. Panesar R, Kaur S, Panesar PS. Production of microbial pigments utilizing agro-industrial waste: a review. Curr Opin Food Sci. 2015 Feb;1:70–6.

29. Markou G, Wang L, Ye J, Unc A. Using agro-industrial wastes for the cultivation of microalgae and duckweeds: Contamination risks and biomass safety concerns. Biotechnol Adv. 2018 Jul;36(4):1238–54.

30. Yadav M, Singh A, Balan V, Pareek N, Vivekanand V. Biological treatment of lignocellulosic biomass by *Chaetomium globosporum*: Process derivation and improved biogas production. Int J Biol Macromol. 2019 May;128:176–83.

31. Prasertsan P. Recycling of Agro-Industrial Wastes Through Cleaner Technology. :11.

32. Dahiya S, Kumar AN, Shanthi Sravan J, Chatterjee S, Sarkar O, Mohan SV. Food waste biorefinery: Sustainable strategy for circular bioeconomy. Bioresour Technol. 2018 Jan;248:2–12.

33. Liu L, You Y, Deng H, Guo Y, Meng Y. Promoting hydrolysis of apple pomace by pectinase and cellulase to produce microbial oils using engineered *Yarrowia lipolytica*. Biomass Bioenergy. 2019 Jul;126:62–9.

34. Nielsen J. Yeast Systems Biology: Model Organism and Cell Factory. Biotechnol J. 2019 Sep;14(9):1800421.

35. Schmitz K, Protzko R, Zhang L, Benz JP. Spotlight on fungal pectin utilization—from phytopathogenicity to molecular recognition and industrial applications. Appl Microbiol Biotechnol. 2019 Mar;103(6):2507–24.

36. Ajila CM, Brar SK, Verma M, Tyagi RD, Godbout S, Valéro JR. Bio-processing of agro-byproducts to animal feed. Crit Rev Biotechnol. 2012 Dec;32(4):382–400.

37. Peters D. Raw Materials. In: Ulber R, Sell D, editors. White Biotechnology [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2007 [cited 2021 Oct 18]. p. 1–30. Available from: http://link.springer.com/10.1007/10_031

38. Joanna B. Chapter 13 - Sugar Beet Pulp as a Source of Valuable Biotechnological Products. :34.

39. Martins LC, Monteiro CC, Semedo PM, Sá-Correia I. Valorisation of pectin-rich agro-industrial residues by yeasts: potential and challenges. Appl Microbiol Biotechnol. 2020 Aug;104(15):6527–47.

40. Kennedy M, List D, Lu Y, Foo LY, Newman RH, Sims IM, et al. Apple Pomace and Products Derived from Apple Pomace: Uses, Composition and Analysis. In: Linskens HF, Jackson JF, editors. Analysis of Plant Waste Materials [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 1999 [cited 2021 Nov 15]. p. 75–119. Available from: http://link.springer.com/10.1007/978-3-662-03887-1_4

41. Vendruscolo F, Albuquerque PM, Streit F, Esposito E, Ninow JL. Apple Pomace: A Versatile Substrate for Biotechnological Applications. Crit Rev Biotechnol. 2008 Jan;28(1):1–12.

42. Hang YD. Management and Utilization of Food Processing Wastes. J Food Sci. 2006 May 31;69(3):CRH104–7.

43. de la Torre I, Martin-Dominguez V, Acedos MG, Esteban J, Santos VE, Ladero M. Utilisation/upgrading of orange peel waste from a biological biorefinery perspective. Appl Microbiol Biotechnol. 2019 Aug;103(15):5975–91.

44. Mohnen D. Pectin structure and biosynthesis. Curr Opin Plant Biol. 2008 Jun;11(3):266–77.

45. Zhang C. Lignocellulosic Ethanol: Technology and Economics. In: Yun Y, editor. Alcohol Fuels - Current Technologies and Future Prospect [Internet]. IntechOpen; 2020 [cited 2021 Oct 19]. Available from: https://www.intechopen.com/books/alcohol-fuels-current-technologies-and-future-prospect/lignocellulosic-ethanol-technology-and-economics

46. Palmqvist E, Hahn-Hägerdal B. Fermentation of lignocellulosic hydrolysates. II: inhibitors and mechanisms of inhibition. Bioresour Technol. 2000 Aug;74(1):25–33.

