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

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Abstract

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

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

Introduction

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

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

The utilization of organic waste residues, derived from crops and plant biomass, as substrates to produce added-value products allows the decrease of raw materials costs favouring environmentally friendly strategies, by saving and reutilizing resources. This may lead to the implementation of a circular bioeconomy, in which the metabolic versatility and the resistance to stresses shown by some non-conventional (non-*Saccharomyces*) yeasts point them as valuable cell factories of high potential for biorefineries [10,11].

Saccharomyces cerevisiae is still the most important cell factory in the Biotechnological Industry [12]. The metabolic engineering of *S. cerevisiae* is allowing the production of many compounds not naturally produced by this yeast species, and the use of pectin-rich agro-industrial sugars [6,12,13]. However, there are non-conventional yeast species that present industrial advantages in terms of metabolic pathways peculiarities, wider range of carbon sources of efficient assimilation, plus higher tolerance to some environmental stresses and cytotoxic compounds, that function as growth inhibitors [14,15]. Also, they are capable to produce the most diverse added-value products, beyond bioethanol, namely sugar alcohols (xylitol and arabitol) [16,17], lipids [18], enzymes [19–21], pigments (carotenoids) [22–24], among others. Different species and even strains differ in their interesting products synthesized, as well as in production

rates and yields. Still, due to these relevant traits, some non-conventional yeasts are becoming so common, with their genome sequences and genetic tools available, that it is expected that will lose the current designation and become useful cell factories in the near future [2,14,15,25]. Examples of non-conventional yeast species with interesting features and production ability are *Meyerozyma guilliermondii* [17], *Rhodotorula mucilaginosa* [22] and *Kluyveromyces marxianus* [19].

The main goals of this work were to isolate and identify non-conventional yeast strains from pectin-rich sources, determine the potential of selected yeast species to utilize SBP hydrolysates as a substrate for added-value products, and secondarily to identify the effects of acetic acid in yeast growth profiles, as well as the effects of temperature and pH as different culture conditions.

Materials and Methods

Strains and culture media

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

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

Isolation of yeast stains potentially interesting for pectin-rich residues bioconversion from SBP

Dry sugar beet pulp (SBP), obtained from the Belgian sugar company Tiense Suiker (https://www.tiensesuiker.be), was hydrated (50 g of pulp in 500 mL water, corresponding to 10 %(w/v)) supplemented with peptone (10 g/L), and 100 µg/mL chloramphenicol and were incubated during 3 weeks at 30ºC, 250 rpm. Each two days, samples were collected. For macerated cherries and fresh grapes, they were washed in sterile water, and samples of the water extract were collected. 100 µL of these samples were spread on YPD agar plates, supplemented with 100 µg/mL chloramphenicol. The utilization of chloramphenicol allowed the control of growth of the bacteria also present in the samples used for yeast isolation. Plates were incubated for 2 to 4 days, at 30ºC. Single colonies morphologically distinct were obtained from different samples, and were streaked on new YPD agar plates, incubated for 2 to 4 days at 30ºC.

Molecular identification of the yeast isolates

Total yeast DNA from the isolates was extracted with 1:1 volume (200-300 µL) of phenol/water plus the corresponding volume of glass beads to disrupt the cells. After 2 minutes vortex, the mixtures were centrifuged (14000 rpm, 5 minutes, 4ºC) in a microcentrifuge MiniSpin Plus (Eppendorf). The supernatant was resuspended in 1:1 volume (300 µL) phenol/aqueous phase, vortexed by 10 seconds and centrifuged in the same conditions. The upper phase was collected, and 1:1 volume (300 µL) ether/aqueous phase was added, being vortexed for 20 seconds and centrifuged. After collection of the lower phase, DNA was precipitated with 1mL of absolute ethanol, incubated at 20ºC for 15 minutes and centrifuged. The pellet was washed with 0.5 mL of 70 %(v/v) ethanol, dried using a SpeedVac Concentrator Plus (Eppendorf) (45ºC, 15 minutes), and resuspended in 100 µL of sterile water. Quantification of the extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer. Amplification of yeast ribosomal DNA was performed by PCR with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-GCATATCAATAAGCGGAGGA-3') [26], which amplify rDNA of ITS 1 and 2 regions, respectively, and primers NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGT TTCAAGACGG-3') [26], that amplify large-subunit 26S D1/D2 region from rDNA. PCR products were separated by electrophoresis in 0.8% agarose gel. DNA bands were cut in the transilluminator and purified with NZYGelpure kit (NZYTech). Quantified DNA by NanoDrop spectrophotometry was sequenced by STABVIDA, Lda. The sequencing results were submitted to BLASTN analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch), being compared to the ones deposited in the National Center for Biotechnology Information (NCBI) database, to identify the yeasts species. After correct identification, aliquots of yeast cultures in YPD medium with 20 % glycerol were stored at -80ºC.

