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A novel FTIR-based approach to evaluate the interactions between lignocellulosic inhibitory compounds and their effect on yeast metabolism

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Inhibitors commonly found in lignocellulosic hydrolysates impair yeast metabolism and growth, reducing the productivity of the overall bioethanol production process. FTIR spectroscopy was used to analyze the metabolomic alterations induced by acetic and formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (HMF) on yeast metabolism, using three *Saccharomyces cerevisiae* strains with different sensitivities. IR spectrum alterations were summarized with synthetic descriptors to rapidly visualize the kinds of molecules displaying the more intense reactions and to evaluate the type of interaction between inhibitors in a mixture, at concentrations close to those found at the industrial scale. The four inhibitors induced different levels of mortality and metabolomic changes. The metabolomic response was proportional to the different strain resistance level, further supporting their original classification. Inhibitor mixtures severely hindered the cell viability with the exception of the lowest concentration tested, which was partially biocidal. Furthermore, for the first time, this study revealed antagonistic interactions exerted by inhibitor mixtures on microbial metabolism, closely strain- and dose-dependent. This confirms that yeast strain resistance to single inhibitors cannot be used to predict behaviour on exposure to mixtures. This finding is worth further studies to explain the underlying antagonistic mechanism and to support the selection of highly tolerant strains.

Introduction

The depletion of fossil fuels together with increased environmental awareness has resulted in a strong drive towards developing eco-friendly biofuel technologies. Bioethanol is considered one of the most promising routes.[1,2] The ideal raw substrate for bioethanol is represented by non-edible lignocellulosic biomass, such as energy crops, spruce or birch, as well as agricultural by-products.[3–8] Because lignocellulose is highly refractory to degradation, pre-treatments are needed to make the cellulose more accessible to subsequent enzymatic saccharification.[1,9] However, pre-treatments also result in the co-production of inhibitory compounds from hemicellulose (mainly furfural and acetic acid), lignin (phenolic compounds) and cellulose (5-hydroxymethyl-2-furaldehyde, HMF). The amount and nature of degradation products is directly related to the pre-treatment method and conditions. Nevertheless, the most common and abundant inhibitors are furans, like HMF and furfural, and weak acids, such as acetic and formic acid.[10–12]

Inhibitors cause multiple negative effects on yeast cells by (i) suppressing the biosynthesis of macromolecules, (ii) denaturing the cytoplasmic proteins, (iii) reducing the activity of glycolytic enzymes, disturbing the processes of ion and metabolite transport across the plasma membrane and (iv) altering the lipid composition of the membranes,[13] thus reducing the productivity of the overall process.[10,13]

A variety of detoxification strategies have been developed to lower the inhibitor concentration from pre-treated lignocellulose. Nevertheless, these methods are far from being techno-economically feasible.[14] Several alternatives to detoxification were proposed, such as the selection or the development of less recalcitrant feedstock, the application of mild pre-treatment settings[5,15] and the development of yeast strains with high inhibitors tolerance.

Advanced improvements in the optimisation of yeast robustness may require novel metabolic engineering tools, such as protein engineering and rational metabolic engineering, already elegantly described.[16] However, strains exhibiting multiple tolerance to high temperature and inhibitors levels have not been developed yet. Furthermore, the majority of the

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engineered and/or evolved strains is obtained so far in haploid laboratory yeast, which generally display suboptimal fermentation performances and poor robustness, making them unsuitable for use in industrial applications.[17]

Screening or selection surveys for wild type tolerant *Saccharomyces cerevisiae* strains were mainly focused on single stress condition, such as high temperature,[18,19] weak acids or furans and phenolics.[13] The search for multiple tolerant yeast has received much less attention.[6,20,21] The resistance to weak acids, furans and phenolics in *S. cerevisiae* is strain-specific and highly dependent on tested concentrations.[13,22] This requires laborious and time-consuming screening procedures.[23] Moreover, these costly researches often focused on single and different physiological parameters (mainly growth, ethanol or biomass yield in the presence of inhibitors) to estimate the ability of the strain to withstand inhibitors, making difficult the comparison between the studies. Therefore, a complete dataset of the responses given by yeast strains exposed to inhibitory compounds is needed for the selection process.

Checkerboard-based methods, normally employed in pharmacology and toxicology to assess the combined effects of two drugs, cannot be employed for this purpose because they (i) do not distinguish the difference between mortality and inhibition, (ii) properly show synergic or additive effects only at lower concentrations than those typically present in lignocellulosic ethanol plants, (iii) give only a synthetic result without any hint of the metabolomic compartments involved.

