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Published in:
Industrial Crops and Products

Link to article, DOI:
10.1016/j.indcrop.2021.113799

Publication date:
2021

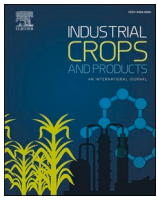
Document Version
Publisher's PDF, also known as Version of record

Industrial Crops & Products 170 (2021) 113799

Contents lists available at ScienceDirect

Industrial Crops & Products

journal homepage: www.elsevier.com/locate/indcrop



Effects of inhibitory compounds derived from lignocellulosic biomass on the growth of the wild-type and evolved oleaginous yeast *Rhodosporidium toruloides*

Zhijia Liu [a], Marlene Fels [a], Giuliano Dragone [b], Solange I. Mussatto [b,*]

[a] Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, Kemitorvet, Building 220, 2800, Kongens Lyngby, Denmark
[b] Department of Biotechnology and Biomedicine, Technical University of Denmark, Søltofts Plads, Building 223, 2800, Kongens Lyngby, Denmark

ARTICLE INFO

Keywords:
Lignocellulosic biomass
Inhibitors
Cell growth
Oleaginous yeast
Adaptive laboratory evolution
Rhodosporidium toruloides

ABSTRACT

The presence of toxic compounds in lignocellulosic hydrolysates is one of the main problems affecting the efficiency of hydrolysate-based fermentation processes. Understanding the effects of biomass-derived inhibitors on the performance of the microbial strain is essential to develop strategies able to result in an improved fermentation performance. In the present study, efforts were done to elucidate the effects of the main biomass-derived inhibitors on the growth of the oleaginous yeast *Rhodosporidium toruloides*. Furfural, 5-hydroxymethylfurfural (5-HMF), acetic acid, levulinic acid, benzoic acid, *p*-coumaric acid, ferulic acid, vanillic acid, vanillin and syringaldehyde were the inhibitory compounds investigated. Assays were performed using a wild-type and an evolved strain of *R. toruloides*. For the individual inhibitors, more than 3 mM benzoic acid completely inhibited the growth of both strains, while acetic acid and levulinic acid were less toxic, presenting IC50 values of 16 and 15 mM, respectively. In a subsequent step, assays were performed in media containing different combinations of inhibitory compounds and a Placket-Burman experimental design was used to identify the most severe inhibitors in mixture, as well as combination of inhibitors, affecting the yeast growth. Results revealed that in mixture, furfural was the most potent inhibitor affecting the growth of *R. toruloides,* followed by vanillin and 5-HMF. The combination of furfural, 5-HMF, vanillin, vanillic acid and ferulic acid resulted in the most severe inhibition to this yeast. The evolved strain showed an improved ability to tolerate toxic compounds, suggesting that the adaptive laboratory evolution is a potential strategy to obtain oleaginous yeasts with improved ability to grow in biomass hydrolysates.

1. Introduction

Due to its renewable nature, abundance (global production estimated in 181.5 billion tones/year (Dahmen et al., 2019)), low cost and rich composition, lignocellulosic biomass is considered a sustainable feedstock, suitable for use in the production of chemicals, fuels, food and feed ingredients and materials. Sugars in the form of cellulose and hemicellulose polysaccharides may represent more than 60 % by weight of the biomass composition, which makes biomass very attractive for use in bioprocesses. The remaining amount of the composition is mainly constituted by lignin, a polyphenolic macromolecule that provides structural support to the plant cell wall (Mussatto and Dragone, 2016).

The complex and recalcitrant structure of biomass hinders the access of chemicals and enzymes to its surface. To overcome the recalcitrance of these materials and release fermentable sugars, pretreatment and hydrolysis processes are required. High temperature, pressure and extreme pH conditions are often used as effective pretreatment methods. However, under such harsh conditions, some toxic compounds are also generated, which may significantly affect the growth performance of microorganisms when biomass hydrolysates are used as fermentation medium (Mussatto and Roberto, 2004a). Although the formation of toxic compounds in hydrolysates largely depends on the type of lignocellulosic biomass and pretreatment conditions used, the major groups of inhibitors found in lignocellulosic biomass hydrolysates include furan derivatives, weak aliphatic acids and phenolic compounds (Wang et al., 2018). The most common furan derivatives found in biomass hydrolysates are furfural and 5-hydroxymethylfurfural (5-HMF), which originate from the degradation of pentose and hexose sugars, respectively.

* Corresponding author.
E-mail addresses: smussatto@dtu.dk, solangemussatto@hotmail.com (S.I. Mussatto).

https://doi.org/10.1016/j.indcrop.2021.113799
Received 4 April 2021; Received in revised form 16 June 2021; Accepted 1 July 2021
Available online 12 July 2021

Aliphatic acids like acetic acid, levulinic acid and formic acid are formed from the acetyl groups and sugar molecules (Du et al., 2010). Finally, phenolic compounds, including acids, alcohols, and aldehydes, are released from the lignin structure (Wang et al., 2018).

Rhodosporidium toruloides is an oleaginous yeast that has attracted great interest due to its ability to use glucose and xylose, the main sugars present in lignocellulosic hydrolysates, as carbon source to produce lipids and carotenoids (Liu et al., 2020). Lipids produced by oleaginous yeasts can reach up to 70 % of dry cell weight and have been proposed as ideal substitutes of vegetable oils for conversion into biodiesel (Vasconcelos et al., 2019). Biodiesel has been considered as an important renewable fuel and its market price has reached $1630/t (NESTE, 2020). Carotenoids are valuable nutritional compounds, able to promote several benefits to human health such as decreasing the risk of inflammation, heart disease and type 2 diabetes, preventing cancer, improving eye-health, and protecting neurons (Novoveská et al., 2019). Due to these benefits, the antioxidant properties and attractive color, carotenoids have been widely utilized in food, feed, cosmetics and nutraceutical industries, and their global market is estimated to reach $2.00 billion by 2026 (MarketsandMarkets, 2020).

