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Santos, Marisa V; Faria, Nuno T; Fonseca, César; Ferreira, Frederico Castelo

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Production of mannosylerythritol lipids from lignocellulose hydrolysates: tolerance thresholds of *Moesziomyces antarcticus* to inhibitors

Moesziomyces antarcticus tolerance to inhibitors in biosurfactants production

Marisa Viegas Santos [a], *Nuno Torres Faria* [a,b], *César Fonseca* [c*], *Frederico Castelo Ferreira* [a,d*]

[a] Department of Bioengineering and IBB-Institute for Biotechnology and Bioengineering, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

[b] Laboratório Nacional de Energia e Geologia, I.P., Unidade de Bioenergia, Estrada do Paço do Lumiar 22, 1649-038 Lisboa, Portugal

[c] Department of Chemistry and Bioscience, Section for Sustainable Biotechnology, Aalborg University, A. C. Meyers Vaenge 15, 2450 Copenhagen SV, Denmark

[d] The Discoveries Centre for Regenerative and Precision Medicine, Lisbon Campus, Universidade de Lisboa, Portugal.

[*] Corresponding author: frederico.ferreira@tecnico.ulisboa.pt, csf@bio.aau.dk

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Abstract:

BACKGROUND: *Moesziomyces antarcticus* is an efficient producer of mannosylerythritol lipids (MEL), a biosurfactant with wide range of potential applications. The use of lignocellulosic biomass can contribute to sustainable MEL production. While lignocellulosic sugars (e.g. D-glucose and D-xylose) can be converted to MEL, the required pretreatment of lignocellulosic biomass releases by-products that are potentially inhibitory for yeasts. A design of experiment (DoE) was performed to evaluate the effect of furfural, acetate and formate on *M. antarcticus* and their capacity to produce MEL from lignocellulose hydrolysates.

RESULTS: Furfural presented a higher inhibitory effect on MEL production than the two dissociated weak acids. The DoE was developed for 7-days D-glucose cultures with inhibitors up to 0.7 $g.L^{-1}$ furfural, 2.0 $g.L^{-1}$ acetate and 1.7 $g.L^{-1}$ formate. The model equations relate D-glucose consumption rate and the production of cell biomass, lipids and MEL with the concentration of inhibitors. For example, MEL titre is reduced in 25% when 0.08 $g.L^{-1}$ furfural, 0.29 $g.L^{-1}$ acetate and 0.25 $g.L^{-1}$ formate were used. The model was validated in D-glucose and used to study MEL production in D-glucose and D-glucose/D-xylose mixtures. The use of D-xylose showed a positive effect on MEL production in the presence of inhibitors since similar MEL titres were attained with (0.08 $g.L^{-1}$ furfural, 0.29 $g.L^{-1}$ acetate and 0.30 $g.L^{-1}$ formate) or without inhibitors when using a D-glucose/D-xylose mixture.

CONCLUSION: This study provides insight on the conditions required by *M. antarcticus* for MEL production from lignocellulosic hydrolysates and points towards further process and strain development requirements.

Keywords: lignocellulose hydrolysates, inhibitors, acetic acid, acetate, formic acid, formate, furfural, design of experiment, mannosylerythritol lipids, *Moesziomyces antarcticus*

Abbreviations: design of experiment – DoE, mannosylerythritol lipids – MEL, cell dry weight – CDW, TCA – Krebs cycle

Introduction

Moesziomyces spp. are haploid and anamorphic basidiomycetous yeasts classified under the *Ustilaginaceae* family.[1-2] The *Moesziomyces* spp. are known for the synthesis of mannosylerythritol lipids (MEL), glycolipids containing a 4-O-β-D-mannopyranosyl-meso-erythritol as the hydrophilic moiety and two short-chain fatty acids (usually C_8 to C_{12}) as the hydrophobic groups.[3] MELs are designated as MEL-A, -B, -C, and -D according to their degree of acetylation at C4 and C6 position on the mannosyl unit (Figure S1). MEL-A corresponds to the diacetylated compound, MEL-B and MEL-C are monoacetylated at C6 and C4, respectively and MEL-D represents the deacetylated molecule.[3]

These biosurfactants have interesting properties such as low toxicity, high biodegradability, good performance at extreme temperatures and pH and mild production conditions when compared with chemical surfactants.[3-4] They have potential to be used in a wide range of applications such as bioremediation, cosmetics, health care and food processing industries.[4-5] The production of these biosurfactants from lignocellulosic materials can lead to a decrease in the use of petrochemical resources.