Over the last few years, Fourier transform infrared (FTIR) spectroscopy has become a powerful high-throughput technique in biophysical and biochemical research, for its sensitivity in the detection of metabolomic changes of cells and tissues.[24] Furthermore, FTIR has been successfully applied for the development of quick bioassays to evaluate the stress-induced cell status in response to different chemicals or to various environmental signals.[25–27]

In the current study, we propose a FTIR-based approach to characterize the metabolomic alterations induced by inhibitory compounds on *S. cerevisiae* metabolism and to evaluate inhibitors interactions at concentrations close to those found in the industrial bioethanol production. Four well-known inhibitors, alone and in quaternary mixtures, were employed to test the possibility offered by this method: acetic acid, formic acid, furfural and 5-hydroxymethyl-2-furaldehyde (HMF). The analysis was carried out using three *S. cerevisiae* strains, chosen among 160 previously screened, as representative for the uppermost, medium and low inhibitors tolerance.

Experimental

Cultures and growth conditions

The *S. cerevisiae* Fm17 and Fp84 were isolated and characterized in terms of inhibitor tolerance together with the type strain *S. cerevisiae* DSM70449, as benchmark.[6,28] Each strain was inoculated at Optical Density (OD_{600}) = 0.5 in 500 mL bottles containing 50 mL YPD medium (yeast extract 1%, peptone 1% and dextrose 2% Difco Laboratories, USA) and grown for 18 h at 25 °C, with 150 rpm shaking.

Stressing agents

Acetic acid, formic acid, furfural and HMF were all obtained from Sigma and used at increasing concentrations in distilled sterile water (Table 1). Each tested concentration was reported as relative concentration (RC) of the third assessed level considered as the highest concentration of the studied inhibitors in lignocellulosic hydrolysates. Inhibitors were formulated also into four mixtures (RC_{25}, RC_{50}, RC_{100}, RC_{200}) obtained adding increasing doses of every toxic compound (Table 1).

FTIR and UV-vis spectrophotometers

The FTIR experiments were carried out with a TENSOR 27 FTIR spectrometer, equipped with HTS-XT accessory for rapid automation of the analysis (Bruker Optics GmbH, Ettlingen, Germany). Cell densities were measured with a Jasco V-530 Spectrophotometer (http://www.jascoinc.com).

FTIR analysis and spectra preprocessing

Cells suspensions, prepared as detailed in "Cultures and growth conditions" section, were centrifuged (3 min at 5300 × *g*), washed twice with distilled sterile water and re-suspended in polypropylene tubes with an appropriate amount of distilled water (standardized OD_{600} = 12). Inhibitors were added to the test tubes in order to obtain the relative concentrations reported in Table 1. The control (0% relative inhibitor concentration) was obtained by re-suspending the cells directly in distilled sterile water. All tests were carried out in triplicate. Tubes were incubated 1 h at 25 °C in a shaking incubator set at 50 rpm. After the incubation, 1.5 mL suspension was taken from each sample, centrifuged (5 min at 5300 × *g*) washed twice with distilled sterile water and re-suspended in 1.5 mL HPLC (High Performance Liquid Chromatography) grade water. 105 μL suspension was sampled for three independent FTIR readings (35 μL each, according to the technique suggested by Essendoubi and colleagues).[29] FTIR measurements were performed in transmission mode. All spectra were recorded in the range between 4000 and 400 cm^{-1}. Spectral resolution was set at 4 cm^{-1}, sampling 256 scans per sample in order to adequately study band intensities and shifts. The software OPUS version 6.5 (BRUKER Optics GmbH, Ettlingen, Germany) was used to carry

Table 1 Inhibitors concentrations used in this study as single compounds or as quaternary mixtures

	Concentration (mM)			
Inhibitor	RC_{25}	RC_{50}	RC_{100}	RC_{200}
Acetic acid[a]	30	60	120	240
Formic acid[a]	13	27	53	106
Furfural[b]	7	14	28	56
HMF[b]	7	15	30	59

[a] pH values of each solution of single acid ranged between 2.4 and 2.8. [b] HMF or furfural formulations have pH values of 6.5. pH values of inhibitors mixtures RC_{25}, RC_{50}, RC_{100}, were 2.6, 2.5, 2.4, 2.2, respectively.

out the quality test, baseline correction and vector normalization.