Production of lipids and carotenoids by oleaginous yeasts from lignocellulosic biomass is considered an attractive and sustainable approach. However, yeast growth in biomass hydrolysates can be affected by the presence of inhibitory compounds in these media. Understanding the effects of these inhibitors on yeast growth is essential for the development of strategies capable of overcoming this obstacle, resulting in an efficient production process. The present work aims to fulfill this gap by providing a detailed study on the effect of several inhibitory compounds commonly found in lignocellulosic hydrolysates on the growth of the oleaginous yeast *R. toruloides*. Both, the individual and combined effects of the inhibitory compounds were evaluated. To assess the combined effects, a Placket-Burman experimental design was used to identify the most severe inhibitors in a mixture as well as combinations of inhibitors that strongly affect the yeast growth. Moreover, to get a better understanding on the potential applications and limitations of this yeast and find solutions to improve its tolerance towards biomass-derived inhibitors, assays were performed using the wild-type and an evolved strain of *R. toruloides*.

2. Materials and methods

2.1. Microorganism, medium composition and inoculum conditions

The wild-type oleaginous yeast used in this study, *Rhodosporidium toruloides* NRRL Y-1091(WT), was obtained from the Agricultural Research Culture Collection, USA. The evolved strain of *R. toruloides* (EV) with improved performance to grow in the presence of inhibitors found in biomass hydrolysates was obtained by previous experiments of adaptive laboratory evolution performed in our laboratory using wheat straw hydrolysate as cultivation medium (Liu et al., 2021). Both strains were preserved in 20 % of glycerol at – 80 °C.

A synthetic complete (SC) medium (Sigma-Aldrich) supplemented with 0.1 g/L leucine and 20 g/L glucose, pH 5.0, was used for inoculum cultivation as well as for the experiments to determine the threshold concentrations of inhibitors that affect cell growth. For inoculum cultivation, the wild and evolved strains were grown in 250-mL Erlenmeyer flasks at 30 °C, 250 rpm, for 48 h. Afterwards, the cells were spread on 1.6 % agarose SC plates and incubated at 30 °C for 5 days to get single colonies. The plates were stored at 4 °C for further use.

2.2. Inhibitor threshold concentration test

The experiments to assess the effect of inhibitor concentration on growth of *R. toruloides* were performed in a Growth Profiler 960 (Enzyscreen, Heemstede, Netherlands). Initially, a calibration curve was set to convert G-values obtained from the Growth Profiler into optical density of yeast cells at 600 nm (OD_{600}). To perform the calibration, overnight cultures of WT and EV strains were prepared from single colonies previously cultivated in 50 mL SC medium at 30 °C and 250 rpm (Liu et al., 2020). The OD_{600} of the culture was measured after 16 h and then adjusted with SC medium to different levels. The following OD_{600} values were used to set the calibration curve: 0, 0.2, 0.8, 1.5, 2.5, 3.5, 5, and 8. Each level was prepared in duplicate. Then, a 300 µL volume of each culture with different OD_{600} value was transferred to a transparent 96 well-plate (CR1496d Enzyscreen, Heemstede, Netherlands) and the bottom of the plate was scanned by a camera. The Growth Profiler software was used to calculate the density of yeast cells in each well from the G-values generated. The following equation (Eq.1) resulted from the calibration:

$$OD_{600} = 2.1 \times 10^{-5} \times (\text{G-value})^{3.2627} \quad (1)$$

The inhibitory compounds tested in this study included furfural, 5-HMF, acetic acid, levulinic acid, benzoic acid, *p*-coumaric acid, syringaldehyde, ferulic acid, vanillic acid, and vanillin (all from Sigma-Aldrich). The concentration range evaluated to each compound (Table 1) was based on the average composition reported for wheat straw hydrolysate (Liu et al., 2021). Similar concentration values have also been reported for hydrolysates produced from other types of biomass (Li et al., 2019; Wang et al., 2016).

To each inhibitor, a stock solution was prepared by dissolving the compound in absolute ethanol to a certain concentration. Then, the solution was sterilized using 0.2 µm filter and stored in brown autoclaved bottles at 4 °C. For the experiments, the stock solutions were diluted in SC medium to obtain the desired concentration. Cell growth experiments were carried out in 96 well-plates inoculated with an initial OD_{600} of 0.5. After inoculation, the plates were sealed with sandwich covers, which limit evaporation of medium in plates to 2% and prevent cross-contamination during vigorous shaking. Control experiments containing only SC medium and inoculum (without inhibitors) were also performed. The plates were incubated in the Growth Profiler at 30 °C, 250 rpm for 72 h–96 h. The bottom of these plates was scanned by the Growth Profiler's camera at 20 min intervals to obtain G-values.

2.3. Determination of growth rate and lag phase

For each assay, the specific growth rate of the yeast was calculated via a MATLAB code adapted from Hemmerich et al. (2017). The output was given as µ (h^{-1}). The growth rate of the control experiments (without inhibitors) represented the μ_{max}. IC50 was then calculated as the concentration of an inhibitor at which the growth rate of the yeast was inhibited to 50 % of μ_{max}. The lag phase duration of each strain in the control cultures and media containing inhibitors was determined as described by Buchanan and Cygnarowicz (1990).

2.4. Plackett-Burman experimental design

To identify the most potent inhibitors affecting the growth of

Table 1
Concentrations of each inhibitor evaluated individually on growth of wild-type and evolved strains of *R. toruloides*.

Inhibitor	Concentration (mM)
Furfural	4, 6, 8
5-Hydroxymethylfurfural	1, 2, 3, 4, 5
Acetic acid	8, 16
Levulinic acid	5, 10, 15, 20
Benzoic acid	1, 2
p-Coumaric acid	2, 5, 7
Ferulic acid	1, 2, 3, 4, 10
Syringaldehyde	1, 3, 4, 5, 7
Vanillin	0.5, 1, 2, 3
Vanillic acid	5, 7, 10, 12

R. toruloides, a Plackett-Burman experimental design was performed. The design of experiments was created by combining ten independent factors (ten different inhibitors) at two levels each, with two dummy factors, which resulted in 28 experiments (Table 2). The two levels corresponded to the absence (-1) and presence (+1) of the inhibitor in the medium. The dummy factors were included to estimate the experimental error, and the reduction in the growth rate of the strain was the response evaluated. Since the WT and EV strains presented similar IC50 values in individual experiments, only the EV strain was considered for the Plackett-Burman design.