47. Taherzadeh M, Karimi K. Pretreatment of Lignocellulosic Wastes to Improve Ethanol and Biogas Production: A Review. Int J Mol Sci. 2008 Sep 1;9(9):1621–51.

48. Yapo BM, Robert C, Etienne I, Wathelet B, Paquot M. Effect of extraction conditions on the yield, purity and surface properties of sugar beet pulp pectin extracts. Food Chem. 2007 Jan;100(4):1356–64.

49. Petrovič U. Next-generation biofuels: a new challenge for yeast: Next-generation biofuels: a new challenge for yeast. Yeast. 2015 Sep;32(9):583–93.

50. Mahlia TMI, Syazmi ZAHS, Mofijur M, Abas AEP, Bilad MR, Ong HC, et al. Patent landscape review on biodiesel production: Technology updates. Renew Sustain Energy Rev. 2020 Feb;118:109526.

51. Patel A, Arora N, Mehtani J, Pruthi V, Pruthi PA. Assessment of fuel properties on the basis of fatty acid profiles of oleaginous yeast for potential biodiesel production. Renew Sustain Energy Rev. 2017 Sep;77:604–16.

52. Meng X, Yang J, Xu X, Zhang L, Nie Q, Xian M. Biodiesel production from oleaginous microorganisms. Renew Energy. 2009 Jan;34(1):1–5.

53. Li Q, Du W, Liu D. Perspectives of microbial oils for biodiesel production. Appl Microbiol Biotechnol. 2008 Oct;80(5):749–56.

54. Karathia H, Vilaprinyo E, Sorribas A, Alves R. *Saccharomyces cerevisiae* as a Model Organism: A Comparative Study. de Polavieja G, editor. PLoS ONE. 2011 Feb 2;6(2):e16015.

55. Goddard MR, Greig D. *Saccharomyces cerevisiae*: a nomadic yeast with no niche? FEMS Yeast Res [Internet]. 2015 May 1 [cited 2021 Oct 18];15(3). Available from: http://academic.oup.com/femsyr/article/doi/10.1093/femsyr/fov009/545611/Saccharomyces-cerevisiae-a-nomadic-yeast-with-no

56. Goddard MR. Quantifying the Complexities of *Saccharomyces cerevisiae*'s Ecosystem Engineering via Fermentation. Ecology. 2008;89(8):2077–82.

57. Radecka D, Mukherjee V, Mateo RQ, Stojiljkovic M, Foulquié-Moreno MR, Thevelein JM. Looking beyond *Saccharomyces*: the potential of non-conventional yeast species for desirable traits in bioethanol fermentation. Nielsen J, editor. FEMS Yeast Res. 2015 Sep;15(6):fov053.

58. Steensels J, Verstrepen KJ. Taming Wild Yeast: Potential of Conventional and Nonconventional Yeasts in Industrial Fermentations. Annu Rev Microbiol. 2014 Sep 8;68(1):61–80.

59. Sitepu IR, Garay LA, Sestric R, Levin D, Block DE, German JB, et al. Oleaginous yeasts for biodiesel: Current and future trends in biology and production. Biotechnol Adv. 2014 Nov;32(7):1336–60.

60. Martorell P, Stratford M, Steels H, Fernández-Espinar MT, Querol A. Physiological characterization of spoilage strains of *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* isolated from high sugar environments. Int J Food Microbiol. 2007 Mar;114(2):234–42.

61. Xue S-J, Chi Z, Zhang Y, Li Y-F, Liu G-L, Jiang H, et al. Fatty acids from oleaginous yeasts and yeast-like fungi and their potential applications. Crit Rev Biotechnol. 2018 Oct 3;38(7):1049–60.

62. Sreeharsha RV, Mohan SV. Obscure yet Promising Oleaginous Yeasts for Fuel and Chemical Production. Trends Biotechnol. 2020 Aug;38(8):873–87.

63. Valdés G, Mendonça RT, Aggelis G. Lignocellulosic Biomass as a Substrate for Oleaginous Microorganisms: A Review. Appl Sci. 2020 Oct 30;10(21):7698.

64. Tehlivets O, Scheuringer K, Kohlwein SD. Fatty acid synthesis and elongation in yeast. Biochim Biophys Acta BBA - Mol Cell Biol Lipids. 2007 Mar;1771(3):255–70.