Spectra analyses

The script MSA (Metabolomic Spectral Analysis) employed for stress analysis was developed in "R" language to carry out the following operations on the matrices of spectral data exported as ASCII text from OPUS 6.5. The analytical procedure could be outlined as follows:

(1) Each single spectrum was normalized in order to have the range spanning from 0 to 1 in a way already suggested in ref. 24. Average spectra from the three repetitions were calculated.

(2) Response spectra (RS) were calculated as difference between each average spectrum and the average spectrum of the same cells maintained in water (defined as control RS). Response spectra of each agent were found to be positive and plotted with the exclusion of the control RS, which is by definition a straight line with RS = 0.

(3) Synthetic stress indexes (SI) were calculated as Euclidean distances of the RS under stress and the control RS. SI of the whole spectrum and of the five different spectral regions individuated by Kümmerle and colleagues[30] were calculated. The five regions were defined as follows: fatty acids (W1) from 3000 to 2800 cm^{-1}, amides (W2) from 1800 to 1500 cm^{-1}, mixed region (W3) from 1500 to 1200 cm^{-1}, carbohydrates (W4) from 1200 to 900 cm^{-1} and typing region (W5) from 900 to 700 cm^{-1}. The typing region was not considered in the analysis because its response did not correlate with the specific stressing conditions tested.

Biocidal activity test

The biocidal activity tests were carried out in parallel with the FTIR-based stress bioassay to compare the metabolomic damages with the loss of viability. 100 μL of each cells suspension prepared for the FTIR analysis were serial diluted to determine the viable cell counting, in triplicate, on YPDA + chloramphenicol (0.5 g L^{-1}) plates. The biocidal effect of the tested compounds was highlighted as cell mortality induced at different concentrations. The cell mortality (M) was calculated as $M = (1 - C_v/C_t) \times 100$, where C_v is the number of viable cells in the tested sample and C_t the number of viable cells in the control suspension.

Measure of the effects of inhibitors mixtures on yeast viability and metabolism

The mortality values induced by the inhibitors mixtures (observed mortality, OM) were compared with that expected at the same concentration (expected mortality, EM).

Since OM values were distributed in a hyperbolic dose–effect curve in all the tested conditions, the fractional product method[31] has been used to estimate EM values, using the following equation:

$$EM_{RC\text{-}th} = 1[(1 - m_1)(1 - m_2)\ldots(1 - m_n)] \quad (1)$$

where $EM_{RC\text{-}th}$ is the EM of each inhibitors mixture (RC_{25}, RC_{50}, RC_{100}, RC_{200}) and m_i is the mortality of the i_{th} inhibitor.

Data obtained were subjected to one-way analysis of variance (ANOVA) and pair comparison was achieved by Tukey's procedure. Additive effect could be postulated with OM = EM, synergistic when OM > EM and antagonistic when OM < EM.

A similar approach was adopted to assess how the yeast metabolome reacts to inhibitors mixture. An Absolute Reduction Indicator (ARI) was calculated as difference between the sum of the metabolomic responses induced separately by each inhibitor and that of the mixture. Positive ARI values indicate antagonism, negative synergism and figures close to 0, additivity.

Study of inhibitors chemical interaction(s) and reaction(s)

The inhibitors potential reactions were studied in water solutions at concentrations of 240, 106, 56 and 59 mM for acetic acid, formic acid, furfural and HMF, respectively. All the combinations of the two acids with furfural and HMF were analyzed. The mixtures were prepared dissolving the inhibitors in water and then left at room temperature for 24 h under magnetic stirring. The samples were then diluted for adequate UV-vis readings (maximum absorbance < 1 a.u.). The spectra registered were superimposed and compared with the spectra of the pure compounds at the same concentrations. The experiments with HCl were performed dissolving furfural and HMF in HCl water solutions at different acid concentrations (pH = 6, 5, 4, 3, 2, 1). The spectral readings were performed in the same manner of the previous experiments and acquired with a Jasco V-530 Spectrophotometer (http://www.jascoinc.com).

