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

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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

1. Introduction

Agro-industrial activities have the potential to be one of the main sectors of a country's economy, especially those who have high production yields in the agricultural sectors (1). 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 such as 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) (2,3). 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 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 (4). These discarded residues are rich in several nutrients according to their origin, for example: fiber, minerals, carbohydrates, and proteins (5–7). Agro-wastes are also attractive alternative sources of renewable energy since they do not compete with food sources (8). 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 (9,10).

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 (11). 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) (12). Biofuels is a great alternative to petro derived fuels since 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 (13). Therefore the use of these agro-forest residues to produce biodiesel through oleaginous yeasts is an environmentally responsible approach and creates a greener circular bio-economy (14,15).

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 to accumulate under optimized conditions up to 80% of cellular biomass (16). 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 (17). 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 (18). 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 (19,20). This intraspecific variation highlights the importance to isolate and examine different strains even if belonging to the same species, since their phenotypic characteristics and industrial potential may differ greatly (18). Therefore, 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 further tested in this work to evaluate their potential in the referred context.

2. Materials and Methods

2.1. 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. 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.

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

Yeast cells of *R. babjevae* (strains IST537, IST549, IST550), *Rhodotorula sp.* IST323, *R. mucilaginosa* (strains IST390, IST423, IST461), *R. toruloides* IST536, *R. glutinis* IST535, *Meyerozyma caribbica* (strains IST494, IST541, IST542, IST601), *Meyerozyma guilliermondii,* (strains IST502, IST587, IST591), *Meyerozyma* sp. IST595, *Debaryomyces hansenii* (strains IST586, IST334, IST339) and *Cystobasidium slooffiae* IST544, 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.

2.3. 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 *Candida boidinii* (strains IST509, IST599), *Kodamaea ohmeri* IST538, *Cryptococcus flavescens* IST325, *Cystobasidium slooffiae* IST547, *Meyerozyma guillermondii* (strains IST369, IST502), *Lipomyces starkeyi* (strains IST532, IST533) and *Lipomyces tetrasporus* IST531, 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.

2.4. 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) (21). 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 µL 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.

2.5. Assessment of the accumulated lipids in yeast cells using Nile Red fluorescence staining and microscopic observation of yeasts' lipid droplets

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 associated to RFU.

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 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).

3. Results and Discussion

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

In this assay, a total of 23 yeast strains from 8 different promising oleaginous species, *R. babjevae* (strains IST537, IST549, IST550), *Rhodotorula sp.* IST323, *R. mucilaginosa* (strains IST390, IST423, IST461), *R. toruloides* IST536, *R. glutinis* IST535, *Meyerozyma caribbica* (strains IST494, IST541, IST542, IST601), *Meyerozyma guilliermondii,* (strains IST502, IST587, IST591), *Meyerozyma* sp. IST595, *Debaryomyces hansenii* (strains IST586, IST334, IST339) and *Cystobasidium slooffiae* IST544 from the IST yeast culture collection were tested to evaluate their potential for the catabolization of different carbon sources (D-glucose, D-xylose, L-arabinose, D-raffinose, D-galactose or D-galacturonic acid) present in hydrolysates of different biomass residues. The growth curves were followed based on the periodic measurement of the culture OD at 600nm and 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 is shown in **Table 1**.

Table 1 - 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, L-arabinose, 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-glucose					D-xylose					L-arabinose					L-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	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}	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	3.8	6.6	-	9.8^{A}	0.5	0.7	1.4	-	2.1^{A}	0.5	0.4	0.3	-	0.4^{A}	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	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}	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	3.3	6.2	-	8.9^{A}	0.5	0.7	0.9	-	2.1^{A}	0.5	0.4	0.4	-	0.4^{A}	0.5	3.5	5.1	-	4.3^{A}	0.5	1.9	3.5	-	5.2^{A}	0.5	0.4	0.4	-	0.4^{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}	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	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^{B}	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	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}	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	6.5	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}	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	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}	0.5	4.5	6.6	6.2	4.7^{A}	0.5	1.4	1.3	2.7	4.9^{A}	0.5	7.3	4.8	5.0	4.9^{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}	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	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}	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	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}	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	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}	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	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}	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	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}	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	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}	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	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}	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	14.4	-	-	18.8^{D}	0.5	0.2	-	-	14.5^{D}	0.5	0.4	-	-	6.2^{D}	0.5	7.4	-	-	8.7^{D}	0.5	0.7	-	-	4.1^{D}	0.5	0.4	-	-	0.6^{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}	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	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}	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	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}	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

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 (22). 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 (21), confirming the potential of *Rhodotorula* species for the bioconversion of these hydrolysates (21,23). The results regarding *R. mucilaginosa* (strains IST390, IST423 and IST461) also showed the importance of screening different strains within the same species due to intraspecific variability, a characteristic already reported in literature for this species due to its genetic and phenotypic diversity (24). 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. Considering the results previously obtained for *R. mucilaginosa* IST390 (21) 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. 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. 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 (25).