Regarding to the concentration of each inhibitor used in the experiments, since the combination of more than one inhibitor can cause a stronger cell growth inhibition than a single inhibitor, each independent factor was evaluated at a concentration of a quarter of their IC50 values (Table 3). For those inhibitors where the IC50 value was not obtained in the previous experiments, a quarter of the maximum concentration at which cell growth was observed was the value considered for the Plackett-Burman design.

2.5. Statistical analysis

Differences among samples were analyzed by one-way analysis of variance (ANOVA) at a significance level of 0.05, using the software OriginPro 2018b (OriginLab, USA). Statistical and graphical analyses of the Plackett-Burman design were performed using the software Statistica version 10.0 (Statsoft Inc., Tulsa, OK, USA) at a significance level of 0.2.

3. Results and discussions

3.1. Effect of furan derivatives on the growth rate and lag phase of *R. toruloides*

Furfural and 5-HMF are the most common furan derivatives found in lignocellulosic hydrolysates and have been reported as being toxic to many microorganisms. Their toxic effect is related to the inhibition of the activity of enzymes such as pyruvate, acetaldehyde and alcohol dehydrogenases (Wang et al., 2018), reduction of the levels of intracellular ATP and NAD(P)H, and change in the direction of cell energy to fixing damages (Almeida et al., 2007). Specifically, the presence of furfural in fermentation medium can result in the accumulation of reactive oxygen species, which causes damages to mitochondrial and vacuole membranes of yeast cells, nuclear chromatin and actin cytoskeleton (Allen et al., 2010).

The effect of furfural and 5-HMF on the growth rate and lag phase of WT and EV strains of *R. toruloides* is shown in Fig. 1. As can be seen, the lag phase of both strains was significantly longer in presence of 4 mM of furfural when compared with the controls, and higher concentrations of furfural had a measurable effect on the adaptation time (Fig. 1a). The lag phase of both yeasts reached the highest values (70.8 ± 1.8 h for WT, 38.0 ± 2.5 h for EV) in the presence of 8 mM of furfural. On the other hand, the growth rates of both strains did not decrease significantly ($p < 0.05$) for furfural concentrations higher than 4 mM. Therefore, in the range of concentrations studied (up to 8 mM), an IC50 value was not reached for any of the strains. To define the IC50, higher concentrations of furfural should be tested.

Furfural had a significant effect on the lag phase of both WT and EV strains of *R. toruloides*. However, the EV strain showed a shorter lag phase than the WT strain. This could be explained by the fact that furfural and 5-HMF can be converted into furfuryl alcohol and HMF

Table 2
Coded levels of factors and response obtained from Plackett-Burman design used to assess the synergistic effect of biomass-derived inhibitors on the growth of evolved *R. toruloides* strain.

Run	Factors *												Response
	FA	VN	VA	BA	CA	LA	AA	5-HMF	FF	SGH	D1	D2	Growth rate (h^{-1}) **
1	1	1	1	−1	−1	−1	1	1	−1	1	−1	−1	0.321
2	−1	−1	−1	1	1	−1	1	1	1	1	−1	−1	0.272
3	1	−1	−1	1	−1	−1	−1	−1	1	1	1	−1	0.248
4	1	1	−1	−1	1	1	1	1	−1	−1	−1	−1	0.276
5	1	1	1	−1	−1	−1	1	−1	1	−1	−1	1	0.313
6	1	1	−1	1	1	1	−1	−1	−1	−1	−1	1	0.423
7	−1	−1	1	−1	1	−1	−1	−1	1	1	−1	1	0.498
8	1	−1	1	−1	1	1	−1	1	1	−1	1	1	0.310
9	−1	1	−1	−1	1	−1	1	−1	−1	−1	1	1	0.342
10	−1	1	1	1	1	−1	1	1	−1	1	1	−1	0.335
11	1	1	−1	1	1	−1	−1	1	1	1	1	1	0.250
12	−1	−1	1	−1	−1	1	−1	1	−1	1	−1	1	0.328
13	−1	1	1	−1	1	1	1	−1	1	1	1	1	0.299
14	1	−1	−1	−1	1	−1	−1	1	−1	1	−1	1	0.410
15	−1	−1	1	1	−1	−1	1	−1	−1	−1	1	1	0.313
16	1	−1	1	1	1	1	−1	−1	−1	−1	1	−1	0.329
17	−1	1	1	1	1	1	−1	−1	−1	1	−1	−1	0.254
18	−1	−1	−1	1	−1	1	1	1	1	−1	−1	1	0.253
19	−1	−1	−1	−1	1	1	1	1	1	−1	1	−1	0.267
20	1	1	1	−1	−1	−1	−1	1	1	−1	1	−1	0.241
21	−1	1	−1	−1	−1	1	−1	−1	1	1	1	−1	0.278
22	−1	1	−1	1	−1	−1	−1	1	−1	−1	1	1	0.301
23	−1	1	1	1	−1	1	−1	1	1	−1	−1	−1	0.310
24	1	−1	1	1	−1	−1	−1	−1	−1	−1	−1	−1	0.590
25	1	1	−1	1	−1	1	1	−1	1	1	−1	1	0.288
26	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.334
27	1	−1	−1	1	−1	1	1	1	−1	1	1	1	0.354
28	1	−1	−1	−1	−1	1	1	−1	−1	1	1	−1	0.313

* FA: ferulic acid, VN: vanillin, VA: vanillic acid, BA: benzoic acid, CA: *p*-coumaric acid, LA: levulinic acid, AA: acetic acid, 5-HMF: 5-hydroxymethyl furfural, FF: furfural, SGH: syringaldehyde, D1: Dummy 1, D2: Dummy 2.