Results and discussion

Metabolomic analysis

FTIR spectroscopy was used to characterize the ability of three different strains of *S. cerevisiae* (Fm17, Fp84 and DSM70449) to withstand increasing concentrations of inhibitors at their early stationary phase, as it is in lignocellulosic bioethanol processes, either during SSF (Simultaneous Saccharification and Fermentation) or CBP (Consolidated BioProcessing) systems.[1,32]

Cells were exposed for 1 hour to four dosages of formic acid, acetic acid, furfural and HMF, alone and in quaternary mixtures. The alterations induced by these chemicals on the IR spectrum were summarized with synthetic stress metrics (Stress Indexes, SIs), allowing to rapidly and simply visualize which spectral regions, and therefore what types of molecules, displayed the most intense responses in each specific stressing condition. SIs were obtained as normalized Euclidean distances between the response spectra of cells under stress and those of cells maintained in water, as previously described in Materials and methods section – Spectra analyses. These metrics have been calculated for the whole spectrum (GSI) and for each specific spectral areas involved in the stress response, namely: fatty acids (W1), amides (W2), mixed region (W3) and carbohydrates (W4).[24]

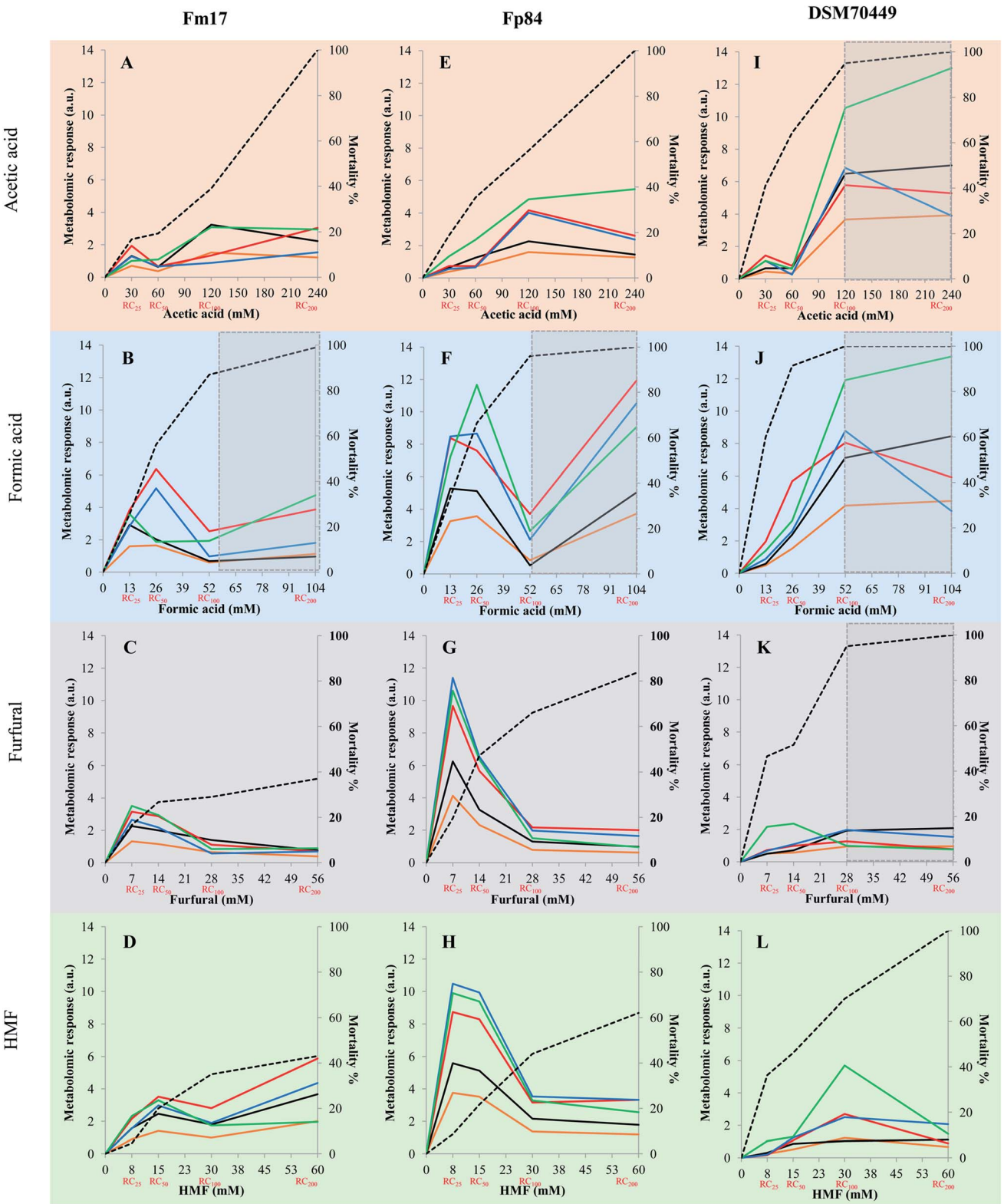

Fig. 1 Stress indexes of *S. cerevisiae* Fm17, Fp84 and DSM70449 cells subjected to increasing RCs of acetic acid, formic acid, furfural and HMF. Orange line represents the whole spectrum (GSI), black line W1 region, red line W2 region, blue line W3 region, green line W4 region, dashed line represents mortality. The degree of variability between replicas throughout the FTIR spectra ranged around 2.7×10^{-2}. Figures in red on the *x* axes represent relative concentrations in respect to the corresponding mmol concentration. *Post mortem* chemical reaction of cellular components is indicated by a grey box. a.u. stands for "arbitrary units".