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

In this assay, 10 strains from 7 different species, *Candida boidinii* (strains IST509, IST599), *Kodamaea ohmeri* IST538, *Cryptococcus flavescens* IST325, *Cystobasidium slooffiae* IST547, *Meyerozyma guillermondii* (strains IST369, IST502), *Lipomyces starkeyi* (strains IST532, IST533) and *Lipomyces tetrasporus* IST531, were also grown in a synthetic medium mimicking lignocellulosic biomass hydrolysates, composed by YNB 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, to evaluate their ability to use the various carbon sources when present simultaneously in the culture medium. 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 *Cystobasidium slooffiae* IST547, *Meyerozyma guillermondii*, IST502, *Lipomyces starkeyi* IST532 and *Lipomyces tetrasporus* IST531 are shown in **Figure 1**

Due to carbon catabolite repression (CCR) regulation, the sequential use of multiple sugars in biomass hydrolysates is a limitation (26). 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 (27,28). 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 (29), 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 (30), or, lastly, yeasts can co-consume freely glucose and other carbon sources like pentoses (31).

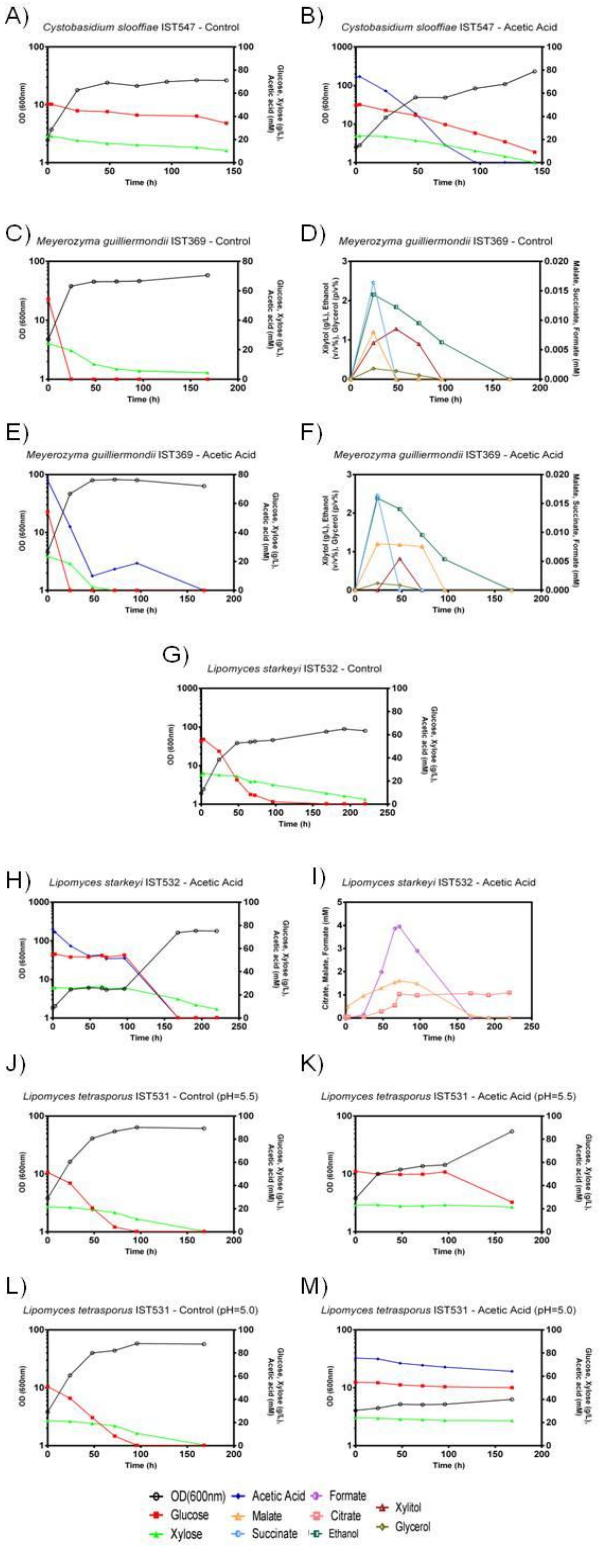

Figure 1 - Growth curves, carbon source utilization, and by-products' synthesis by *Cy. slooffiae* IST547, *Meyerozyma guilliermondii* IST369, *L. starkeyi* IST532 and *L. tertrasporus* IST531. Yeast cells were cultivated in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5, containing 50 g/L D-glucose and 25 g/L D-xylose (the control for acetic acid effect on yeast) (A, C, D, G, J, L) or in the same medium supplemented with 4.5 g/L (75 mM) acetic acid (B, E, F, H, I, K, M). Yeast growth (OD (600 nm)), D-glucose, D-xylose and acetic acid are represented in A, B C, E, G, H, J, K, L, M, and by-products synthesized are represented in D, F, I.