** Growth rate for the control (without inhibitors): 0.73 h^{-1}.

Table 3
Concentration of each inhibitor used for the Placket-Burman experimental design.

Inhibitor	IC50 or maximum concentration (mM) *	Concentration tested in the Placket-Burman design (mM)
Ferulic acid	10 (max)	2.5
Vanillin	3 (max)	0.75
Vanillic acid	10 (IC50)	2.5
Benzoic acid	2 (IC50)	0.5
p-Coumaric acid	7 (IC50)	1.75
Levulinic acid	15 (IC50)	3.75
Acetic acid	16 (IC50)	4.2
5-Hydroxymethylfurfural	3 (IC50)	0.75
Furfural	8 (max)	2
Syringaldehyde	4 (IC50)	1
Dummy 1	–	–
Dummy 2	–	–

* Maximum concentration of each inhibitor at which cell growth was observed.

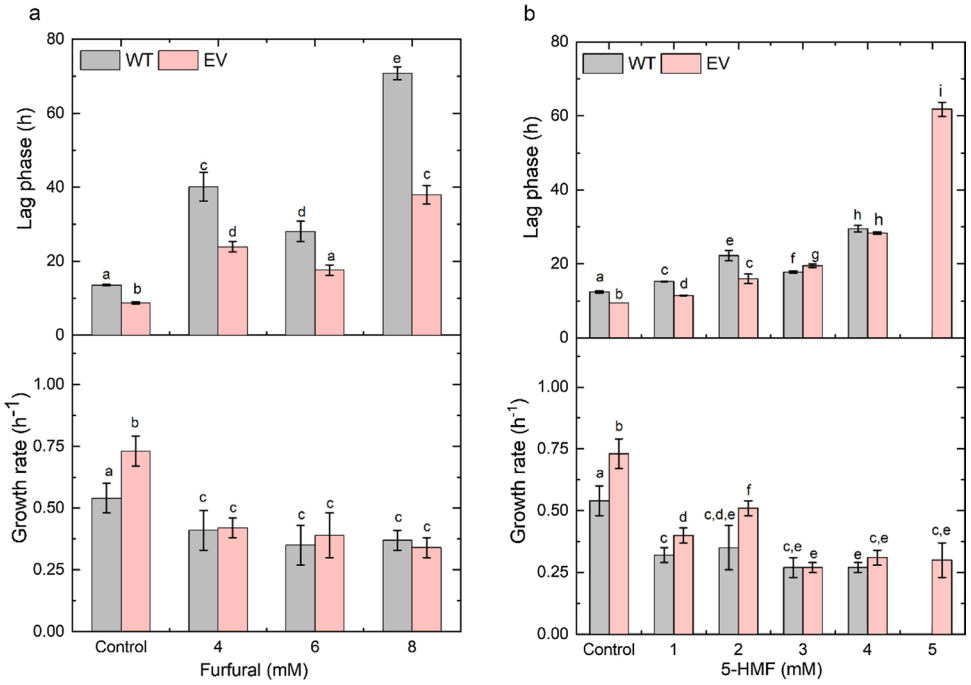


Fig. 1. Effects of furan derivatives on growth rate and lag phase of wild-type (WT) and evolved (EV) strains of *R. toruloides.* a) Furfural; b) 5-hydroxymethylfurfural (5-HMF). To each plot, bars marked with different letters mean statistically significant differences at $p < 0.05$.

alcohol respectively by a series of dehydrogenases in oleaginous yeasts. The toxicity of these two compounds could be reduced by further oxidation reactions, which convert furfuryl and HMF alcohol into furoic acid and HMF acid as final products. Both degradation processes occur during the initial stage of fermentation (Wang et al., 2016); therefore, the duration of lag phase observed in media containing furfural and 5-HMF could indicate the detoxification abilities of yeast strains toward these two compounds. The EV strain may hold a stronger detoxification ability than that of WT strain. Once the strains adapted to these toxic environments, growth rates of both were maintained in certain levels.

When assessing the effect of 5-HMF, it was observed a slight elongation of the lag phase for both strains in comparison with those of the controls, up to 3 mM (Fig. 1b). On the other hand, at the maximum 5-HMF concentration tested (5 mM), the WT strain could not start its exponential growth, while the lag phase of the EV strain lasted 61 ± 1.9 h. Growth rates of the WT and EV strains were also significantly affected by the presence of 5-HMF in the medium. At 1 mM 5-HMF, the growth rates of both yeasts decreased by more than 30 % ($p < 0.05$) when compared to the growth rates of the controls. The IC50 of 5-HMF for WT and EV strains was reached at 3 mM, but EV grew up to 5 mM at the same growth rate (0.27 h^{-1}).

In summary, furfural and 5-HMF had strong effects on the lag phase of both strains. It is worth noting that the WT and EV strains could enter in exponential growth in presence of 8 mM of furfural, while 5 mM of 5-HMF totally inhibited the WT growth. These results suggest that 5-HMF presents a more severe individual effect on *R. toruloides* than furfural when considering the same concentration, which differs from the results previously reported for *Saccharomyces cerevisiae,* where 5-HMF was found to be less inhibitory than furfural (Almeida et al., 2007).

3.2. *Effects of aliphatic acids*

Aliphatic acids usually found in lignocellulosic biomass hydrolysates include acetic acid, levulinic acid, and formic acid. Acetic acid is formed from the acetyl groups present in the side chain of hemicellulose, while levulinic acid results from the degradation of 5-HMF, and formic acid from the degradation of both furfural and 5-HMF (Mussatto and Roberto, 2004a). The inhibition mechanism of aliphatic acids in yeasts is explained by uncoupling and intracellular anion accumulation (Russell, 1992). Most undissociated aliphatic acids with low molecular weight are able to diffuse through the plasma membrane into the cytosol and then dissociate due to the higher intracellular pH, which results into

decreasing cytosolic pH and accumulation of potentially toxic levels of anionic species of the weak acids (Wang et al., 2018).