Mortality and spectral alterations due to single inhibitors. Cellular stress induces very fast changes in terms of cell metabolites, all detectable through an accurate metabolomic analysis, such as FTIR, as early as in the first hours of exposure.[25] The four inhibitors considered in this study caused different levels of mortality and metabolomic alterations on the tested *S. cerevisiae* strains, as shown by the SIs evolution of Fig. 1. The metabolomic response was dissected in two components on the basis of the indication provided by the mortality data: the response of living cells that actively react to the stressing agent (*pre mortem* response) and that typical of dead cells, hereafter referred to as *post mortem*, resulting from an increase of membrane permeability after cell death or by an enzymatic activity consequent with the loss of cell compartmentalization, as already suggested by Corte and colleagues.[36]

Fm17 strain (Fig. 1A–D) displayed the least mortality and metabolomic response. Most of the metabolomic changes were due to 25% and 50% of each inhibitor Relative Concentration (RC), hereinafter named as RC_{25} and RC_{50} (see Materials and methods – Stressing agents). Major responses were in the amides (W2), mixed (W3) and carbohydrates (W4) regions. More specifically, acetic acid (Fig. 1A) induced less mortality with less metabolomic alterations than formic acid (Fig. 1B), which caused a strong reaction at RC_{25} and RC_{50}. The metabolomic response to formic acid at higher concentrations was clearly due to the *post mortem* chemical reaction of cellular components, as indicated by the grey box. Both furfural and HMF induced a maximum of about 40% mortality, but different metabolomic alterations. Namely, cells actively responded to furfural at RC_{25} while HMF prompted the maximum alterations at RC_{50} and RC_{200} (Fig. 1C and D).

Metabolomic responses displayed by Fp84 strain were similar to those of Fm17, but of greater intensity (Fig. 1E–H). Acetic acid exposure determined the least metabolomic changes (Fig. 1E) while the other inhibitors caused a strong and similar response at RC_{25} and RC_{50}, three-fold than that displayed by Fm17 strain (Fig. 1F–H). Formic acid showed the highest biocidal efficacy by inducing 100% mortality already at RC_{100} (Fig. 1F). These data indicated that cells actively coped with low RCs of formic acid, furfural and HMF trying to counter the effect exerted by inhibitors. Conversely, at higher concentrations, the inhibitors rapidly killed the cells hampering any reaction.

Metabolomic analysis confirmed DSM70449 as the most sensitive of the three tested strains (Fig. 1I–L). Cells challenged by weak acids showed high mortality and metabolomic response, with all SIs curves increased until RC_{100}, following the mortality trend. The response to formic acid was moderately stronger than to acetic acid. After death, over RC_{100}, cells displayed similar chemical intracellular reactions (Fig. 1I and J). On the contrary, this strain did not actively react at low RCs of furans, although these inhibitors induced over 40% mortality already at RC_{25} (Fig. 1K and L).

In general, mortality values over 50% were observed for all strains challenged by weak acids. Interestingly, in all these experimental conditions, the Global Stress Index (GSI), represented by the orange line, reached values around or higher than 1.0 a.u. (arbitrary units), confirming an unhealthy cell state as previously observed with other stressing compounds.[27,33,34] Weak acids have been reported to contribute to ATP depletion, toxic anion accumulation and inhibition of aromatic amino acids uptake.[13,14,22] All these effects could be justified by the stoppage of catabolism with the reduction and then the extinction of all ATP-depending metabolic activities, such as the export of ions from the cell.[35] The high mortality induced in our experiments by formic acid in all strains, and by acetic acid mainly in DSM70449, confirmed this hypothesis. Furthermore, the mortality and the low metabolomic responses of Fm17 and Fp84 strains challenged by acetic acid suggested a decrease of the metabolic activity that cannot contrast the toxic effect exerted by this inhibitor.