Screening of yeast strains for growth ability in distinct carbon sources

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

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

Aerobic and microaerophilic assays

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

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

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

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

The collected samples were centrifuged (10000 rpm, 3 minutes) in a microcentrifuge MiniSpin Plus (Eppendorf) and 100 µL of supernatant were pipetted into a high-performance liquid chromatography (HPLC) vial and diluted in 900 µL 5 mM H_2SO_4. The concentration of glucose, arabinose, galactose, galacturonic acid, acetic acid, ethanol and arabitol in each sample was determined by HPLC (Hitachi LaChrom Elite), using a column Aminex HPX-87H (Bio-Rad). Sugars and alcohols were quantified through refractive index detection, while the acids were quantified through UV/visible detection. The elution of the compounds was performed at 65ºC and 0.6 mL/min flow rate, with 5 mM H_2SO_4, for 30 minutes. The respective concentrations were calculated through calibration curves constructed for each compound. In aerobic assays, volumetric consumption rates [27,28], as well as maximum ethanol and arabitol yields were determined [27,29]. The respective formulas are:

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

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

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

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

Carotenoids production assays

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

Extraction and quantification of total carotenoids

The extraction and quantification of carotenoids was performed as described before [22], with some modifications. Cells were centrifuged (8000 rpm, 3 minutes) in a centrifuge 5804 R (Eppendorf), to remove supernatant, and the pellet was washed three times with distilled water. Light exposure was prevented using aluminium foil. Biomass pellets were frozen at -20ºC and lyophilized in Scanvac CoolSafe Freeze Dryer (LaboGene), for 3 days. Before extraction, cells dry weight was determined. Then, the pellet was resuspended in 2 mL acetone, and cells were disrupted using zirconia beads, releasing intracellular carotenoid to acetone phase. This suspension was vortexed for 5 minutes and centrifuged (8000 rpm, 3 minutes). The supernatant was collected, and 2 mL acetone was added, repeating this step until total pellet colourless was achieved. Acetone extracts were collected, and all acetone evaporated using RapidVap Vacuum Dry Evaporator (Labconco). Dried carotenoids were resuspended in 1 mL acetone and their absorbance was measured at 452 nm (β-carotene maximum absorbance wavelength in acetone phase), with an extinction coefficient $E^{1\%}_{450}$ = 2500 [30], in a spectrophotometer. Total carotenoids concentration was calculated using the expression:

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

Influence of acetic acid in glucose and arabinose growth profiles

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

Influence of temperature and pH in SBP hydrolysates growth curves

Rhodotorula mucilaginosa IST390, *Meyerozyma guilliermondii* IST369, *Kluyveromyces marxianus* IST389, *Kluyveromyces*

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

Results and Discussion

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

The isolation work of yeast strains from sugar beet pulp, fruits and beverages was performed in collaboration with the MSc student Paula Semedo. In total, 10 strains were isolated from hydrated SBP, fruits and beverages, with 5 strains of different species being identified in this work. From hydrated SBP with peptone samples, 3 distinct colonies were identified: *Pichia kudriavzevii, Clavispora lusitaniae* and *Rhodotorula mucilaginosa* (99 % identity). These species are found in multiple environments, including fresh and rotten fruits and trees, fermented food and beverages and pectin-containing residues [14,31,32]. From cherries, one strain was identified as *Metschnikowia pulcherrima* (96 % identity) and from grapes, one strain of *Hanseniaspora opuntiae* was identified (99 % identity). These species are respectively, common in the isolation environments [33,34]. Despite low pectin content of cherries and grapes [35], they are interesting sources to discover strains with different carbon sources catabolization, metabolic pathways and robustness.

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

The performance of 19 yeast strains to catabolise glucose, arabinose and xylose and grow in SBP hydrolysate was first screened, also in collaboration with MSc student Paula Semedo. SBP hydrolysates were prepared, sterilized and supplied by the German partner of the EraNet Project YEASTPEC. The composition of each hydrolysate tested in this work is detailed in Table 1. The strains that exhibit higher OD values in the majority of growth substrates were considered relevant. According to these results and literature data collected, the most interesting non-conventional yeast strains for our study were considered to be *K. marxianus* IST389, *M. guilliermondii* IST369 and *R. mucilaginosa* IST390. [22,29,36–40]. Also, *S. cerevisiae* Ethanol Red presented the highest OD values compared with other 3 *S. cerevisiae*. In addition to the selected yeast strains, other strains were subsequently added to this study: *R. mucilaginosa* IST423, *K. marxianus* CBS712, and *S. cerevisiae* CEN.PK 122.

Aerobic cultures in SBP hydrolysates H8 and H11

Selected strains were evaluated for performance in aerobic conditions in the sterile SBP hydrolysates H8 and H11 (Table 1). OD values were measured (Figure 1), and samples collected during growth were analysed by HPLC (Figure 2). *M. guilliermondii* IST369 was the strain that exhibited the highest final biomass of all strains in all conditions. The highest maximum specific growth rates were exhibited by *S. cerevisiae* Ethanol Red and *K. marxianus* IST389. Additionally, maximum specific growth rates were higher in H11 than in H8, except in the case of *R. mucilaginosa* IST390.