Currently MEL are mainly produced from vegetable oils by *Moesziomyces* spp.[6-7] but can also be produced from other substrates such as sugars,[8] glycerol,[9] or hydrocarbons[10]. *Moesziomyces antarcticus* was described as the most efficient MEL

producer from sugars (e.g. D-glucose and D-xylose), when compared with other *Moesziomyces* spp.[8] Although the highest MEL titres and yields are achieved using vegetable oils as substrate, the increasing raw material cost, the competition with the food supply chain, and the requirements of extensive downstream processing limit MEL sustainable production. Lignocellulosic materials can be used as alternative to vegetable oils and refined sugars. The major components in this raw material are cellulose, hemicellulose and lignin, with the composition varying according with the type of lignocellulose source.[11] The pretreatment of lignocellulosic biomass has the purpose of accessing the sugar content available, often decreasing cellulose crystallinity and hydrolysing hemicellulose, while minimizing chemical degradation of sugars.[12] The cellulose fraction is composed of D-glucose units, which are usually obtained by a subsequent step of enzymatic hydrolysis. The hemicellulose hydrolysate can contain hexoses (D-glucose, D-galactose and D-mannose), but in many cases are rich in pentoses (mainly D-xylose), organic acids (acetic acid), sugar and lignin degradation products, such as furans. The furans divide in 5-hydroxymethyl-2-furaldehyde (HMF) and furfural and are formed due to dehydration of hexoses and pentoses, respectively [12]. When hydrothermal pretreatment is applied to agricultural residues and hardwoods, HMF is virtually absent and furfural is formed [12]. Acetic, formic and levulinic acid are the main organic acids present in lignocellulosic hydrolysates. Acetic acid is formed by de-acetylation of hemicellulose whereas formic and levulinic acids are products of HMF breakdown.[13-14] At acidic conditions and high temperatures, formic acid can also be formed from furfural.[13] Above certain concentrations, some organic acids and furan derivatives are known inhibitors of yeast growth [15] and for that reason their effect on MEL production must be assessed.

The design of experiment (DoE) is a helpful technique when assessing effect of multiple independent variables on a complex system. In this work we used a DoE for a systematic study aiming to establish a quantitative effect of multiple hydrolysate inhibitory derivatives (independent variables), in D-glucose consumption rate and production of cell biomass, lipids and MEL (dependent variables). This approach allows the execution of a small set of experiments, still with statistical significance, to create a model valid to produce MEL in presence of specific inhibitory compounds. Furfural and acetic and formic acid in their dissociated form were selected for this study as these compounds are the most common and abundant microbial inhibitors found in lignocellulose hydrolysates.

This is the first study performed to test the impact of inhibitory compounds found in lignocellulose hydrolysates on *M. antarcticus* and its capacity to produce MEL.

Materials and Methods

Microorganisms and maintenance

Moesziomyces antarcticus PYCC 5048^{T} (CBS 5955) was provided by the Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Caparica, Portugal. Strains were plated on YMA (yeast extract 3 g.L^{-1}, malt extract 3 g.L^{-1}, peptone 5 g.L^{-1}, D-glucose10 g.L^{-1} and agar 20 g.L^{-1}) and incubated for 3 days at 30 °C. Cultures were kept at 4 °C and renewed every week and stored at −80 ºC in 20% (w/v) glycerol to be recovered when necessary.

Media and cultivation conditions

Inoculum was prepared in Erlenmeyer flasks with 1/5 working volume of medium containing 3 g.L^{-1} $NaNO_3$, 0.3 g.L^{-1} $MgSO_4$, 0.3 g.L^{-1} KH_2PO_4, 1 g.L^{-1} yeast extract, 40

g.L^{-1} D-glucose, and incubated at 27 °C, 250 rpm, for 48 h. Batch cultivations were performed in Erlenmeyer flasks containing 1/5 working volume of mineral medium (0.3 g.L^{-1} $MgSO_4$, 0.3 g.L^{-1} KH_2PO_4, 1 g.L^{-1} yeast extract) supplemented with 35 g.L^{-1} D-glucose. The experiment started by transferring 10% *(v/v)* inoculum, corresponding to approx. 0.6 g.L^{-1} of cell dry weight (CDW), followed by incubation at 27 °C, 250 rpm, for 14 days. Samples were taken to quantify monosaccharides, biomass (CDW), lipids and MEL at days 0, 4, 7, 10 and 14. Three inhibitory compounds, acetic acid (0 – 2.0 g.L^{-1}), formic acid (0 – 1.7 g.L^{-1}) and furfural (IUPAC name: furan-2-carbaldehyde) (0 – 0.7 g.L^{-1}), were added to the cultures at day 0 and the pH corrected to 6. Since culture pH was above the pKa of acetic (4.76) and formic (3.77) acids, the acids prevail in their dissociated form, acetate and formate. The tests were performed using single or multiple inhibitors at different concentrations. Cultures without inhibitors were used as control.

For additional studies, cultures were incubated for 14 days under similar conditions to those described for batch cultivations with mineral medium, supplemented with 35 g.L^{-1} D-glucose or mixtures of 35 g.L^{-1} D-glucose and 12-13 g.L^{-1} of D-xylose.