65. Takaku H, Matsuzawa T, Yaoi K, Yamazaki H. Lipid metabolism of the oleaginous yeast *Lipomyces starkeyi*. Appl Microbiol Biotechnol. 2020 Jul;104(14):6141–8.

66. Fakankun I, Mirzaei M, Levin DB. Impact of Culture Conditions on Neutral Lipid Production by Oleaginous Yeast. In: Balan V, editor. Microbial Lipid Production [Internet]. New York, NY: Springer New York; 2019 [cited 2021 Oct 20]. p. 311–25. Available from: http://link.springer.com/10.1007/978-1-4939-9484-7_18

67. Jiru TM, Groenewald M, Pohl C, Steyn L, Kiggundu N, Abate D. Optimization of cultivation conditions for biotechnological production of lipid by *Rhodotorula kratochvilovae* (syn, *Rhodosporidium kratochvilovae*) SY89 for biodiesel preparation. 3 Biotech. 2017 Jun;7(2):145.

68. Qadeer S, Khalid A, Mahmood S, Anjum M, Ahmad Z. Utilizing oleaginous bacteria and fungi for cleaner energy production. J Clean Prod. 2017 Dec;168:917–28.

69. Gonçalves FAG, Colen G, Takahashi JA. *Yarrowia lipolytica* and Its Multiple Applications in the Biotechnological Industry. Sci World J. 2014;2014:1–14.

70. Pfeiffer T, Morley A. An evolutionary perspective on the Crabtree effect. Front Mol Biosci [Internet]. 2014 Oct 21 [cited 2021 Oct 21];1. Available from: http://journal.frontiersin.org/article/10.3389/fmolb.2014.00017/abstract

71. Tripodi F, Nicastro R, Reghellin V, Coccetti P. Post-translational modifications on yeast carbon metabolism: Regulatory mechanisms beyond transcriptional control. Biochim Biophys Acta BBA - Gen Subj. 2015 Apr;1850(4):620–7.

72. Turcotte B, Liang XB, Robert F, Soontorngun N. Transcriptional regulation of nonfermentable carbon utilization in budding yeast. FEMS Yeast Res. 2010 Feb;10(1):2–13.

73. van Maris AJA, Abbott DA, Bellissimi E, van den Brink J, Kuyper M, Luttik MAH, et al. Alcoholic fermentation of carbon sources in biomass hydrolysates by *Saccharomyces cerevisiae*: current status. Antonie Van Leeuwenhoek. 2006 Nov;90(4):391–418.

74. Flores C-L, Rodríguez C, Petit T, Gancedo C. Carbohydrate and energy-yielding metabolism in non-conventional yeasts: Figure 1. FEMS Microbiol Rev. 2000 Oct;24(4):507–29.

75. Kim J-H, Roy A, Jouandot D, Cho KH. The glucose signaling network in yeast. Biochim Biophys Acta BBA - Gen Subj. 2013 Nov;1830(11):5204–10.

76. Perez-Samper G, Cerulus B, Jariani A, Vermeersch L, Barrajón Simancas N, Bisschops MMM, et al. The Crabtree Effect Shapes the *Saccharomyces cerevisiae* Lag Phase during the Switch between Different Carbon Sources. Winston FM, editor. mBio [Internet]. 2018 Nov 7 [cited 2021 Oct 21];9(5). Available from: https://journals.asm.org/doi/10.1128/mBio.01331-18

77. De Deken RH. The Crabtree Effect: A Regulatory System in Yeast. J Gen Microbiol. 1966 Aug 1;44(2):149–56.

78. Simpson-Lavy K, Kupiec M. Carbon Catabolite Repression in Yeast is Not Limited to Glucose. Sci Rep. 2019 Dec;9(1):6491.

79. Pfeiffer T, Schuster S, Bonhoeffer S. Cooperation and Competition in the Evolution of ATP-Producing Pathways. Sci New Ser. 2001;292(5516):504–7.

80. Piskur J, Rozpedowska E, Polakova S, Merico A, Compagno C. How did *Saccharomyces* evolve to become a good brewer? Trends Genet. 2006 Apr;22(4):183–6.

81. Agarwal B, Singh LK. Sugar and Sugar Alcohols: Xylitol. In: Saran S, Babu V, Chuabey A, editors. High Value Fermentation Products [Internet]. Hoboken, NJ, USA: John Wiley & Sons, Inc.; 2019 [cited 2021 Oct 20]. p. 285–307. Available from: https://onlinelibrary.wiley.com/doi/10.1002/9781119460053.ch12

82. Jagtap SS, Rao CV. Microbial conversion of xylose into useful bioproducts. Appl Microbiol Biotechnol. 2018 Nov;102(21):9015–36.