Furans induced a lower mortality than weak acids and triggered the metabolomic response only at low RCs (GSI ranging around 1.0 a.u.), with the exception of the sensitive strain, unable to actively react. These compounds have been described to inhibit glycolysis acting specifically on alcohol dehydrogenase (ADH), pyruvate dehydrogenase (PDH) and aldehyde dehydrogenase (ALD).[13] Altogether, these effects suggested that catabolism was stopped or slowed, with a possible involvement of the carbohydrates region, as supported in our data by the prominence of the W4 SI.

Moreover, the comparison between metabolomic and mortality data at low inhibitors concentrations (RC_{25} and RC_{50}), enabled to define three different types of response, corresponding to the different tolerance levels of the strains tested. The yeast Fm17 disclosed low mortality values and relatively low metabolomic responses, the typical behavior of a resistant strain (Fig. 1A–D). On the contrary, in DSM70449, inhibitors exerted a strong and immediate action that prevented the metabolome reaction, as normally in a sensitive strain. Finally, *S. cerevisiae* Fp84 displayed a strong response and low mortality values, indicating an effort of the cells to produce endometabolites to contrast the inhibitors toxicity. The above observations corroborated that this FTIR bioassay allows to characterize the resistance of microbial strains to stressors, as already reported for other toxic agents.[36]

Mortality and spectral alterations due to quaternary mixtures. Inhibitors mixtures severely reduced cell viability with the exception of RC_{25}, a partially biocidal concentration causing 26, 44 and 64% mortality in Fm17, Fp84 and DSM70449, respectively (Fig. 2). The evolution of SIs indicated that the metabolomic response was proportional to the different strain resistance level, confirming what already discussed for single inhibitors and further supporting the original classification of these three strains as resistant, intermediate and sensitive, respectively.[6] More in detail, data reported in Fig. 2 for RC_{25} pointed out the similar strain specific pattern detected in the analysis of the metabolomic alterations induced by single inhibitors (Fig. 1). In fact, the sensitive DSM70449 strain was not able to contrast the high mortality rate inferred by inhibitors mixture (GSI = 0.6; 64% mortality), the tolerant Fm17 showed low metabolomic response (GSI: 0.9 a.u.) and low mortality (26%), while the intermediately tolerant Fp84 displayed a high metabolomic reaction (GSI: 2.4 a.u.) together with

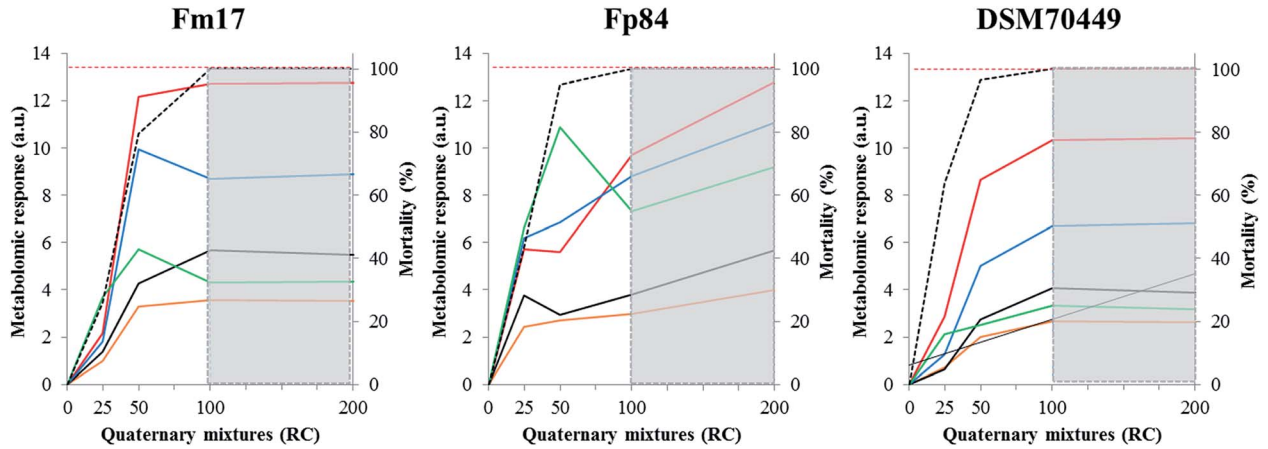

Fig. 2 Stress indexes of *S. cerevisiae* Fm17, Fp84 and DSM70449 cells subjected to increasing RCs of quaternary mixtures. Orange line represent the whole spectrum (GSI), black line W1 region, red line W2 region, blue line W3 region, green line W4 region, dashed line represents mortality. The degree of variability between replicas throughout the FTIR spectra ranged around 2.7×10^{-2}. *Post mortem* chemical reaction of cellular components is indicated by a grey box. a.u. stands for "arbitrary units".

a mortality of 44%. Conversely, all the metabolomic alterations detected at RC_{50}, RC_{100} and RC_{200} were attributable to a *post mortem* cells reaction, since, at these concentrations, the cell mortality ranged from 80% to 100%.