Growth and biomass determination

Cell growth was followed by measuring cell dry weight (CDW). CDW was determined from 1 mL culture broth by centrifugation at 13000 rpm for 10 min, washing with deionized water (twice) and drying at 60 °C for 48 h.

High performance liquid chromatography (HPLC) analysis

Culture broth samples were centrifuged, at 13000 rpm for 10 min, and the supernatants were filtered through a 0.22 µm-pore-size filter. D-Glucose, D-xylose, acetic acid, formic acid and furfural were quantified in a high-performance liquid chromatography

(HPLC) system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany) and a Rezex^{TM} ROA Organic Acid H^+ (8%) column (300 mm × 7.8 mm, Phenomenex), at 65 °C. Sulfuric acid (5 mM) was used as mobile phase at 0.5 mL.min^{-1}.

Gas chromatography (GC) analysis

The fatty-acid composition of biological samples was determined by methanolysis and GC analysis of methyl esters.[17] Pure methanol (20 mL) was cooled down to 0 °C and 1 mL acetyl chloride was added to generate a water-free HCl/methanol solution. Culture broth samples (3 mL) were freeze-dried, weight and mixed with 2 mL HCl/methanol solution and incubated for 1 h at 80 °C for transesterification into methyl esters. Heptanoic acid was used as internal standard. The resulting product was extracted with hexane (1 mL) and 1 µL of the organic phase was injected in a GC system (Hewlett-Packard, HP5890), equipped with a FID detector and an Agilent HP Ultra2 capillary column (L 50 m × I.D. 0.32 mm, df 0.52 µm). The oven was programmed to an initial temperature of 140 °C and three temperature gradients were defined: 140 to 170 °C at 15 °C.min^{-1}, 170 to 210 °C at 40 °C.min^{-1} and 210 to 310 °C at 50 ºC.min^{-1}. A final time of 3 min at 310 °C was defined. Carrier gas was used with a split of 1/25. MEL were quantified through the amount of C_8, C_{10} and C_{12} fatty acids considering a molecular weight between 574 and 676 g.mol^{-1} depending on the length of the two acyl chains (C_8–C_{12}) and the degree of acetylation.[8]

Inhibitory compounds tested

The effect of selected inhibitory compounds on *M. antarcticus* cultures was assessed using a Design of Experiment (DoE). Media containing acetic acid, formic acid and

furfural, inhibitors commonly present in lignocellulose hydrolysates, were prepared. Individual and combined inhibition was evaluated using a central composite face-centred (CCF) design. The maximum concentrations of inhibitors used for the model were defined according to concentrations commonly found in wheat straw hydrolysates after hydrothermal pretreatment – acetic acid 2.0 $g.L^{-1}$; formic acid 1.7 $g.L^{-1}$; furfural 0.7 $g.L^{-1}$.[18]

DoE implementation

Experimental design was performed using StatSoft *Statistica 10* DoE tool. A full quadratic model, for each dependent variable (-q_G, CDW, lipids and MEL), containing all factors (acetate x_1, formate x_2, furfural x_3) and interactions ($x_1^2, x_1x_2, x_1x_3, x_2^2, x_2x_3, x_3^2$) was fitted using the DoE tool of the statistical software. Statistical significance of all independent variables was accessed through analysis of variance (ANOVA) using Fisher statistical test.

ANOVA results can be visualized through Pareto charts, in which the absolute value of the magnitude of the standardized estimated effect (the estimate effect divided by the standard error) of each factor was plotted in decreasing order and compared to the minimum magnitude of a statistically significant factor with a confidence of 95% (p=0.05). In summary, the longer bars on the chart illustrate the variables with more cumulative effect on the system (supplementary data).

Detailed experimental data used in the design of the different models, one for each parameter (-q_G, CDW, lipids and MEL) is shown in ***Table 1***.

Results and discussion

Selection of conditions and parameters for the DoE

The response variables studied in the DoE were D-glucose consumption rate ($-q_G$) and production of cell biomass, as cell dry weight (CDW), lipids and MEL. These parameters were chosen to help understanding the effect that common inhibitory compounds have in yeast metabolism and ultimately define threshold concentrations of these inhibitory compounds for MEL production. The $-q_G$ and CDW were modelled to evaluate if the selected inhibitory compounds influence the cell growth and sugar consumption rate, which can eventually influence MEL production. The accumulation and consumption of lipids (mainly in the form of molecules containing C_{16}-C_{18} fatty acids) play a key role in MEL production as they are the precursors of shorter fatty acids incorporated in the glycolipid. In fact, an accumulation of C_{16}-C_{18} lipids during sugar consumption and cell growth usually leads to a subsequent MEL production.[8]

Data was collected at day 7 of the cultivation since, under the standard cultivation conditions applied, at such time, typically the yeast has already concluded its growth phase, while sugar is in the great majority consumed, cell biomass and lipids are typically close to their maximum and MEL is already being produced at maximum productivity. [8] At this time point, it is expected that any effect of compounds potentially inhibitory of yeast metabolism and MEL production are visible.