83. Fonseca C, Romão R, Rodrigues de Sousa H, Hahn-Hägerdal B, Spencer-Martins I. L -Arabinose transport and catabolism in yeast: L -Arabinose transport and catabolism in yeast. FEBS J. 2007 Jul;274(14):3589–600.

84. McMillan JD, Boynton BL. Arabinose utilization by xylose-fermenting yeasts and fungi. Appl Biochem Biotechnol. 1994 Mar;45–46(1):569–84.

85. Granot D, Snyder M. Carbon source induces growth of stationary phase yeast cells, independent of carbon source metabolism. Yeast. 1993 May;9(5):465–79.

86. Paulo JA, O'Connell JD, Gaun A, Gygi SP. Proteome-wide quantitative multiplexed profiling of protein expression: carbon-source dependency in *Saccharomyces cerevisiae*. Solomon MJ, editor. Mol Biol Cell. 2015 Nov 5;26(22):4063–74.

87. Sellick CA, Campbell RN, Reece RJ. Chapter 3 Galactose Metabolism in Yeast—Structure and Regulation of the Leloir Pathway Enzymes and the Genes Encoding Them. In: International Review of Cell and Molecular Biology [Internet]. Elsevier; 2008 [cited 2021 Oct 28]. p. 111–50. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1937644808010034

88. Kuorelahti S, Kalkkinen N, Penttilä M, Londesborough J, Richard P. Identification in the Mold *Hypocrea jecorina* of the First Fungal D -Galacturonic Acid Reductase. Biochemistry. 2005 Aug 1;44(33):11234–40.

89. Kuorelahti S, Jouhten P, Maaheimo H, Penttila M, Richard P. l-galactonate dehydratase is part of the fungal path for d-galacturonic acid catabolism. Mol Microbiol. 2006 Aug;61(4):1060–8.

90. Richard P, Hilditch S. d-Galacturonic acid catabolism in microorganisms and its biotechnological relevance. Appl Microbiol Biotechnol. 2009 Mar;82(4):597–604.

91. Strijbis K, Distel B. Intracellular Acetyl Unit Transport in Fungal Carbon Metabolism. Eukaryot Cell. 2010 Dec;9(12):1809–15.

92. Krivoruchko A, Zhang Y, Siewers V, Chen Y, Nielsen J. Microbial acetyl-CoA metabolism and metabolic engineering. Metab Eng. 2015 Mar;28:28–42.

93. Gong Z, Shen H, Zhou W, Wang Y, Yang X, Zhao ZK. Efficient conversion of acetate into lipids by the oleaginous yeast *Cryptococcus curvatus*. Biotechnol Biofuels. 2015 Dec;8(1):189.

94. Papanikolaou S, Aggelis G. Lipids of oleaginous yeasts. Part I: Biochemistry of single cell oil production. Eur J Lipid Sci Technol. 2011 Aug;113(8):1031–51.

95. Verduyn C, Postma E, Scheffers WA, Van Dijken JP. Effect of benzoic acid on metabolic fluxes in yeasts: A continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast. 1992 Jul;8(7):501–17.

96. Pampulha ME, Loureiro-Dias MC. Combined effect of acetic acid, pH and ethanol on intracellular pH of fermenting yeast. Appl Microbiol Biotechnol. 1989 Oct;31–31(5–6):547–50.

97. Viegas CA, Sa-Correia I. Activation of plasma membrane ATPase of *Saccharomyces cerevisiae* by octanoic acid. J Gen Microbiol. 1991 Mar 1;137(3):645–51.

98. Thirumurugan D, Cholarajan A, Raja SSS, Vijayakumar R. An Introductory Chapter: Secondary Metabolites. In: Vijayakumar R, Raja SSS, editors. Secondary Metabolites - Sources and Applications [Internet]. InTech; 2018 [cited 2021 Oct 20]. Available from: http://www.intechopen.com/books/secondary-metabolites-sources-and-applications/an-introductory-chapter-secondary-metabolites

99. Vohra M, Manwar J, Manmode R, Padgilwar S, Patil S. Bioethanol production: Feedstock and current technologies. J Environ Chem Eng. 2014 Mar;2(1):573–84.