Analysis of the interactions between inhibitors

Mortality analysis. To evaluate whether positive (synergistic or additive) or negative (antagonistic) interactions occurred between inhibitors affecting yeast cell viability, the observed mortality (OM) of cells challenged with the inhibitors mixtures was compared with the expected mortality (EM), according to those caused by increasing concentrations of single inhibitory compounds. Since mortality induced by each inhibitor has hyperbolic curve (data not shown), EM was calculated according to the eqn (1) and reported, together with the OM values, in Fig. 3 for RC_{25} and RC_{50}. Data for RC_{100} and RC_{200} were not presented because the EM reached values over 100%.

Additive effect could be postulated with OM = EM, synergistic when OM > EM and antagonistic when OM < EM. ANOVA revealed that exposure to inhibitors mixtures resulted in statistically significant OM < EM values, indicating that some sort of mechanism induces antagonism among inhibitors. This phenomenon was evident mainly at RC_{25} where the most tolerant strain Fm17 exhibited the highest antagonistic effect, with an OM/EM ratio of about 0.51, meanwhile the intermediate yeast Fp84 and the sensitive strain DSM70449 showed lower antagonistic effects with 0.65 and 0.70 OM/EM ratios, respectively. At RC_{50}, antagonism was still detectable for *S. cerevisiae* Fm17 (OM/EM ratio = 0.79) and slightly observable for the other two strains (OE/EM ratio nearly 0.97).

Metabolomic absolute reduction indicator (ARI). Mortality data can be applied to evaluate the interactions between two or more chemicals only when the sum of the mortalities induced does not exceed 100%. This approach cannot be employed when the aggressors need to be tested at concentrations that saturate the cell mortality, such as for RC_{100} and RC_{200} of inhibitors

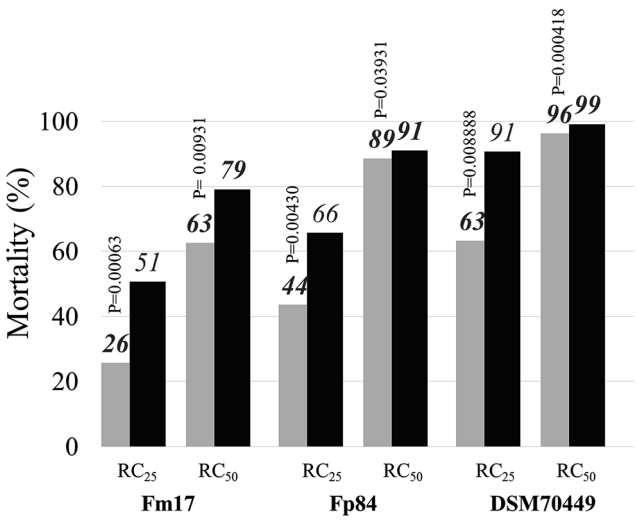

Fig. 3 Comparison of observed mortality values (grey bars) and expected (black bars) after exposure of the strains Fm17, Fp84 and DSM70449 to RC_{25} and RC_{50} mixtures. Observed and expected values of mortality are reported on each bar. Expected mortality values was estimated according to the fractional product method.[31] Data obtained were subjected to one-way analysis of variance (ANOVA), pair comparison was achieved by Tukey's procedure and probability values (*p*) are reported.

quaternary mixture. Conversely, FTIR analysis can bypass the analytical limits linked with the use of mortality data taking into account the metabolomic alterations induced by inhibitors both *pre*- and *post*-cell death.