Model output and statistical analysis

The output of the model was represented in Pareto charts (Figure S5). The factors without statistical significance at a confidence level of 95% were ignored two at a time from the model and pooled into the error term to obtain the new reduced models.

ANOVA performed on the reduced models showed their statistical significance for the listed factors with *p*-values much lower than 0.05 (data not shown). A lack of fit test (***Table 2)***, which compares the residual error (error associated with the fitted model) to the pure error, was also evaluated and a non-significant p-value higher than 0.05 was obtained, proving that the experimental data fits the models. The proportion of total variation attributed to the fit could be evaluated by the *R*-square value. For the $-q_G$ and CDW variables, the respective values of 0.87 and 0.81 were obtained, indicating an acceptable correlation between the models and experimental results. The values of 0.99 and 0.97 were obtained, respectively, for lipid and MEL concentration variables indicating a good correlation between the models and the experimental results.

Effect of inhibitors on glucose consumption rate ($-q_G$) and cell biomass production (CDW)

The response surface regarding the area contours for $-q_G$ as function of formate and furfural was plotted using the reduced model expressed by ***Equation 1***. The plot (***Figure 1***, A), showed that $-q_G$ was highly sensitive to the presence of furfural and highly tolerant to the formate present in culture. For fixed concentrations of acetate and low concentrations of furfural, the $-q_G$ remained constant even for the maximum formate value tested in culture.

The response surfaces regarding the area contours for CDW as function of acetate and furfural or acetate and formate were plotted using the reduced model expressed by ***Equation 2***. The plots (***Figure 1***, B) showed that the production of cell biomass was very sensitive to the presence of furfural in culture but also to the combined presence of acetate and formate. For a fixed value of formate concentration, the yeast was still able to tolerate relatively high concentrations of furfural in the presence of low values of

acetate. For a fixed concentration of furfural, cell biomass seemed to be more affected by the increase of acetate than formate concentration in the culture. When increasing formate concentration in the presence of low acetate concentrations, only a slight decrease on CDW was observed.

$$-q_G\ (g.L^{-1}.day^{-1}) = 4.53 - 4.40x_3 - 1.62x_2x_3$$

Equation 1

$$CDW\ (g.L^{-1}) = 5.99 - 3.31x_3 - 0.15x_1x_2 - 3.42x_1x_3$$

Equation 2

Effect of inhibitors on lipids and mannosylerythritol lipids (MEL) production

Using the reduced model expressed by *Error! Reference source not found.* and ***Equation*** , for lipid and MEL concentrations, respectively, the response surfaces regarding the area contours were plotted, as function of formate and furfural or acetate and furfural concentrations (***Figure 1***, C and D). Like the model estimates, lipid metabolism was less affected by the presence of furfural in culture than MEL production (***Figure 1***, C). For a given concentration of acetate, at low concentration of inhibitors, lipid production was estimated to have higher tolerance to increasing concentrations of formate and furfural than MEL. However, an increase in concentrations of formate and furfural was estimated to have higher inhibition on lipids than MEL production. As it was observed for MEL, the lipid concentration was only slightly affected by the variation of acetate in the culture. The increase in furfural and acetate concentrations for a given formate concentration was estimated to have a lower impact in lipids than on MEL production. The model estimated that MEL production was highly sensitive to the presence of furfural in culture (***Figure 1***, D), with MEL

production decreasing steeply with increase in furfural concentration. For a fixed concentration of acetate and low furfural concentrations, MEL production decreases with increase in formate concentration in the culture broth, being this effect more stringent as furfural concentration increases (***Figure 1***, D1). On the other hand, also at fixed formate and low furfural concentrations, MEL productions were only slightly affected by variation in acetate concentration (***Figure 1***, D2). Interestingly, this trend follows the opposite observation for CDW production, which was more affected by increases on concentrations of acetate than formate.

Regarding all dependent variables tested, $-q_G$ seemed to be the less sensitive to the inhibitors presence followed by CDW, lipids and MEL.

$$Lipids\ (g.L^{-1}) = 2.16 - 1.19x_1 + 0.48x_1^2 - 0.30x_2^2 - 1.94x_3 + 0.31x_1x_2 - 0.98x_1x_3 + 0.74x_2x_3$$

Equation 3

$$MEL\ (g.L^{-1}) = 2.08 - 0.60x_1 + 0.26x_1^2 - 0.28x_2^2 - 4.31x_3 + 1.77x_3^2 - 0.30x_1x_3 + 1.23x_2x_3$$

Equation 4

Model validation and inhibitors concentration thresholds

The empirical equations obtained allowed the theoretical estimation of the effect that *M. antarcticus* inhibitors have on -qG, CDW, lipids and MEL production. The model validation was focused on the formation of the product of interest, MEL. Three experiments, combining different concentrations of inhibitory compounds, were performed. MEL concentration was determined after 7 days of culture, the same culture time used when developing the model. The results obtained for the model validation are

represented in ***Table 3***. The differences between experimental and theoretical values are 9.5%, 8.3% and -1.3%, respectively, for experiments 1, 2 and 3 illustrating the model validity, as the differences between theoretical and experimental MEL values were below ±10%.