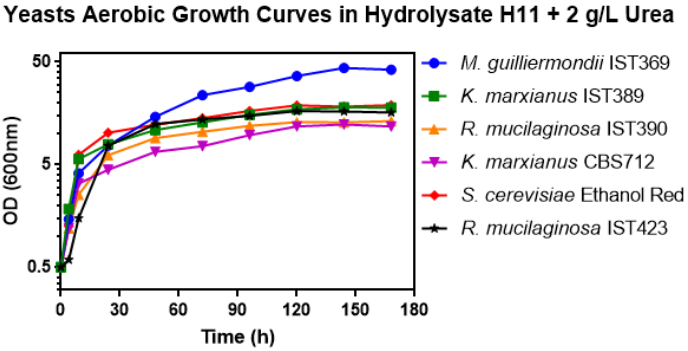

Figure 1 – Growth curves of the studied yeasts in SBP hydrolysate H11. Growth profiles were based on OD measurements of M. *guilliermondii* IST369 (dark blue circle), *K. marxianus* IST389 (green square), K. marxianus CBS712 (purple inverted triangle), *R. mucilaginosa* IST390 (orange triangle), *R. mucilaginosa* IST423 (black star) and *S. cerevisiae* Ethanol Red (red diamond), at 30ºC, pH 5.0 and 130 rpm.

In terms of carbon sources consumption, *S. cerevisiae* Ethanol Red and *K. marxianus* IST389 presented the highest glucose and galactose consumption rates. Acetic acid was co-consumed with glucose by *M. guilliermondii* and *R. mucilaginosa*, but not by *S. cerevisiae* and *K. marxianus* strains. A previous study also reported the co-consumption of acetic acid with glucose, xylose and arabinose by *M. guilliermondii*, not suffering glucose catabolite repression as occurs in *S. cerevisiae* [154].

Arabinose was only metabolized by *M. guilliermondii* and *K. marxianus* strains, corresponding to the only arabitol producers' strains. Nevertheless, *M. guilliermondii* was the only strain that fully metabolized arabinose during the culture time. A study comparing *M. guilliermondii* and *Candida arabinofermentans* showed that *C. arabinofermentans* leads to a much lower accumulation of arabitol and to traces of ethanol production in arabinose medium, due to a more efficient arabinose catabolic pathway [41]. Arabinose metabolic pathway starts with arabinose conversion into arabitol by an NAD(P)H-dependent L-arabinose reductase (aldose reductase) (AR). It is posteriorly oxidized to L-xylulose by L-arabitol-4-dehydrogenase (LAD), which is converted into xylitol by L-xylulose reductase (LXR). Xylitol, a common metabolite of arabinose and xylose pathways, is then converted to D-xylulose by xylitol dehydrogenase (XDH) [42]. When arabitol is not a desired product, it represents one of the major drawbacks to an efficient arabinose fermentation and metabolization, due to its accumulation resulting from a cofactor imbalance (AR prefers NADPH, while LAD prefers NAD^+) [41,42]. Subsequently, it was confirmed by analysis of the kinetics of arabinose pathway enzymes of

Table 1 – Composition of SBP hydrolysates utilized in this study. Sugar beet pulp hydrolysates were prepared by enzymatic hydrolysis and supplied by the German partner of the EraNet Project YEASTPEC. GalA: Galacturonic acid.

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

each strain, that *C. arabinofermentans* enzymes showed much higher catalytic efficiency (higher V_{max} and lower K_m) compared with *M. guilliermondii*, especially the enzyme responsible for arabitol oxidation (LAD) [43]. Therefore, the studied *M. guilliermondii* IST369 seems to have a more efficient arabinose metabolism when compared to *K. marxianus* strains IST389 and CBS712, since these last strains accumulates higher arabitol concentrations and were unable to fully metabolize arabinose during culture duration.

Galacturonic acid (GalA) revealed to be a carbon source very difficult to be metabolized by most yeasts. One study reports that the yeast *Rhodosporidium toruloides* possess an efficient D-GalA metabolism, similar to D-glucose and D-xylose metabolism in MM at 30ºC and pH 5.5 [44], and the same authors state that *R. mucilaginosa* also possesses an efficient D-GalA metabolic pathway, that it is not described yet (unpublished results) [45]. *R. mucilaginosa* strains IST390 and IST423 did not metabolized arabinose and GalA in SBP hydrolysates during the culture time, but in H11 cultures, both strains showed a slight decrease of 1 g/L to 2 g/L in arabinose and GalA concentration in the last 24 hours of culture. Since both *R. mucilaginosa* strains exhibit low carbon source consumption rates (similar in both hydrolysates) compared with other strains. So, it is possible that if the cultures were maintained during a longer period of time, both GalA and arabinose would possibly be metabolized. When compared with MSc student Paula Semedo study, IST390 and IST423 strains showed arabinose and GalA consumption in MM with concentrations of carbon sources resembling the hydrolysates, with preference for arabinose over GalA, especially when acetic acid (a carbon source preferred over arabinose a GalA, was not supplemented to the medium. These cultures were maintained for 268 h, and only in the medium without acetic acid, the 10 g/L arabinose and GalA present were almost exhausted. This is consistent with the observation that *R. mucilaginosa* strains were capable to grow using GalA as sole concentration source, although only around 30 % of the initial concentration was used.