The effect of acetic acid and levulinic acid on the growth of WT and EV strains of *R. toruloides* is shown in Fig. 2. When compared to the controls, addition of 8 mM acetic acid did not increase the lag phase significantly ($p < 0.05$) for both strains. However, when this concentration was doubled to 16 mM, the lag phase of both strains was significantly ($p < 0.05$) longer (by 9.7 h and 9.9 h for WT and EV, respectively) (Fig. 2a). Regarding the growth rate, the μ_{max} for the control cultures was 0.54 ± 0.06 h^{-1} (WT) and 0.73 ± 0.06 h^{-1} (EV). Addition of 8 mM or 16 mM acetic acid resulted in no significant ($p < 0.05$) decrease in the growth rate of WT. On the other hand, the growth rate of EV decreased to 0.46±0.13 h^{-1} and 0.35 ± 0.06 h^{-1} in the presence of 8 mM and 16 mM acetic acid, respectively. Therefore, the IC50 of acetic acid for EV was found at 16 mM, whereas this value was not identified for the WT strain.

In the case of levulinic acid, increasing the concentration of this acid in the medium resulted in a gradual increase of the lag phase for both strains. At the highest concentration tested (20 mM), the WT strain was not able to grow, while the EV strain grew after a lag phase of 19.6 ± 2.9 h (Fig. 2b). Levulinic acid also had a significant influence on the growth rate of both strains at concentrations higher than 5 mM. The growth rate of WT and EV strains was similarly inhibited in presence of 5 mM, 10 mM and 15 mM levulinic acid, and fell below the IC50 at 15 mM. However, the EV strain was able to grow in presence of 20 mM levulinic acid, with a specific growth rate (0.28 ± 0.01 h^{-1}) comparable to that observed at 15 mM ($p < 0.05$).

Although the inhibition mechanism of levulinic acid is similar to that of acetic acid, their toxicities on yeast cells were different. While 5 mM of levulinic acid led to a significant increase in the lag phase and decrease in growth rate for the WT strain, the presence of 8 mM acetic acid had lesser influence on yeast performance. This could be explained by the fact that the pKa of levulinic acid (4.64) is lower than that of acetic acid (4.76) (Jönsson et al., 2013); thus, the level of undissociated levulinic acid was higher in comparison to that of acetic acid.

3.3. Effects of phenolic compounds

Phenolic compounds are released from the lignin structure during the pretreatment of lignocellulosic biomass. Due to the complex structure of lignin, its degradation forms a large group of inhibitors, including acids, alcohols and aldehydes. The ability of phenolic compounds to penetrate into cells' membrane is considered as the main reason of toxicity of phenolic compounds to microorganisms (Mussatto and Roberto, 2004a). Phenolic compounds can change the fluidity and hydrophobicity of cell membrane, resulting in damages in the cellular membrane and leakage of intracellular components. Another theory about the inhibition mechanism of phenolic compounds is that their presence can increase the generation of reactive oxygen species, and the interaction of these species with proteins and enzymes can cause DNA mutagenesis and cell death (Ibraheem and Ndimba, 2013).

Benzoic acid is a lignin degradation product usually found in biomass hydrolysates and it can be harmful to microorganisms due to the formation of cytoplasmic benzoate upon entering the cell. The reduction of benzoate requires large amount of energy, which in turn is not available for growth or other cellular processes (Warth, 1988). In this study, it was found that the presence of benzoic acid in the medium affected the growth of *R. toruloides*. The lag phase of the WT strain increased about 60 % in comparison to the control, when benzoic acid was present in the medium in concentration higher than 1 mM (Fig. 3a). The same trend was observed for the EV strain, with the lag phase reaching 15.3 ± 1.7 h at 2 mM of benzoic acid. Concentrations of benzoic acid in the range of 1 mM–2 mM also significantly affected the growth rate of the EV strain, being possible to observe the IC50 in this case, at 2 mM.

In the case of *p*-coumaric acid, the toxicity of this acid (like other weak acids) has been related to the fact that it can diffuse through the plasma membrane into the cell, where it is degraded by phenolic acid decarboxylase to other toxic vinyl derivatives (Lentz and Harris, 2015). Previous studies have reported inhibitory effects of this acid on the growth of *S. cerevisiae* (Adeboye et al., 2014). Similarity, it was found that *p*-coumaric acid also affected the growth of *R. toruloides* in the present study. Both the WT and EV strains exhibited longer lag phase in presence of *p*-coumaric acid (Fig. 3b). Concentrations up to 7 mM caused a lag phase of approximately 20 h for both strains. However, at 2–5 mM,