100. Arshad M, Zia MA, Shah FA, Ahmad M. An Overview of Biofuel. In: Arshad M, editor. Perspectives on Water Usage for Biofuels Production [Internet]. Cham: Springer International Publishing; 2018 [cited 2021 Oct 20]. p. 1–37. Available from: http://link.springer.com/10.1007/978-3-319-66408-8_1

101. Sarris D, Papanikolaou S. Biotechnological production of ethanol: Biochemistry, processes and technologies. Eng Life Sci. 2016 May;16(4):307–29.

102. Voordeckers K, Kominek J, Das A, Espinosa-Cantú A, De Maeyer D, Arslan A, et al. Adaptation to High Ethanol Reveals Complex Evolutionary Pathways. Zhang J, editor. PLOS Genet. 2015 Nov 6;11(11):e1005635.

103. Bai FW, Anderson WA, Moo-Young M. Ethanol fermentation technologies from sugar and starch feedstocks. Biotechnol Adv. 2008 Jan;26(1):89–105.

104. Chidi BS, Bauer FF, Rossouw D. Organic Acid Metabolism and the Impact of Fermentation Practices on Wine Acidity: A Review. South Afr J Enol Vitic [Internet]. 2018 Sep [cited 2021 Oct 20];39(2). Available from: http://www.journals.ac.za/index.php/sajev/article/view/3172

105. Goold HD, Kroukamp H, Williams TC, Paulsen IT, Varela C, Pretorius IS. Yeast's balancing act between ethanol and glycerol production in low-alcohol wines. Microb Biotechnol. 2017 Mar;10(2):264–78.

106. Godswill AC. Sugar Alcohols: Chemistry, Production, Health Concerns And Nutritional Importance Of Mannitol, Sorbitol, Xylitol, And Erythritol. 2017;3(2):36.

107. Mäkinen KK, Söderling E. Effect of Xylitol on Some Food-Spoilage Microorganisms. J Food Sci. 1981 May;46(3):950–1.

108. Goli JK, Panda SH, Linga VR. Molecular Mechanism of d-Xylitol Production in Yeasts: Focus on Molecular Transportation, Catabolic Sensing and Stress Response. In: da Silva SS, Chandel AK, editors. D-Xylitol [Internet]. Berlin, Heidelberg: Springer Berlin Heidelberg; 2012 [cited 2021 Nov 16]. p. 85–107. Available from: http://link.springer.com/10.1007/978-3-642-31887-0_4

109. Mohamad NL, Mustapa Kamal SM, Mokhtar MN. Xylitol Biological Production: A Review of Recent Studies. Food Rev Int. 2015 Jan 2;31(1):74–89.

110. Joye IJ. Acids and Bases in Food. In: Encyclopedia of Food Chemistry [Internet]. Elsevier; 2019 [cited 2021 Nov 16]. p. 1–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780081005965215825

111. Zelle RM, de Hulster E, van Winden WA, de Waard P, Dijkema C, Winkler AA, et al. Malic Acid Production by *Saccharomyces cerevisiae* : Engineering of Pyruvate Carboxylation, Oxaloacetate Reduction, and Malate Export. Appl Environ Microbiol. 2008 May;74(9):2766–77.

112. Shimizu K. Metabolic Regulation Analysis and Metabolic Engineering. In: Comprehensive Biotechnology [Internet]. Elsevier; 2011 [cited 2021 Nov 16]. p. 407–20. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780080885049001173

113. Cheng K-K, Wang G-Y, Zeng J, Zhang J-A. Improved Succinate Production by Metabolic Engineering. BioMed Res Int. 2013;2013:1–12.

114. McNeil JB, Bopart AL. *In vivo* Analysis of Folate Coenzymes and Their Compartmentation in Saccharomyces cer&ae. :11.

115. Kennedy CJ, Boyle PM, Waks Z, Silver PA. Systems-Level Engineering of Nonfermentative Metabolism in Yeast. Genetics. 2009 Sep 1;183(1):385–97.

116. Peters-Wendisch P, Stolz M, Etterich H, Kennerknecht N, Sahm H, Eggeling L. Metabolic Engineering of *Corynebacterium glutamicum* for L -Serine Production. Appl Environ Microbiol. 2005 Nov;71(11):7139–44.