In this study, glucose repression was observed in *L. starkeyi* IST532 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 *L. starkeyi* IST532. 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.

Remarkably, the presence of acetic acid seemed to promote D- xylose consumption by *Cy. slooffiae* IST547, *M. guilliermodii* IST369. 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 (32). 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 (33). 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 (32,34). This process allows for the co-utilization of xylose and acetate, as well as acting as a redox sink for excess NADH (26). It is likely that *Cystobasidium slooffiae* IST547, *Meyerozyma guilliermodii* IST369 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.

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 were achieved and D-glucose were 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 (36–38).

3.3. 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 cells stained with Nile Red was determined and shown in **Figure 2**.

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. 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 (39). 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 (40).

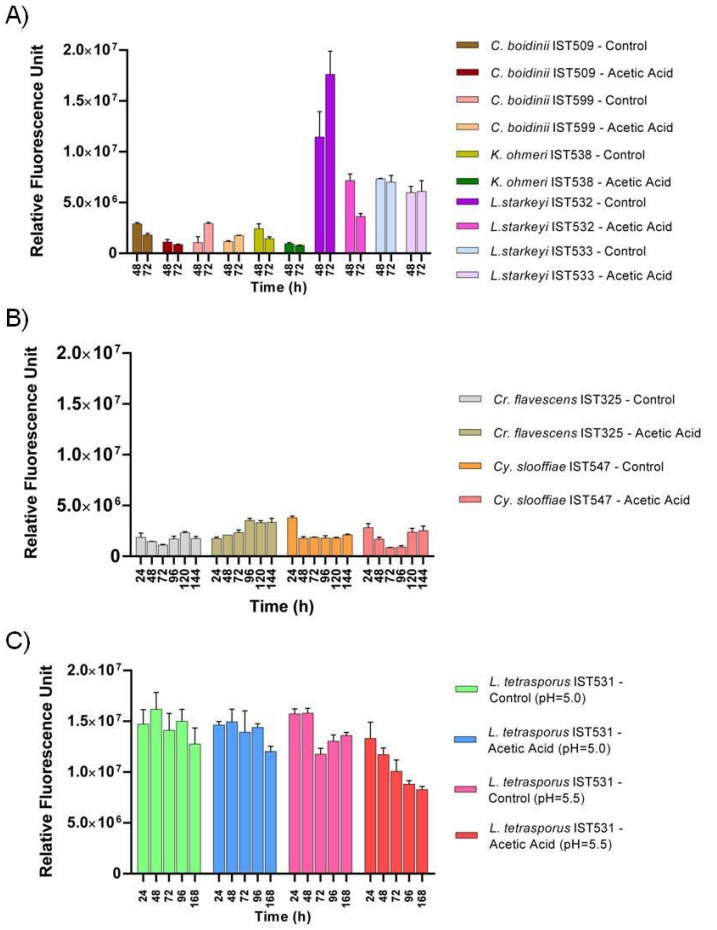

Figure 2 - Relative Fluorescence Units (RFU) of *C. boidinii* (strains IST509 and IST599), *K. ohmeri* IST538, *Cr. flavescens* IST325, *Cy. slooffiae* IST547, *L. starkeyi* (strains IST532 and IST533) and *L. tetrasporus* IST531 cells. Relative Fluorescence Units (RFU) for *C. boidinii* strains IST509 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 acetic acid; B) RFU for *Cr. flavescens* IST325 and *Cy. slooffiae* IST547 cells, in the same media before mentioned, after 24 h, 48 h, 72 h, 96 h, 120 h and 144 h of cultivation; C) RFU 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.