Considering arabinose metabolic pathway, reductases prefer NADPH as cofactor, whereas dehydrogenases are dependent on NADH, which generates a double cofactor redox imbalance [17,42,46]. Similarly, GalA metabolic pathway of *Rhodosporidium toruloides,* as the ones in filamentous fungi, generates a double redox imbalance. GalA is firstly reduced to L-galactonate by a NADPH-specific D-GalA reductase, being then transformed into 3-deoxy-L-threo-hex-2-ulosonate by a dehydratase, and posteriorly into L-glyceraldehyde and pyruvate by an aldolase. L-glyceraldehyde is reduced to glycerol, a central metabolite, by a NADPH-dependent glyceraldehyde [44]. Cofactor imbalance is another and most probable reason for the arrestment of GalA catabolism. Despite two NADPH being generated during ethanol and acetate catabolization, which should solve this problem, cells may utilize NADPH in other processes, since this is the preferred cofactor in biosynthetic reactions [47].

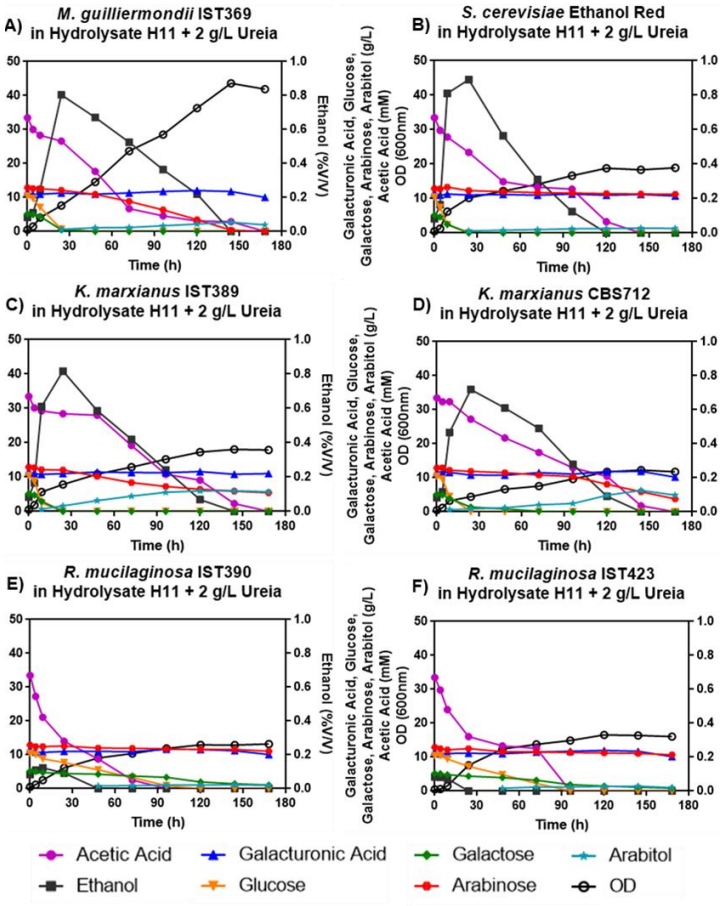

Figure 2 – Sugars and acids consumption and ethanol and arabitol production profiles obtained in SBP hydrolysate H11 + 2 g/L urea by *M. guilliermondii* IST369 (A), *S. cerevisiae* Ethanol Red (B), *K. marxianus* IST389 (C), *K. marxianus* CBS712 (D), *R. mucilaginosa* IST390 (E) and *R. mucilaginosa* IST423 (F). The samples were collected from the cultivations at several time points (0, 4, 9, 24, 48, 72, 96, 120, 144 and 168 h). The compounds evaluated were galacturonic acid (dark blue triangles), glucose (orange inverted triangles), galactose (green diamonds), arabinose (red hexagons), acetic acid (purple closed circles), ethanol (grey squares) and arabitol (light blue stars). The ODs (linear scale) are also represented in the graphs (black open circles).

The sequential consumption of carbon sources in H11 was slightly different than in H8, which may be dependent on initial carbon sources concentrations. The low carbon sources concentration may determine that these profiles are not the real ones that would be observed with higher

concentrations. In H8, *S. cerevisiae* and *K. marxianus* strains consumed glucose > galactose > ethanol > acetic acid/ arabinose. *M. guilliermondii* preferentially consumed glucose/acetic acid > galactose > ethanol > arabinose. In H11, *M. guilliermondii* preferred glucose/acetic acid > galactose > ethanol/arabinose, while *S. cerevisiae* glucose > acetic acid/ galactose > ethanol and for *K. marxianus* strains the preference was glucose > galactose > acetic acid/ ethanol/ arabinose. In both substrates, *R. mucilaginosa* strains catabolized glucose/ acetic acid/ ethanol > galactose. Ethanol, but especially acetic acid, are important respirable substrates in the SBP hydrolysate that are preferred by yeasts over arabinose, and by some of the yeasts tested, also over galactose.