117. Aksu Z, Eren AT. Carotenoids production by the yeast *Rhodotorula mucilaginosa*: Use of agricultural wastes as a carbon source. Process Biochem. 2005 Sep;40(9):2985–91.

118. Yoo AY, Alnaeeli M, Park JK. Production control and characterization of antibacterial carotenoids from the yeast *Rhodotorula mucilaginosa* AY-01. Process Biochem. 2016 Apr;51(4):463–73.

119. Cheng Y-T, Yang C-F. Using strain *Rhodotorula mucilaginosa* to produce carotenoids using food wastes. J Taiwan Inst Chem Eng. 2016 Apr;61:270–5.

120. Kot AM, Błażejak S, Gientka I, Kieliszek M, Bryś J. Torulene and torularhodin: “new” fungal carotenoids for industry? Microb Cell Factories. 2018 Dec;17(1):49.

121. Violle C, Enquist BJ, McGill BJ, Jiang L, Albert CH, Hulshof C, et al. The return of the variance: intraspecific variability in community ecology. Trends Ecol Evol. 2012 Apr;27(4):244–52.

122. Richardson JL, Urban MC, Bolnick DI, Skelly DK. Microgeographic adaptation and the spatial scale of evolution. Trends Ecol Evol. 2014 Mar;29(3):165–76.

123. West-Eberhard MJ. Phenotypic Plasticity and the Origins of Diversity. :32.

124. Hill WG, Caballero A. Artificial Selection Experiments. :24.

125. Sampaio JP. *Rhodotorula*. In: The Yeasts [Internet]. Elsevier; 2011 [cited 2021 Nov 15]. p. 1873–927. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780444521491001555

126. Kot AM, Błażejak S, Kurcz A, Gientka I, Kieliszek M. *Rhodotorula glutinis*—potential source of lipids, carotenoids, and enzymes for use in industries. Appl Microbiol Biotechnol. 2016 Jul;100(14):6103–17.

127. Kurtzman CP. *Meyerozyma* Kurtzman & M. Suzuki (2010). In: The Yeasts [Internet]. Elsevier; 2011 [cited 2021 Nov 16]. p. 621–4. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780444521491000471

128. Suzuki M, Prasad GS, Kurtzman CP. *Debaryomyces* Lodder & Kreger-van Rij (1952). In: The Yeasts [Internet]. Elsevier; 2011 [cited 2021 Nov 16]. p. 361–72. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780444521491000240

129. Breuer U, Harms H. *Debaryomyces hansenii* — an extremophilic yeast with biotechnological potential. Yeast. 2006 Apr 30;23(6):415–37.

130. Meyer SA, Payne RW, Yarrow D. *Candida* Berkhout. In: The Yeasts [Internet]. Elsevier; 1998 [cited 2021 Nov 16]. p. 454–573. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780444813121500691

131. M. S, R. J. The biotechnological production of sorbitol. Appl Microbiol Biotechnol. 2002 Jan 1;59(4–5):400–8.

132. Fonseca Á, Boekhout T, Fell JW. *Cryptococcus* Vuillemin (1901). In: The Yeasts [Internet]. Elsevier; 2011 [cited 2021 Nov 16]. p. 1661–737. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780444521491001385

133. Smith MTh, Kurtzman CP. *Lipomyces* Lodder & Kreger-van Rij (1952). In: The Yeasts [Internet]. Elsevier; 2011 [cited 2021 Nov 16]. p. 545–60. Available from: https://linkinghub.elsevier.com/retrieve/pii/B9780444521491000434

134. Martins LC, Palma M, Angelov A, Nevoigt E, Liebl W, Sá-Correia I. Complete Utilization of the Major Carbon Sources Present in Sugar Beet Pulp Hydrolysates by the Oleaginous Red Yeasts *Rhodotorula toruloides* and *R. mucilaginosa*. J Fungi. 2021 Mar 17;7(3):215.

135. Romi W, Keisam S, Ahmed G, Jeyaram K. Reliable differentiation of *Meyerozyma guilliermondii* from *Meyerozyma caribbica* by internal transcribed spacer restriction fingerprinting. BMC Microbiol. 2014 Dec;14(1):52.

136. Niehus X, Casas-Godoy L, Vargas-Sánchez M, Sandoval G. A Fast and Simple Qualitative Method for Screening Oleaginous Yeasts on Agar. J Lipids. 2018 Jul 29;2018:1–8.