A maximum decrease in MEL production of 25% in *M. antarcticus* cultures, compared to the production in control cultures, without inhibitors, was established as an illustrative boundary to acceptable impact on MEL production when using lignocellulosic hydrolysates. Under such boundary, the model for MEL production represented by *Error! Reference source not found.* was used to define thresholds of the inhibitory compounds. To facilitate visualisation of the different effects of the three inhibitors considered, for fixed concentrations of each inhibitor, the relation of concentrations of the other two inhibitors was plotted (***Figure 2***). For example, ***Figure 2***A shows that for furfural and formate concentrations below 0.03 $g.L^{-1}$ and 0.43 $g.L^{-1}$, respectively, the decreases in MEL production were always less than the 25% boundary within acetate concentrations used in the DoE (0 to 2 $g.L^{-1}$). Further reduction of furfural to 0.02 $g.L^{-1}$ or absence of this compound will lead to the same impact on MEL production, as long as formate concentrations stays below 0.59 and 0.79 $g.L^{-1}$, respectively. For furfural concentrations higher than 0.03 $g.L^{-1}$ (see examples of furfural at 0.04, 0.06 or 0.08 $g.L^{-1}$), the impact of acetate becomes more relevant. Even in the absence of formate, 25% MEL production decrease was estimated for acetate concentrations of 1.1 $g.L^{-1}$, 0.6 $g.L^{-1}$, or 0.4 $g.L^{-1}$ for furfural at 0.04, 0.06 or 0.08 $g.L^{-1}$, respectively.

Interestingly, MEL production was more affected by formate presence at intermediate acetate concentrations. As expected, as acetate concentration increases from 0 to about 1.1 $g.L^{-1}$, lower formate concentrations have a higher negative impact on MEL

production (***Figure 2***, A). However, when increasing acetate concentrations from 1.1 to 2.0 $g.L^{-1}$, this compound seems to contribute to an increasing tolerance of the yeast to formate and furfural in respect to this yeast's ability to produce MEL (***Figure 2***, A). Again, the same behaviour was observed for interdependency of furfural and acetate, with MEL productions more affected with increases of furfural concentrations as acetate concentrations increases up to 1.1 $g.L^{-1}$, but again above this range of concentration the presence of acetate seemed to become beneficial for MEL production, decreasing the negative effect of furfural on yeast (***Figure 2***, B). The same profile observed for MEL on ***Figure 2***, B can be visualized for lipid production (***Figure 1,*** C2), although with a less prominent inhibitory effect, showing a direct relation between MEL and lipid production.

Figure 2C, illustrates the synergistic effect of furfural and formate for different fixed acetate concentrations, showing the boundaries for concentrations of these compounds that are acceptable in culture without surpassing the thresholds defined for MEL production.

Selected thresholds determined using the reduced model equation for MEL were then experimentally confirmed in cultures using D-glucose as carbon source and production yields from day 7 of culture. A cultivation with 0.29 $g.L^{-1}$ acetate, 0.25 $g.L^{-1}$ of formate and 0.08 $g.L^{-1}$ of furfural (condition T1) was performed and 1.69 ± 0.07 $g_{MEL}.L^{-1}$ was obtained (***Table S1***), against 2.05 ± 0.03 $g_{MEL}.L^{-1}$ of MEL produced without inhibitors, corresponding to a ratio of 0.82 (***Figure 3*** A), confirming the forecasted effect of these inhibitors on MEL production.

Knowledge of the model applied to MEL production from D-glucose/D-xylose mixtures

The utilization of the model was further extended to MEL production from D-glucose and D-glucose/D-xylose mixtures. Using the thresholds of inhibitors concentrations provided by the DoE, three conditions were selected to evaluate the effect of inhibitors on $-q_G$, CDW, and lipid and MEL production. A control condition without inhibitors and two conditions with inhibitors were used: (T1) with 0.29 $g.L^{-1}$ acetate, 0.30 $g.L^{-1}$ formate and 0.09 $g.L^{-1}$ furfural; and (T2) with a stringent increase of acids to concentrations between 0.7-1.0 $g.L^{-1}$ and an increase in furfural to 0.13 $g.L^{-1}$. The relevant kinetic parameters summarized in ***Table S1*** were determined for day 14, when the highest MEL titres were observed. MEL concentration and productivities were also presented for day 7 when the highest MEL productivities were typically achieved. In ***Figure 3*** is presented the ratio of MEL produced with inhibitors (T1 and T2) over the MEL produced without inhibitors (control).