In terms of products generation, ethanol was considered to be produced from glucose and galactose by *M. guilliermondii* IST369 and *K. marxianus* IST389, and only from glucose by the other strains. Ethanol yields obtained in this study varied between 0.32 $g_{ethanol}/g_{sugars}$ and 0.44 $g_{ethanol}/g_{sugars}$ for *K. marxianus* strains IST389 and CBS712, *M. guilliermondii* IST69 and *S. cerevisiae* Ethanol Red, showing similar maximum concentrations produced (0.2 to 0.3 %(v/v) in H8 and 0.7 to 0.8 %(v/v) in H11). The obtained yields are similar to those reported in studies with lignocellulosic and pectin-containing residues. *K. marxianus* yield was approximately 0.40 g/g in sugar beet molasses (100 g/L reducing sugars) [19]. *S. cerevisiae* Ethanol Red attained 0.40 g/g in soybean hull rich in glucose [48]. On the other hand, *R. mucilaginosa* IST390 only fermented glucose, showing lower ethanol concentrations in H8 (0.1 %(v/v)), and almost no production occurred in H11, consistently with *R. mucilaginosa* classification as obligate aerobic and generally non-fermentative specie [32]. Still, it produces high pigmentation in these hydrolysates. Nevertheless, since SBP hydrolysates contain lower concentrations of fermentable sugars (e.g. glucose and galactose), this substrate is not a suitable for ethanol production, but it can be useful for obtaining other products, such as arabitol (mostly produced from arabinose) or carotenoids.

K. marxianus strains IST389 and CBS712 showed the highest accumulation of arabitol (2 g/L to 4 g/L in H8, and 6 g/L in H11) and production yields (0.77 to 0.90 $g_{arabitol}/g_{arabinose}$ in both hydrolysates) in aerobiosis, indicating that SBP hydrolysates complex composition and arabinose metabolism of these strains favour arabitol production. Only one study showed that *K. marxianus* was able to produce arabitol in 20 g/L arabinose medium at 150 rpm, with yields approximately 0.27 g/g at 30ºC, and 0.48 g/g at 40ºC [49], much lower than those obtained in this study. On the other hand, *M. guilliermondii* IST369 presented low arabitol accumulation (maximum concentration of 3 g/L, and production yield of 0.22 $g_{arabitol}/g_{arabinose,}$ obtained in H11), which is related with the described efficient arabinose metabolic pathway that does not favour arabitol accumulation. However, there are other reports in which diverse *M. guilliermondii* strains were examined describing the species as a great arabitol producer [41,42,50].

Generally, supplementation with urea slightly increase final biomass, maximum specific growth rates, consumption and ethanol and arabitol production concentration. However, the great differences are observer between H8 and H11, in which yeasts' performance was significantly better, except acetic acid consumption rates, which remain similar. This increase is related with higher initial carbon sources concentrations, while acid concentration is approximately the same.

Microaerophilic assays in SBP hydrolysates H8 and H11

Complementary to the cultures performed in oxygenated conditions, microaerophilic conditions were also tested. When compared with aerobiosis, no significant improves in ethanol amounts were observed in microaerophilia (maximum concentrations of 0.5 %(v/v) by *M. guilliermondii* IST369 in H8 and 0.9 %(v/v) by K. marxianus IST389). Only *K. marxianus* IST389 showed a considerable arabitol concentration (2.6 g/L), still being much lower than in aerobic assays Additionally, no consumption of acetic acid, arabinose and GalA was observed in this condition, and most carbon sources need to be respired to be properly catabolized, namely acetic acid and ethanol [52]. Aerobic conditions are necessary for maximum carbon sources utilization and arabitol and carotenoids production, which is desirable in industrial applications.

Carotenoid production by *R. mucilaginosa* strains

High pigment production was observed in *R. mucilaginosa* strains when these cells grown aerobically, and specific assays were performed to determine their production capacity in SBP hydrolysate. The final dry biomass of *R. mucilaginosa* IST390 and IST423 was much higher in YPD than in MM, since YPD is a rich medium, while in SBP hydrolysate H11, biomass reached the lowest values. Despite biomass production by IST423 in MM with glucose, the biomass did not present a pink colour, suggesting absence or not significant carotenoid production in this condition.

Table 1 – Total carotenoids produced per biomass units by *R. mucilaginosa* IST390 and IST423 in MM with glucose, YPD and SBP hydrolysate H11. These values were obtained from cultures grown at 30ºC, pH 5.0 and 250 rpm, for 120 h. The concentration is in mg of carotenoids per g of dry biomass.

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

Concerning carotenoid production, *R. mucilaginosa* IST390 produced lower carotenoids in minimal medium MM with glucose, whereas IST423 did not produce detectable levels of carotenoids in this medium (Table 2). Compared with IST390, strain IST423 produced lower

levels of carotenoids in both YPD and SBP hydrolysate but confirming maximum production levels in SBP hydrolysates.

Figure 3 – *R. mucilaginosa* IST390 production of carotenoids observed in MM with 10 g/L arabinose and increasing concentrations of acetic acid (0 mM, 10 mM, 20 mM and 35 mM) after 96 h. These assays were performed at 30ºC, pH 5.0, 130 rpm.

Several studies report the production of carotenoids by *R. mucilaginosa* strains and the most comparable studies to the one performed here present similar values to those obtained. The most similar study was performed using food wastes (4.3 to 5.9 g/L of reducing sugars) with 3 g/L yeast extract and mineral supplementation, reporting a maximum carotenoid production of 376.5 µg/g in ketchup, 268.6 µg/g in molasses and 245.0 µg/g in health drink. Carotenoids production varies among yeast strains, substrates consumed and culture conditions [53]. Metal ions addition (Fe^{2+} > Ca^{2+} > Mg^{2+}) was found to improve both carotenoid and biomass production, and, despite molasses having lower reducing sugar concentrations (4.3 g/L) compared with other substrates (5.9 g/L), they contains heavy metals, proteins, vitamins and fat, that also may favour this production [54], similarly to SBP hydrolysates [55,56].