137. Müller-Maatsch J, Bencivenni M, Caligiani A, Tedeschi T, Bruggeman G, Bosch M, et al. Pectin content and composition from different food waste streams. Food Chem. 2016 Jun;201:37–45.

138. Pryce TM, Palladino S, Kay ID, Coombs GW. Rapid identification of fungi by sequencing the I T S l and ITS2 regions using an automated capillary electrophoresis system. Med Mycol. 2003;13.

139. Badotti F, de Oliveira FS, Garcia CF, Vaz ABM, Fonseca PLC, Nahum LA, et al. Effectiveness of ITS and sub-regions as DNA barcode markers for the identification of Basidiomycota (Fungi). BMC Microbiol. 2017 Dec;17(1):42.

140. Lyman M, Urbin S, Strout C, Rubinfeld B. The Oleaginous Red Yeast *Rhodotorula/Rhodosporidium* : A Factory for Industrial Bioproducts. In: Peixoto Basso T, editor. Yeasts in Biotechnology [Internet]. IntechOpen; 2019 [cited 2021 Oct 27]. Available from: https://www.intechopen.com/books/yeasts-in-biotechnology/the-oleaginous-red-yeast-em-rhodotorula-rhodosporidium-em-a-factory-for-industrial-bioproducts

141. Kong M, Wang F, Tian L, Tang H, Zhang L. Functional identification of glutamate cysteine ligase and glutathione synthetase in the marine yeast *Rhodosporidium diobovatum*. Sci Nat. 2018 Feb;105(1–2):4.

142. Pompella A, Visvikis A, Paolicchi A, Tata VD, Casini AF. The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol. 2003 Oct;66(8):1499–503.

143. Libkind D, Gadanho M, Broock M van, Sampaio JP. Studies on the heterogeneity of the carotenogenic yeast *Rhodotorula mucilaginosa* from Patagonia, Argentina. J Basic Microbiol. 2008 Apr;48(2):93–8.

144. Garay LA, Sitepu IR, Cajka T, Chandra I, Shi S, Lin T, et al. Eighteen new oleaginous yeast species. J Ind Microbiol Biotechnol. 2016 Jul 1;43(7):887–900.

145. Mollea C, Marmo L, Bosco F. Valorisation of Cheese Whey, a By-Product from the Dairy Industry. In: Muzzalupo I, editor. Food Industry [Internet]. InTech; 2013 [cited 2021 Oct 27]. Available from: http://www.intechopen.com/books/food-industry/valorisation-of-cheese-whey-a-by-product-from-the-dairy-industry

146. Domingues L, Guimarães PMR, Oliveira C. Metabolic engineering of *Saccharomyces cerevisiae* for lactose/whey fermentation. Bioeng Bugs. 2010 May;1(3):164–71.

147. Knob A, Izidoro SC, Lacerda LT, Rodrigues A, de Lima VA. A novel lipolytic yeast *Meyerozyma guilliermondii*: Efficient and low-cost production of acid and promising feed lipase using cheese whey. Biocatal Agric Biotechnol. 2020 Mar;24:101565.

148. Heba M. Kanzy, *, N.F. Nasr, , Hoida A.M. El-Shazly, and Olfat S. Barakat. Optimization of Carotenoids production by yeast strains of *Rhodotorula* using salted cheese whey. Int J Curr Microbiol Appl Sci.

149. Valdez Castillo M, Tahmasbi H, Pachapur VL, Brar SK, Vuckovic D, Sitnikov D, et al. Production of aroma and flavor-rich fusel alcohols by cheese whey fermentation using the *Kluyveromyces marxianus* and *Debaryomyces hansenii* yeasts in monoculture and co-culture modes. J Chem Technol Biotechnol. 2021 Aug;96(8):2354–67.

150. Yurkov AM, Kachalkin AV, Daniel HM, Groenewald M, Libkind D, de Garcia V, et al. Two yeast species *Cystobasidium psychroaquaticum* f.a. sp. nov. and *Cystobasidium rietchieii* f.a. sp. nov. isolated from natural environments, and the transfer of *Rhodotorula* minuta clade members to the genus *Cystobasidium*. Antonie Van Leeuwenhoek. 2015 Jan;107(1):173–85.

151. Gao M, Ploessl D, Shao Z. Enhancing the Co-utilization of Biomass-Derived Mixed Sugars by Yeasts. Front Microbiol. 2019 Jan 22;9:3264.