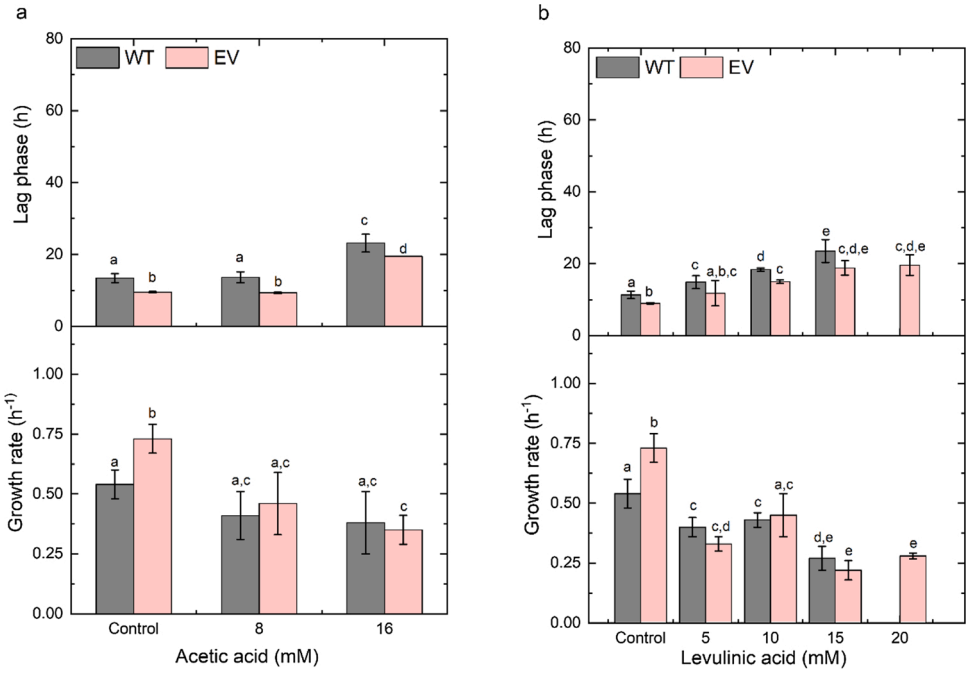


Fig. 2. Effects of weak acids on growth rate and lag phase of wild-type (WT) and evolved (EV) strains of *R. toruloides*. a) Acetic acid; b) levulinic acid. To each plot, bars marked with different letters mean statistically significant differences at $p < 0.05$.

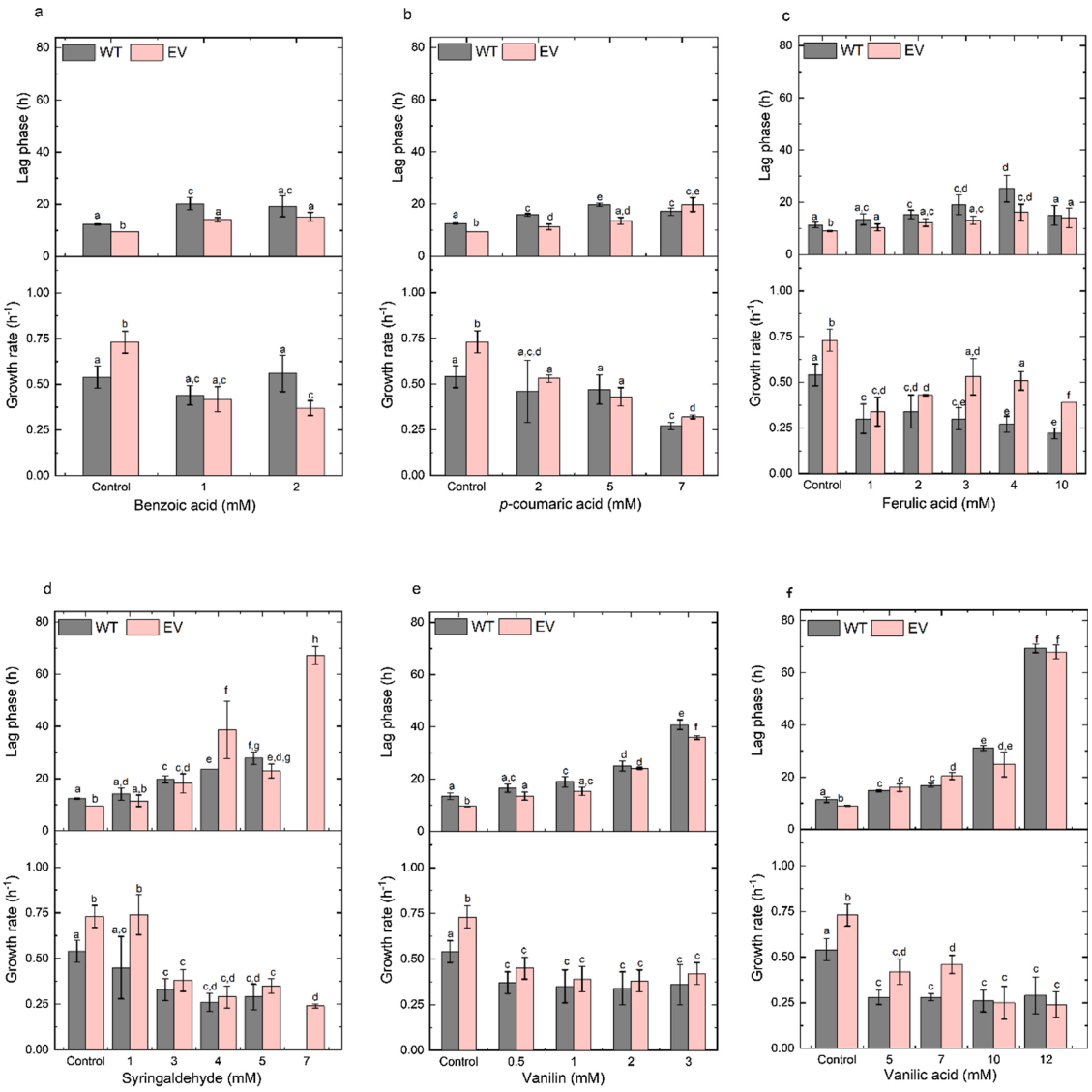


Fig. 3. Effects of phenolic compounds on growth rate and lag phase of wild-type (WT) and evolved (EV) strains of *R. toruloides*. a) Benzoic acid; b) *p*-coumaric acid; c) ferulic acid; d) syringaldehyde; e) vanillin; f) vanillic acid. To each plot, bars marked with different letters mean statistically significant differences at $p < 0.05$.

no significant ($p < 0.05$) inhibition of the growth rate was observed for the WT strain, while the EV strain showed a decrease in growth rate. At the end, a significant reduction of growth rate was observed for both strains at 7 mM *p*-coumaric acid, which theoretically reached the IC50.

Ferulic and *p*-coumaric acids belong to the group of lignin-derived carboxylic acids and have similar inhibition mechanisms. However, it has been reported that among ten types of organic acids derived from lignocellulosic biomass, ferulic acid presented one of the strongest inhibition to the oleaginous yeast *Trichosporon fermentans* (Huang et al., 2012). In the present study, ferulic acid also caused inhibition to *R. toruloides*. The lag phase of both WT and EV strains were longer in media containing this acid. However, the growth rate of the WT strain was in the range of 0.3 h^{-1} for all the ferulic acid concentrations tested (1–10 mM). When compared to the control, the IC50 of ferulic acid was reached at 4 mM for WT. The growth rate of the EV strain was also affected in presence of ferulic acid, but higher growth rates were observed for EV compared to WT for all the concentrations of ferulic acid tested. Since the growth rate of the EV strain was less affected, the IC50 was not reached in this case.