Also, by observation of the cells growing in glucose and arabinose with acetic acid media in shake flasks, it was found that pink pigmentation production increased with increasing concentrations of acetic acid (Figure 3). Despite no carotenoid quantification was obtained, acetic acid concentrations usually present in SBP hydrolysates at pH 5.0 are metabolized as relevant carbon source for pigment generation. Tests using acetic acid as a sole carbon sources would be desirable to examine its potential for carotenoid production. Considering the carbon and nitrogen sources, and other trace elements available in SBP hydrolysates, these pectin-rich substrates are promising also for carotenoids production.

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

Pectin-rich hydrolysates contain several components that may have potential toxic effects in the yeast cells impairing their growth, fermentative and production abilities. Among them, the expected presence of acetic acid is thought to be one the most relevant inhibitors. To determine acetic acid effect in the growth of the examined yeasts, cultures in MM with glucose or arabinose supplemented with increasing concentrations of acetic acid were performed. Those were the sugars present in the hydrolysates that were proven to be metabolized by all the non-conventional yeasts examined. As such, it was considered of interest to assess that influence of yeast growth in both carbon sources.

K. marxianus IST389 and *S. cerevisiae* Ethanol Red exhibit the same profile of growth in all glucose plus acetic acid conditions until 35 mM. Only for 50 mM acetic acid the lowest final OD was attained. As expected, *S. cerevisiae* Ethanol Red did not grow in medium containing only arabinose. *S. cerevisiae* presented the highest tolerance to acetic acid, since it was increasingly growing from this carbon source. *M. guilliermondii* and *R. mucilaginosa* showed to be the most sensible yeasts to acetic acid, which is more accentuated in arabinose than in glucose medium. Still, at 50 mM acetic acid, after cells adaptation, high biomass production was achieved.

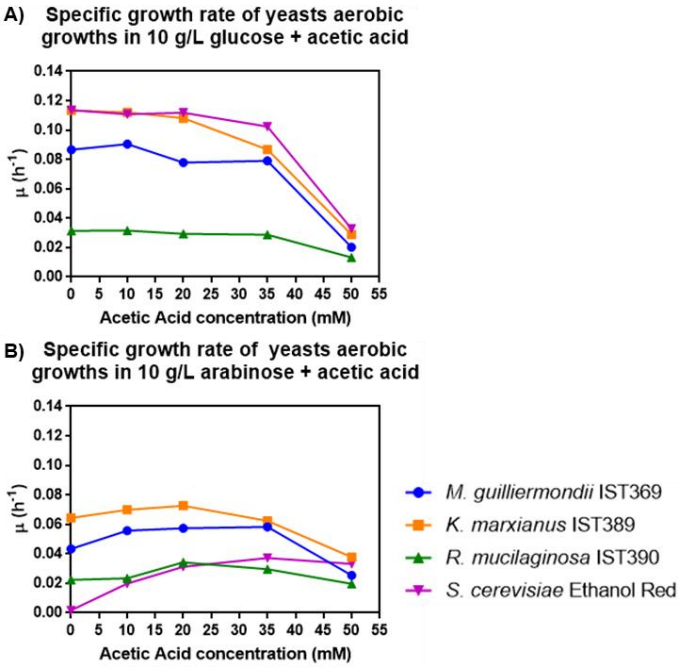

Figure 4 – Maximum specific growth rates (µ) of chosen yeasts in MM with glucose (A) or arabinose (B), in relation with the increasing acetic acid concentration. These values were calculated based on growth curves of *M. guilliermondii* IST369 (dark blue circle), *K. marxianus* IST389 (orange square), *R. mucilaginosa* IST390 (green triangle) and *S. cerevisiae* Ethanol Red (purple inverted triangle.

Acetic acid concentration until 35 mM seem to improve the maximum specific growth rates in arabinose and not causing effect in glucose cultures, that only decrease at 50 mM, to similar values among the medium (Figure 4). This emphasizes the importance of acetic acid as a carbon source, only causing growth inhibition of the yeasts tested at 50 mM, at pH 5.0. Nevertheless, maximum specific growth rates are lower in arabinose than in glucose, except in the case of *R. mucilaginosa*, showing lower maximum specific growth rates in the absence of stress. The maximum specific growth rates obtained in media with acetic acid concentrations from 10 mM to 35 mM slightly increased in arabinose medium, while in glucose media the specific growth rates maintained similar values. This indicates that in glucose media, yeasts' growth in the exponential phase was mostly due to glucose consumption, while in arabinose, acetic acid was also actively metabolized and yeast growth was improved. A previous report on *M.*

guilliermondii cultivation in glucose, xylose and arabinose at pH 5.5, also refer that the addition of 5 g/L acetic acid slightly improved the global specific growth rate compared with an un-supplemented medium and it was proved that acetic acid was co-consumed with all the other carbons sources [154].