For the case study more stringent, T2, which uses higher concentrations of inhibitors, the reduction of MEL production in D-glucose cultures was higher than 25% along the 14 days cultivation. For the condition T1, the MEL production ratio was 0.82 at day 7, but it increased to 0.91 at day 14, suggesting that the cells were initially affected (e.g. longer lag phase) but could recover and produce MEL. Interestingly, in D-glucose/D-xylose mixtures, the level of inhibitors found under condition T1 had no negative impact in MEL production, while under condition T2 (higher inhibitors concentration) the impact on MEL production was seen at days 4 and 7, with ratios of 0.61 and 0.72, respectively. At days 10 and 14 the yeast was able to recover and cope with the presence of inhibitors reaching equivalent MEL titres to the ones observed in the control (ratios of 0.96 and 1.07, respectively).

The carbon source, CDW, lipids and MEL profiles over the 14-day culture, as well as respective kinetic parameters estimated, are given in detail as supplementary data

(***Figure S6*** and ***Table S1***). Control cultures, in which no inhibitors were added using as carbon source either D-glucose (32-33 $g.L^{-1}$) or mixtures of D-glucose/D-xylose (32-33 $g.L^{-1}$/12-13 $g.L^{-1}$), resulted on similar MEL concentrations (e.g. at day 7, 2.05 ± 0.03 and 2.03 ± 0.29 $g.L^{-1}$ respectively) and MEL productivities (e.g. at day 7, 0.29 ± 0.00 and 0.29 ± 0.04 $g_{MEL}.L^{-1}.day^{-1}$ respectively). The sugar consumption rate increased for higher concentrations of inhibitors and biomass to substrate production yield was lower in the presence of inhibitors, especially for condition T2. Such result may be explained by higher maintenance to cope with the presence of inhibitors. In D-glucose/D-xylose mixtures, the MEL specific productivity was also similar or even slightly above for condition T1 when compared to the control condition, but lower for condition T2. As discussed above, MEL production after 14 days was not significantly affected by the presence of the inhibitors, except for condition T2 in D-glucose (***Figure 3***), with a final MEL titre of 1.36 ± 0.12 $g.L^{-1}$. The better performance in D-glucose/D-xylose mixtures was an interesting observation of this study. D-Xylose was consumed mostly from day 10 in the condition T2, after D-glucose depletion (***Figure S5,*** C1). However, for other conditions, including on the absence of inhibitors, after 14 days a significant amount of D-xylose is still present, probably because D-glucose was depleted later in the process. This result was in line with previous work, where D-glucose and D-xylose were sequentially consumed during MEL production from D-glucose/D-xylose mixtures.[8] Also, in previous work we observed that the absence of nitrate in cultivation media contributed to a decrease in the sugar consumption rate.[8] Interestingly, investigating C_{16}-C_{18} lipid concentration from day 10 to day 14, it decreased in D-glucose cultures, but remained constant (***Figure S5,*** A1 and B1) or increased (***Figure S5,*** C1) in D-glucose/D-xylose cultures (the condition at which sharper D-xylose consume was observed from day 10 to 14). The presence and consumption of additional sugar (D-

xylose) may have contributed to the continuation of lipid accumulation and, as a consequence, higher MEL titres at day 14.

Inhibitory effects of furfural and weak acids

The effect of inhibitory compounds derived from pretreatment of lignocellulosic biomass, namely weak acids (in their undissociated or dissociated form) and furans, on yeast growth inhibition is well known.[19] Interactions of different inhibitors have been reported to cause additional negative effects on yeast growth and ethanol production in *Saccharomyces cerevisiae*,[12, 19] and promote a decrease on sugar consumption and cellular growth in the oleaginous yeast *Rhodosporidium toruloides*.[20]

The experimental work developed has shown that, from the three small molecules assessed, furfural was the compound with higher inhibitory effect on MEL production. In comparison to what was observed for *M. antarcticus*, the oleaginous yeast *Rhodosporidium fluviale* DMKU-SP314 was also sensitive to the presence of formate and furfural with higher inhibitory effect on biomass and lipid production when tested individually.[21] In the oleaginous organisms *R. toruloides* and *Trichosporon fermentans*, furfural is known to have a strong inhibitory effect, affecting the activity of alcohol dehydrogenase (*ADH*), aldehyde dehydrogenase (*ALDH*), pyruvate dehydrogenase (*PDH*), among other enzymes involved in glycolysis, with negative impact in specific growth rates and lipid production.[20, 22] Here, we hypothesize that, also for *M. antarcticus*, inhibition of *PDH* could affect the conversion of pyruvate to acetyl-CoA, essential in the synthesis of lipids.