Syringaldehyde is formed by the degradation of sinapyl alcohols from lignin (Du et al., 2010). Fig. 3**d** shows the lag phase and growth rate of the WT and EV strains of *R. toruloides* grown in media with different syringaldehyde concentrations. No significant ($p < 0.05$) impacts on the lag phase and growth rate of both strains were observed at the lowest concentration tested (1 mM). In contrast, more than 67 h were needed for the EV strain to adapt to the medium containing 7 mM syringaldehyde, while the WT strain was unable to grow in this condition. The growth rate of both strains was significantly affected when the concentration of syringaldehyde was increased and, in both cases, the IC50 was reached at 4 mM.

Vanillin has been considered as one of the most potent toxic compounds in biomass hydrolysates as it causes serious DNA damage (Shen et al., 2014) and affects the membrane components and function of microorganisms (Zhang et al., 2014). In this study, vanillin showed inhibitory effects on yeast growth similar to that of furfural in concentrations up to 3 mM. For both strains, a 4 h delay in the lag phase was observed by the presence of just 0.5 mM vanillin in the medium (Fig. 3e). At 3 mM of vanillin, the lag phase of the WT lasted 40.8 ± 1.9 h. Although the lag phases were longer when the concentration of vanillin in the medium was increased, the growth rates of both strains remained almost constant for all vanillin concentrations tested. All concentrations of vanillin reduced the growth rates by approximately one third but remained relatively stable at around 0.4 h^{-1} for both WT and EV strains, and the IC50 was not reached in either case. This

maintenance of a relatively high growth rate indicates that after a prolonged lag phase, no increased inhibition occurred. Hence, the effect of vanillin was clearly stronger on the lag phase than on the growth rates.

The effect of vanillic acid, an intermediate degradation product of vanillin, on the growth of *R. toruloides* was also assessed in the present study. As can be seen in Fig. 3f, a gradual increase in the lag phase occurred for both strains when the concentration of vanillic acid was raised from 5 to 10 mM. However, at 12 mM, vanillic acid caused a distinct lag phase of about 69 h in both strains. The growth rate of the WT yeast was approximately 0.27 h^{-1} ($p < 0.05$) for all the vanillic acid concentrations tested, which resulted in an IC50 at 5 mM. For the EV yeast, 10 mM vanillic acid led to a significant reduction of the growth rate to 0.25±0.09 h^{-1}, reaching the IC50 in this case. These results suggest that once adapted, *R. toruloides* can grow at a stable rate in culture media containing vanillic acid in concentrations up to 12 mM.

3.4. Comparison of growth parameters of wild-type and evolved strains

Table 4 summarizes the maximum inhibitor tolerance, growth rate and lag phase for the WT and EV strains of *R. toruloides* grown in culture media containing different biomass-derived inhibitors. Overall, the maximum concentration of each inhibitor tolerated by the strains was very similar for both WT and EV; however, the EV strain was able to tolerate higher concentration of 5-HMF (5 mM), levulinic acid (20 mM) and syringaldehyde (7 mM) than the WT strain (4, 15, and 5 mM, respectively). In other cases, the EV strain presented significantly shorter lag phase than the WT strain, for the same concentration of toxic compound. This was observed for furfural, acetic acid, benzoic acid, and vanillin. In the case of *p*-coumaric and ferulic acids, the main difference between the strains was the highest growth rate presented by the EV strain compared to the WT, for the same concentration of inhibitory compound. The better performance of the EV strain may be attributed to its higher efficiency to export toxic compounds from intracellular environments and the up-regulation of inhibitor resistance genes naturally present in the WT strain (Liu et al., 2021).

In a recent study, a mutant of *R. toruloides* obtained by combination of evolutionary and metabolic engineering also presented better performance to grow in medium containing acetic acid and furfural when compared to the WT strain (Díaz et al., 2018). Another mutant of *R. toruloides* obtained by plasma mutagenesis also demonstrated improved tolerance and ability to grow in presence of acetic acid, formic acid, vanillin and 5-HMF (Qi et al., 2014). In both cases, the lipid accumulation by the mutant strains was also improved when compared to the WT strain. In the present study, the EV strain of *R. toruloides* was obtained by applying a new strategy of adaptive laboratory evolution using biomass hydrolysate as cultivation medium. Besides presenting better performance to grow in the presence of inhibitory compounds, the EV strain also presented an improved ability to accumulate carotenoids (Liu et al., 2021).

It is worth noting that the reduced lag phase observed for the EV strain in the present study, as discussed above, represents a notable advantage in terms of fitness and industrial application of this yeast. Additionally, a reduced lag phase can also help reducing the overall energy demand of the process since the aeration and agitation required for both yeast propagation and lipid accumulation are important factors contributing to the energy consumption during the process for microbial lipid production (Karlsson et al., 2016).

Table 4
Comparison of maximum inhibitor tolerance, growth rate and lag phase for the wild-type (WT) and evolved (EV) strains of *R. toruloides*.