Higher concentrations until 35 mM lead to higher biomass, except in the case of *K. marxianus*, in arabinose medium, given that 20 mM already seem to affect biomass production. An interesting study on *K. marxianus* showed that acetic acid toxicity at 40 mM is highly pH and substrate dependent, being growth more affected in galactose and lactose medium than in glucose, inulin and fructose [57]. It was hypothesised the interference of acetic acid with lactose cellular uptake, which occurs only by symport, an energetically unfavoured process when cells are responding to acetic acid stress, trying to export protons to counteract cytosol acidification [57]. This is an highly energetically expensive adaptation mechanism, in which cytoplasmic ATP consumption by ATPases increases and inhibits growth and mitochondria function, as reported for *S. cerevisiae* [58]. It is also considered that enzymes of lactose and galactose metabolic pathways may be affected by acetic acid stress [57]. Similarly to galactose, arabinose is mostly transported by symport through high affinity transporters, as reported for several non-conventional yeasts as *M. guilliermondii* [43], while glucose can more easily enter by facilitated diffusion. Possibly, acetic acid affects more *K. marxianus* cells growing in arabinose than in glucose by impairing cellular uptake, and possibly by interfering with enzymes of arabinose metabolic pathway, while for glucose only at 50 mM acetic acid a detectable effect was observed.

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

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

A rapid comparison of the growth profiles was performed in SBP hydrolysates H11 and H13 (Table 1) at 30ºC and 35ºC and pH 4.5 and 5.0. *S. cerevisiae* CEN.PK122 was added to this assays, since *S. cerevisiae* CEN.PK is tolerant to acetic acid [59].

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

At both 30ºC and 35ºC (Figure 5), it is possible to observe that all strains presenting a similar pattern: H11 at pH 5.0 > H11 at pH 4.5 > H13 at pH 5.0 > H13 at pH 5.0.

K. marxianus IST389 and *S. cerevisiae* Ethanol Red presented similar growth curves at both temperatures. *K. marxianus* is a thermotolerant specie, being capable of growing until maximum temperatures of 52ºC [107], so it as expected that a 5ºC increase did not exert negative effects on growth. *S. cerevisiae* is known for having an inherent acetic acid tolerance [7,47], higher than *K. marxianus*. Additionally, Ethanol Red optimal temperature is 34ºC [60]. The conjugated effect of stress factors seems to affect these strains in a similar way, not presenting significative growth impairment.

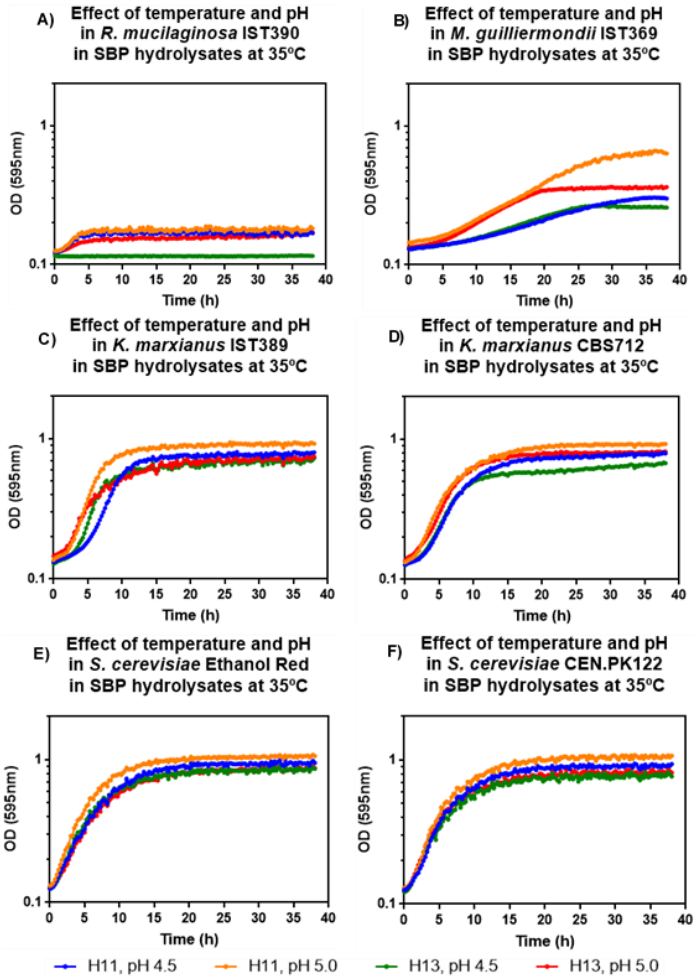

Figure 5 – Growth profiles of the selected yeasts in SBP hydrolysates H11 and H13 at 35ºC. Growths of *R. mucilaginosa* IST390 (A), *M. guilliermondii* IST369 (B), K. *marxianus* IST389 (C), *K. marxianus* CBS712 (D), (orange triangle), *S. cerevisiae* Ethanol Red (E) and *S. cerevisiae* CEN.PK122 (F) were performed in hydrolysate H11, at pH 4.5 (blue line), and pH 5.0 (orange line) and in H13, at pH 4.5 (green line) and pH 5.0 (red line), in a 96-well microplate, and curves were bases in OD measurements at 595nm in a fluorescence microplate reader, in kinetics mode.