Acetic and formic acid, commonly released during pretreatment lignocellulosic materials, have been reported as inhibitors of *S. cerevisiae* growth and product formation.[19,23] In their undissociated form, these organic small acids are able to

passively diffuse across the cell membrane of *S. cerevisiae*.[20,24,25] However, the pH of the fermentation broth used in the current work was 6, well above the pKa of acetic (4.75) and formic acid (3.75), and thus in the culture, these acids were mainly on their dissociated form, acetate (>94.6%) and formate (>99.4%). It was observed for *S. cerevisiae* that acetate can be transported into the cell through an acetate carrier (Ady2) where it will accumulate, causing turgor pressure and oxidative stress. [24] However, for *M. antarcticus*, and some oleaginous yeasts[20,21,22], for culture pH above pKa, a tolerance to acetate has been observed. A previous study, using ^{13}C labelled acetate, report that 36% of the synthesized lipids by the oleaginous yeast *Mortierella isabelline* were derived from acetate added to culture at 2.0 $g.L^{-1}$.[23] MEL production depends on lipid synthesis to yield the lipidic moiety of this glycolipid. Moreover, in the DoE experiments, the effect of acetate in C_{16}-C_{18} lipids and MEL production was similar. Therefore, here it was hypothesized a possible positive effect of acetate on MEL production by *M. antarcticus* through lipids production, concealing negative effects of formate and furfural for higher acetate concentrations.

Conclusions

A fermentation process with lignocellulosic hydrolysate can be implemented if the concentration of inhibitory compounds does not exceed a threshold that affects the metabolic capacity of yeast cells to grow and produce MEL.

The development of a model and its validation for cultures on D-glucose revealed that furfural was the inhibitor with highest negative impact on MEL production by *M. antarcticus*. The model allowed the determination of threshold concentrations of inhibitory compounds for MEL production by *M. antarcticus*, without dramatically compromising MEL tires. Theoretical examples were provided for a 25% reduction in

MEL tires. Experimentally, cultures with D-glucose and 0.29 $g.L^{-1}$ acetate, 0.25 $g.L^{-1}$ formate and 0.08 $g.L^{-1}$ furfural resulted in reductions of MEL tires in 18% at day 7 and 9% at day 14. On the other hand, the increase of inhibitor concentrations to values of 0.74 $g.L^{-1}$ acetate, 0.88 $g.L^{-1}$ formate and 0.13 furfural $g.L^{-1}$ resulted already in dramatic reductions in MEL titres (more than 40% at days 7 and 14). The experimental results showed a positive effect in MEL production when D-glucose was supplemented with D-xylose. In cultures with D-glucose/D-xylose mixtures the yeast can tolerate a concentration of 0.29 $g.L^{-1}$ acetate, 0.30 $g.L^{-1}$ formate and 0.09 $g.L^{-1}$ furfural without negative impacts in MEL production, reaching 2.08 ± 0.15 $g_{MEL}.L^{-1}$ at day 7, compared to the 2.03 ± 0.29 $g_{MEL}.L^{-1}$ achieved for cultures in the same conditions without inhibitors. The increase in inhibitors concentrations to 0.76 $g.L^{-1}$ acetate, 0.91 $g.L^{-1}$ formate and 0.13 $g.L^{-1}$ furfural, affected MEL production in D-glucose/D-xylose cultures, but only at early stages (28% reduction at day 7). These cultures were able to recover, reaching a MEL production higher than the control culture at day 14, with 2.73 ± 0.27 $g_{MEL}.L^{-1}$.

Overall, this study allows the design of integrated processes for MEL production from lignocellulosic hydrolysates, including compatible biomass pretreatment and detoxification of hydrolysates, as well as the design of adequate evolutionary engineering protocols to increase *M. antarcticus* tolerance to these inhibitors.

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Table 1. Glucose consumption rate ($-q_G$), cell dry weight (CDW), Lipids and MEL of *M. antarcticus* cultures in D-glucose after 7 days for each experiment used in the central composition design.

Experiment	$\mathbf{x_1}$ Acetate ($g.L^{-1}$)	$\mathbf{x_2}$ Formate ($g.L^{-1}$)	$\mathbf{x_3}$ Furfural ($g.L^{-1}$)	MEL ($g.L^{-1}$)	Lipids ($g.L^{-1}$)	CDW ($g.L^{-1}$)	$-q_G$ ($g.L^{-1}.day^{-1}$)
1	2.01	0	0	1.97	1.73	5	4.62
2	0	0	0	2.02	2.17	6	2.40
3	0	0	0	2.08	2.15	8	3.56
4	0	0	0.69	0	0.71	5	2.78
5	0.29	0.19	0	1.83	1.66	5	4.43
6	0.25	0.24	0.07	1.76	1.71	5	3.66
7	1.22	0.24	0	1.58	1.36	5	5.66
8	0.29	0.25	0.08	1.62	1.53	4	3.63
9	0.42	0.61	0.12	1.65	1.81	8	4.62
10	0.74	0.87	0.12	1.16	1.51	6	4.01
11	0.74	0.88	0.13	1.20	1.29	6	3.96
12	0	0	0.33	0.72	1.74	9	3.64
13	0.90	0.97	0.71	0	0	0	0
14	1.00	1.00	0.60	0	0	0	0
15	1.60	1.00	0.60	0	0	0	0
16	2.00	1.00	0.60	0	0	0	0
17	2.00	1.00	0.70	0	0	0	0
18	1.00	1.60	0.60	0	0	0	0
19	0	1.74	0	1.21	1.19	4	5.64
20	1.00	2.00	0.60	0	0	0	0
21	1.00	2.00	0.70	0	0	0	0
22	2.00	2.00	0.70	0	0	0	0