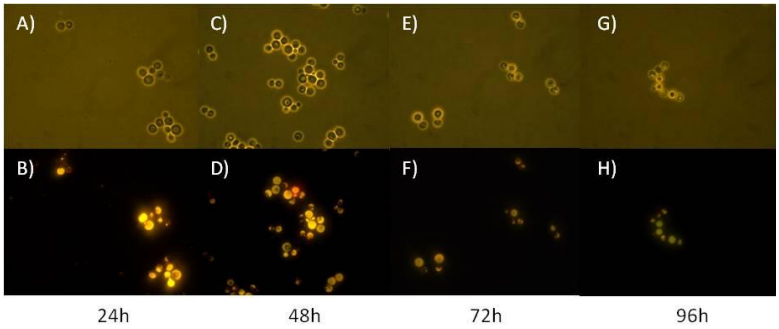

Figure 3 - Microscopic observations of *L. tetrasporus* IST531 cells stained with Nile Red fluorescence dye during cultivation in media mimicking lignocellulosic biomass hydrolysates, at pH 5.5. Cells were observed and photographed, with a total magnification of 1000x, at several time points along the growth curves depicted in **Fig.1K**. A, C, E and G were taken using bright-field microscopy and B, D, F and H were taken using epifluorescence microscopy.

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 performed 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 (35).

4. Concluding Remarks and Future Perspectives

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, such as *L. tetrasporus* IST531 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, genetically engineer *Saccharomyces cerevisiae* to catabolize the sugars present in hydrolysates from agro-forest industrial residues and to increase yeast tolerance to multiple stress factors occurring during the bioconversion of those residues into value-added products.

In *M. guilliermondii* IST369, there was no evidence of carbon catabolite repression in which co-consumption of acetic acid and D-glucose was observed and in *Cy. slooffiae* IST547 and *M. guilliermodii* IST369 the presence of acetic acid at pH 5.5 seemed to promote D-xylose consumption. In future assays, these industrially desirable metabolic characteristics 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* where 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.

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5. References

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

2. Balat M. Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energy Conversion and Management. 2011 Feb;52(2):858–75.

3. 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. Journal of Environmental Management. 2018 Dec;227:395–405.

4. 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.

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

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

7. Panesar R, Kaur S, Panesar PS. Production of microbial pigments utilizing agro-industrial waste: a review. Current Opinion in Food Science. 2015 Feb;1:70–6.

8. Yadav M, Singh A, Balan V, Pareek N, Vivekanand V. Biological treatment of lignocellulosic biomass by *Chaetomium globosporum*: Process derivation and improved biogas production. International Journal of Biological Macromolecules. 2019 May;128:176–83.

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

10. 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 and Bioenergy. 2019 Jul;126:62–9.

11. 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.

12. 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. Renewable and Sustainable Energy Reviews. 2017 Sep;77:604–16.

13. 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.

14. Cristobal-Sarramian A, Atzmüller D. Yeast as a production platform in biorefineries: conversion of agricultural residues into value-added products. 2018;476.4Kb.

15. 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. Available from: http://www.intechopen.com/books/old-yeasts-new-questions/non-conventional-yeasts-in-fermentation-processes-potentialities-and-limitations

16. Ratledge C. Fatty acid biosynthesis in microorganisms being used for Single Cell Oil production. Biochimie. 2004 Nov;86(11):807–15.

17. Rebello S, Abraham A, Madhavan A, Sindhu R, Binod P, Babu AK, et al. Non-conventional Yeast cell factories for sustainable bioprocesses. FEMS Microbiology Letters [Internet]. 2018 Sep 13; Available from: https://academic.oup.com/femsle/advance-article/doi/10.1093/femsle/fny222/5096020

18. 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.

19. 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.

20. 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 in Ecology & Evolution. 2012 Apr;27(4):244–52.

21. 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*. JoF. 2021 Mar 17;7(3):215.

22. 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 Chemistry. 2016 Jun;201:37–45.

23. 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.

24. 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.

25. 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. Biocatalysis and Agricultural Biotechnology. 2020 Mar;24:101565.

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

27. Yaguchi A, Spagnuolo M, Blenner M. Engineering yeast for utilization of alternative feedstocks. Current Opinion in Biotechnology. 2018 Oct;53:122–9.

28. 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.

29. 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*. Bioresource Technology. 2011 Oct;102(20):9705–9.

30. 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 and Bioenergy. 2013 Feb;49:273–8.

31. 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 Expr. 2016 Dec;6(1):58.

32. 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.

33. 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. Biotechnology Advances. 2019 Mar;37(2):271–83.

34. 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.

35. 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.

36. 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.

37. 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.

38. 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.

39. 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.

40. 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.