Inhibitor *	WT strain			EV strain		
	Max (mM)	μ (h^{-1})	Lag phase (h)	Max (mM)	μ (h^{-1})	Lag phase (h)
FF	8	0.37 ± 0.04	70.8 ± 1.8	8	0.34 ± 0.04	38.0 ± 2.5
5-HMF	4	0.27 ± 0.02	29.5 ± 0.9	5	0.30 ± 0.07	61.8 ± 1.9
AA	16	0.38 ± 0.13	23.1 ± 2.5	16	0.35 ± 0.06	19.5 ± 0.0
LA	15	0.27 ± 0.05	23.5 ± 3.2	20	0.28 ± 0.01	19.6 ± 2.9
BA	3	0.56 ± 0.10	19.3 ± 4.0	3	0.37 ± 0.04	15.3 ± 1.7
CA	7	0.27 ± 0.02	17.1 ± 1.4	7	0.32 ± 0.01	19.8 ± 2.8
FA	10	0.22 ± 0.03	15.0 ± 3.8	10	0.39 ± 0.00	14.0 ± 3.8
SGH	5	0.29 ± 0.07	27.8 ± 2.4	7	0.24 ± 0.01	67.3 ± 3.5
VN	3	0.36 ± 0.11	40.8 ± 1.9	3	0.42 ± 0.06	35.9 ± 0.7
VA	12	0.29 ± 0.10	69.3 ± 1.6	12	0.24 ± 0.07	68.0 ± 2.6
Control		0.54 ± 0.06	11.31 ± 1.03		0.73 ± 0.06	8.97 ± 0.16

* FF: furfural, 5-HMF: 5-hydroxymethylfurfural, AA: acetic acid, LA: levulinic acid, BA: benzoic acid, CA: *p*-coumaric acid, FA: ferulic acid, SGH: syringaldehyde, VN: vanillin; VA: vanillic acid.

3.5. Identification of the most severe inhibitors in mixtures and combination of inhibitors

In this step of the study, a Plackett-Burman experimental design was used to identify the most severe inhibitors in a mixture as well as combinations of inhibitors that affect the growth of *R. toruloides*. This design is a useful method for screening of a large number of factors performing a relatively small number of runs. Due to synergistic effects caused by the combination of multiple inhibitors in the culture medium, the concentration of each inhibitor used in the Plackett-Burman design was a quarter of the IC50 value or a quarter of the maximum concentration of each inhibitor at which cell growth was observed (Table 3). The reduction in the growth rate of the strain was the response considered for statistical analysis (Table 2).

The Pareto chart plotted in Fig. 4 shows the standardized effects of the factors (inhibitors) on the response. According to this analysis, furfural was the most potent inhibitor affecting the growth of *R. toruloides* ($p < 0.2$). Therefore, the removal of furfural or the reduction of its concentration can be a key strategy to reduce the toxicity of biomass hydrolysates to *R. toruloides*. Furfural can be removed selectively and with high efficiency (>95 %) from hydrolysates by vacuum

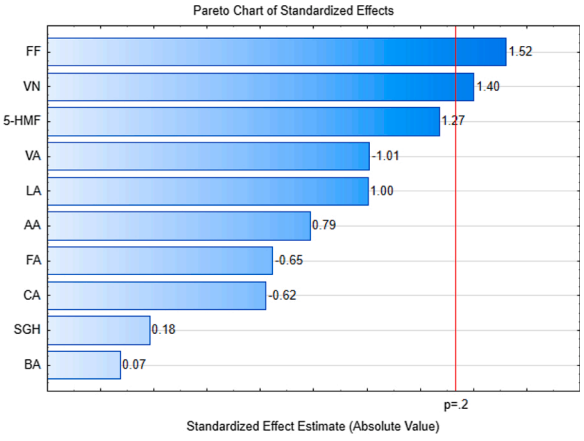


Fig. 4. Pareto chart of standardized effects to estimate the effect of inhibitors on the reduction of the growth rate of evolved *R. toruloides*. Results for $p < 0.2$. FF: furfural, VN: vanillin, HMF: 5-hydroxymethylfurfural, VA: vanillic acid, LA: levulinic acid, AA: acetic acid, FA: ferulic acid, CA: *p*-coumaric acid, SGH: syringaldehyde, BA: benzoic acid.

evaporation at 70 °C (Mussatto and Roberto, 2004b). Vanillin was the second most severe inhibitor compound affecting the growth of *R. toruloides* ($p < 0.2$), followed by 5-HMF ($p < 0.25$). For the studied inhibitory compounds, benzoic acid was found to be the least toxic to *R. toruloides*. In terms of synergistic effect, the combination of furfural, 5-HMF, vanillin, vanillic acid and ferulic acid resulted in the strongest inhibition to *R. toruloides* (assay 20, Table 2). Such compounds can be significantly removed from hydrolysates by adsorption in activate charcoal, for example. This method can remove more than 87 % of the total inhibitors present in biomass hydrolysates (Liu et al., 2020).

4. Conclusion

The presence of inhibitory compounds in lignocellulosic hydrolysates has been considered as one of the most important barriers hindering the production of microbial lipids and carotenoids from biomass hydrolysates. Identifying the most potent inhibitors and understanding their effects on yeast growth is a crucial step to overcome this problem. The present study contributes to advance this area by providing a detailed information on the effects of the main toxic compounds present in biomass hydrolysates on the performance of the oleaginous yeast *R. toruloides*. Furfural was found to be a severe inhibitor of the yeast growth. Removal of this compound from biomass hydrolysate can potentially improve the yeast growth and the production of lipids and carotenoids, as a consequence.

This study also revealed that an evolved strain of *R. toruloides* presented better tolerance to the toxic compounds when compared to the wild-type strain, being able to tolerate higher concentration of inhibitors and/or presenting a reduced lag phase. These results suggest that adaptive laboratory evolution is a potential strategy to obtain microbial strains with improved ability to grow in biomass hydrolysates, which contain a mixture of sugars and inhibitory compounds. Overall, these findings contribute with new knowledge and information to support the development of a sustainable technology for the production of lipids and carotenoids by oleaginous yeasts using lignocellulosic biomass as feedstock for the bioprocess.

CRediT authorship contribution statement

Zhijia Liu: Methodology, Analysis, Data curation, Validation, Writing - original draft. **Marlene Fels:** Methodology, Analysis, Data curation. **Giuliano Dragone:** Conceptualization, Supervision, Writing - review & editing. **Solange I. Mussatto:** Conceptualization, Supervision, Resources, Project administration, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was supported by the Novo Nordisk Foundation (NNF), Denmark (grant number NNF20SA0066233), and the China Scholarship Council (CSC), China (grant number 201708530221).

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