M. guilliermondii IST369 showed the highest differences between conditions. At 30ºC, *M. guilliermondii* growth is favoured in H11 over H13, which may be related with hydrolysates composition (higher glucose and less acetic acid). At 35ºC, pH 5.0 is favoured over pH 4.5,

independently of the hydrolysates, consistent with the higher susceptibility to the conjugated effect of temperature, pH and acetic acid. Acetic acid affects the uptake and metabolization of carbon sources, especially at lower pH (pH < pKa 4.7), by cytosol acidification [162].

R. mucilaginosa IST390 presented the more extended latency phases. At 35ºC, IST390 exhibited a marked decrease in biomass production, with reduced duration of exponential phases, due to a much faster growth stabilization, and in H13, pH 4.5, no growth occurred. The increased temperature only affected especially *R. mucilaginosa*, which is reported to present good growth from 25ºC to 30ºC, and variable growth ability at 35ºC [32], even impairing growth of some strains [61]. IST390 seems to be a temperature sensible strain. Still, *R. mucilaginosa* seems to be the most affected by the conjugated effect of temperature, pH and acetic acid, even more than *M. guilliermondii*.

Conclusions and future perspectives

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

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

M. guilliermondii IST369 produced the highest final biomass concentration and it was the only strain that efficiently catabolized arabinose. Several studies report the successful metabolic engineering of *S. cerevisiae* by expressing filamentous fungi pathways for arabinose (and xylose) utilization [62,63]. It would be interesting to express these metabolic pathways in *S. cerevisiae*, especially from other ascomycete yeast as *M. guilliermondii*. *R. mucilaginosa*, despite being the only species examined that can grow using GalA as the sole carbon source, GalA consumption in the hydrolysates was not possible during 168 h of incubation. This is not only due to the low specific growth rate and sugar utilization of this species strains, but also because GalA, as well as arabinose, are only utilized following the consumption of the more easily catabolized carbon sources. *S. cerevisiae* metabolic engineering with GalA yeast metabolic pathway, instead of using filamentous fungi pathways, as it was already reported [64,65], would also be desirable. For this, a more detailed study of *M. guilliermondii* IST369 and *R. mucilaginosa* strains IST390 and IST423 metabolic pathways of these sugars would be required, as well as the optimization of the cultivation conditions, especially for *R. mucilaginosa*, if to be directly used as cell factory.

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

In terms of production potential, ethanol was produced by *K. marxianus* strains IST389 and CBS712 and M. guilliermondii IST389 at amounts and yields similar to S. cerevisiae. Both *K. marxianus* strains IST389 and CBS712 produce high arabitol concentrations, with high production yields. For these strains, SBP hydrolysates as substrate and culture conditions proved to be suitable for arabitol production.

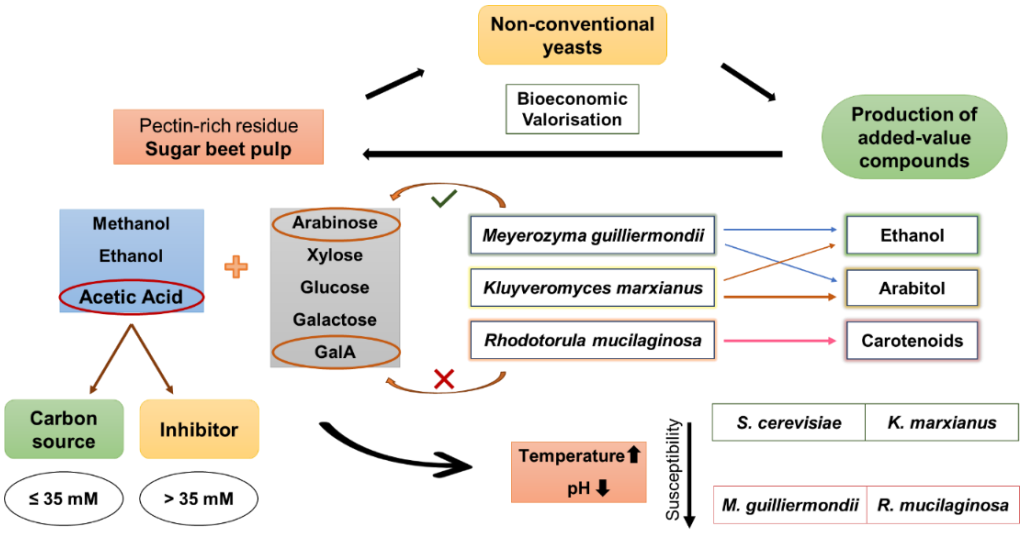

Figure 6 – Schematization of the most relevant conclusions obtained in the present study.

The thermotolerance of *K. marxianus* can be an interesting and advantageous feature to be explored in both ethanol and arabitol production. Despite its low fermentative ability and growth rate, *R. mucilaginosa* strains IST390 and IST423 possess high potential for carotenoid production, especially in SBP hydrolysates.

This thesis provides insights on the metabolization and production potential of new yeast isolates obtained in our laboratory to be explored for the bioconversion of pectin-rich residues, in particular of SBP hydrolysates.

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