152. Yaguchi A, Spagnuolo M, Blenner M. Engineering yeast for utilization of alternative feedstocks. Curr Opin Biotechnol. 2018 Oct;53:122–9.

153. Lane S, Xu H, Oh EJ, Kim H, Lesmana A, Jeong D, et al. Glucose repression can be alleviated by reducing glucose phosphorylation rate in *Saccharomyces cerevisiae*. Sci Rep. 2018 Dec;8(1):2613.

154. Gancedo JM. Yeast Carbon Catabolite Repression. Microbiol Mol Biol Rev. 1998 Jun;62(2):334–61.

155. Huang X, Wang Y, Liu W, Bao J. Biological removal of inhibitors leads to the improved lipid production in the lipid fermentation of corn stover hydrolysate by *Trichosporon cutaneum*. Bioresour Technol. 2011 Oct;102(20):9705–9.

156. Huang C, Chen X, Xiong L, Yang X, Chen X, Ma L, et al. Microbial oil production from corncob acid hydrolysate by oleaginous yeast *Trichosporon coremiiforme*. Biomass Bioenergy. 2013 Feb;49:273–8.

157. Tanimura A, Takashima M, Sugita T, Endoh R, Ohkuma M, Kishino S, et al. Lipid production through simultaneous utilization of glucose, xylose, and l-arabinose by *Pseudozyma hubeiensis*: a comparative screening study. AMB Express. 2016 Dec;6(1):58.

158. Wei N, Quarterman J, Kim SR, Cate JHD, Jin Y-S. Enhanced biofuel production through coupled acetic acid and xylose consumption by engineered yeast. Nat Commun. 2013 Dec;4(1):2580.

159. Kwak S, Jo JH, Yun EJ, Jin Y-S, Seo J-H. Production of biofuels and chemicals from xylose using native and engineered yeast strains. Biotechnol Adv. 2019 Mar;37(2):271–83.

160. Sun L, Jin Y. Xylose Assimilation for the Efficient Production of Biofuels and Chemicals by Engineered *Saccharomyces cerevisiae*. Biotechnol J. 2021 Apr;16(4):2000142.

161. Kamineni A, Shaw J. Engineering triacylglycerol production from sugars in oleaginous yeasts. Curr Opin Biotechnol. 2020 Apr;62:239–47.

162. Alcantara Jerico Z., Mondala Andro H. Effect of Acetic Acid on Succinic Acid Production of Fungal Co-culture in Slurry Fermentation. Chem Eng Trans. 2020 Jun;80:97–102.

163. Sandager L, Gustavsson MH, Ståhl U, Dahlqvist A, Wiberg E, Banas A, et al. Storage Lipid Synthesis Is Non-essential in Yeast. J Biol Chem. 2002 Feb;277(8):6478–82.

164. Leber R, Zinser E, Paltauf F, Daum G, Zellnig G. Characterization of lipid particles of the yeast,*Saccharomyces cerevisiae*. Yeast. 1994 Nov;10(11):1421–8.

165. Uzuka Y, Naganuma T, Tanaka K, Suzuki K. Relation between Neutral Lipid Accumulation and the Growth Phase in the Yeast, *Lipomyces starkeyi* , a Fat Producing Yeast. Agric Biol Chem. 1985 Mar;49(3):851–2.

166. Ruan Z, Hollinshead W, Isaguirre C, Tang YJ, Liao W, Liu Y. Effects of inhibitory compounds in lignocellulosic hydrolysates on *Mortierella isabellina* growth and carbon utilization. Bioresour Technol. 2015 May;183:18–24.

167. Georgieva TI, Mikkelsen MJ, Ahring BK. Ethanol Production from Wet-Exploded Wheat Straw Hydrolysate by Thermophilic Anaerobic Bacterium Thermoanaerobacter BG1L1 in a Continuous Immobilized Reactor. Appl Biochem Biotechnol. 2008 Mar;145(1–3):99–110.

168. Papanikolaou S, Aggelis G. Lipids of oleaginous yeasts. Part II: Technology and potential applications. Eur J Lipid Sci Technol. 2011 Aug;113(8):1052–73.

169. Lopes HJS, Bonturi N, Kerkhoven EJ, Miranda EA, Lahtvee P-J. C/N ratio and carbon source-dependent lipid production profiling in *Rhodotorula toruloides*. Appl Microbiol Biotechnol. 2020 Mar;104(6):2639–49.