Table 2. Results of the lack of fit test performed on the reduced models of $-q_G$, CDW, Lipids and MEL of *M. antarcticus* cultures in D-glucose after 7 days.

		D	***SS***	***MS***	***F-value***	***p-value***
$-q_G$	*Error*	19	13.07	0.69		
	Lack of fit	18	10.75	0.60	0.26	0.940
	Pure error	1	2.32	2.32		
	Total	38	26.14			
CDW	*Error*	18	40.31	2.24		
	Lack of fit	17	38.31	2.25	1.13	0.640
	Pure error	1	2.00	2.00		
	Total	36	80.63			
Lipids	*Error*	14	0.41	0.03		
	Lack of fit	13	0.41	0.03	156.93	0.062
	Pure error	1	0.00	0.00		
	Total	21	0.82			
MEL	*Error*	14	0.15	0.011		
	Lack of fit	13	0.15	0.011	6.30	0.303
	Pure error	1	0.00	0.00		
	Total	21	0.30			

Table 3. Inhibitor concentrations used to validate DoE model of *M. antarcticus* cultures in D-glucose after 7 days. a) Theoretical values of MEL given by the model; b) Experimental values of MEL obtained.

Experiment validation	$\mathbf{x_1}$ Acetate ($g.L^{-1}$)	$\mathbf{x_2}$ Formate ($g.L^{-1}$)	$\mathbf{x_3}$ Furfural ($g.L^{-1}$)	MEL [a] ($g.L^{-1}$)	MEL [b] ($g.L^{-1}$)
1	0.12	0.05	0	2.01	1.82
2	0.77	0.92	0.14	1.09	1.18
3	0.29	0.25	0.08	1.60	1.62

Figure 1. Response surface plots of the central composite design for the combined significant effects of inhibitors on $-q_G$ (A), CDW (B), lipids (C) and MEL (D) of *M. antarcticus* cultures in D-glucose after 7 days. (0) Combined effect of formate and acetate for a value of 0.33 $g.L^{-1}$ furfural. (1) Combined effect of furfural and formate for a value of 0.83 $g.L^{-1}$ acetate. (2) Combined effect of furfural and acetate for a value of 0.80 $g.L^{-1}$ formate.

Figure 2. Effect of the combination of inhibitory compounds (acetate, formate and furfural) in MEL production by *M. antarcticus* cultures in D-glucose, after 7 days. The lines represent the threshold of concentrations contributing to a 25% reduction in MEL production, when compared to MEL produced without the presence of inhibitors. Variation of acetate and formate for furfural at 0, 0.02, 0.03, 0.04, 0.06, 0.08, 1.30 $g.L^{-1}$ (A), Variation of furfural and acetate for formate at 0, 0.3, 0.7, 0.9, 1.2 $g.L^{-1}$ (B), Variation of furfural and formate for acetate at 0, 0.3, 0.7, 0.9, 1.2, 1.8, 2.0 $g.L^{-1}$ (C).

Figure 3. MEL production ratios for condition T1 and T2 with inhibitors in relation to control (without inhibitors) for *M. antarcticus* cultures in D-glucose (G, top panel) or D-glucose/D-xylose (GX, bottom panel), after day 4, 7, 10 and 14. (G) In D-glucose cultures (top panel): T1: 0.29 $g.L^{-1}$ acetate, 0.25 $g.L^{-1}$ formate and 0.08 $g.L^{-1}$ furfural; and T2: 0.74 $g.L^{-1}$ acetate, 0.88 $g.L^{-1}$ formate and 0.13 $g.L^{-1}$ furfural; (GX) in D-glucose/D-xylose cultures (bottom panel): T1: 0.29 $g.L^{-1}$ acetate, 0.30 $g.L^{-1}$ formate and 0.09 $g.L^{-1}$ furfural; and T2 0.76 $g.L^{-1}$ acetate, 0.91 $g.L^{-1}$ formate and 0.13 $g.L^{-1}$ furfural. (Mean ± SD, n=2)