Metabolomic data were analyzed yielding an Absolute Reduction Indicator (ARI), proposed as the difference between the sum of the metabolomic responses induced separately by each inhibitor and that induced by the mixture (Table 2). Positive ARI values indicate antagonism, negative synergism and figures close to 0 additivity. More specifically, considering that variation coefficients (*i.e.* the ratio between standard

Table 2 Distribution of the Absolute Reduction Indicator (ARI) in the spectroscopic regions[a]

	Strain	Mixture RC_{25}	Σ	Absolute reduction index (ARI)	Mixture RC_{50}	Σ	Absolute reduction index (ARI)	Mixture RC_{100}	Σ	Absolute reduction index (ARI)	Mixture RC_{200}	Σ	Absolute reduction index (ARI)
GSI	Fm17	0.97	4.52	3.55	3.31	4.59	1.28	3.56	3.78	0.22	3.51	4.73	1.22
	Fp84	2.43	11.53	9.10	2.71	10.08	7.38	2.99	4.58	1.60	4.00	6.77	2.77
	DSM70449	0.72	1.69	0.96	2.02	2.96	0.94	2.68	10.01	7.33	2.64	10.03	7.39
W1	Fm17	1.37	8.06	6.69	4.29	7.12	2.83	5.68	7.12	1.43	5.47	7.60	2.13
	Fp84	3.76	17.74	13.98	2.94	14.76	11.82	3.81	6.24	2.43	5.69	9.21	3.51
	DSM70449	0.62	2.06	1.44	2.74	4.62	1.88	4.06	16.57	12.51	3.88	18.66	14.78
W2	Fm17	2.16	11.09	8.92	12.16	13.40	1.24	12.73	7.79	−4.94	12.75	13.51	0.75
	Fp84	5.70	27.52	21.82	5.62	22.29	16.67	9.73	13.21	3.49	12.81	19.84	7.03
	DSM70449	2.86	4.30	1.44	8.66	8.57	−0.09	10.33	17.78	7.45	10.40	12.87	2.47
W3	Fm17	1.79	8.34	6.55	9.94	10.99	1.05	8.68	4.33	−4.36	8.90	8.39	−0.51
	Fp84	6.20	30.90	24.70	6.86	25.77	18.91	8.82	11.64	2.82	11.09	17.84	6.75
	DSM70449	1.25	2.90	1.66	5.03	5.21	0.18	6.69	20.09	13.39	6.82	11.35	4.53
W4	Fm17	3.68	10.43	6.74	5.72	9.19	3.47	4.30	7.60	3.30	4.36	10.56	6.20
	Fp84	6.60	29.03	22.42	10.87	29.78	18.91	7.31	12.27	4.96	9.18	18.00	8.82
	DSM70449	2.11	5.70	3.60	2.52	7.53	5.01	3.32	29.14	25.81	3.19	28.60	25.41

[a] SIs values indicate the metabolomic response of the whole spectrum (GSI) and the fatty acids (W1), amides (W2), mixed (W3) and carbohydrate (W4) regions. Positive ARI values indicate antagonism, negative synergism and figures close to 0 additivity.

deviation and the average of a measure) in metabolomic analyses range from 5 to 10%, ARI confidence limits for additivity should range from −0.20 to 0.20.

With very few exceptions, the analysis of the ARI values displayed an antagonistic effect among inhibitors, closely strain- and dose-specific. Fp84 showed the highest ARIs and DSM70449 the lowest at RC_{25} and RC_{50} while, at the highest relative concentrations, we observed an inversion. The spectral region mostly affected by the antagonism was the carbohydrates one, suggesting a possible involvement of these molecules in the phenomenon as well as in the reaction to the single compounds (Fig. 1). This hypothesis, together with the related mechanism(s), needs further studies to be deeply elucidated.

The present work was based on the postulate that FTIR approach could detect the interactions occurring between inhibitors in mixture through the analysis of the alterations that they induced on the cell metabolome. ARI values presented validated this assumption, highlighting that inhibitors mixtures exerted an antagonistic effect on the microbial metabolism, as already suggested by the analysis of the mortality data for RC_{25} and RC_{50} (Fig. 3). Moreover, metabolomic data analysis allowed to assess this phenomenon also at concentrations that saturate the cell mortality (RC_{100} and RC_{200}), similar to those usually present in the lignocellulosic ethanol processing. Finally, antagonism was found to be closely strain- and dose-specific, confirming that the resistance of a yeast strain to single inhibitors cannot be used to predict its behavior when exposed to inhibitory mixtures.

These results are not in accordance with the related literature, that mainly report an additive and synergistic effects of inhibitors on microbial metabolism.[13,22] So far, only two papers have described the antagonistic effects of two inhibitors, acetic acid and furfural, on yeast growth[37] and transcriptome.[38] This underlines the importance of the developing of new approaches, such as the one above proposed, to better understand the strain(s) behavior in real industrial conditions and to guide the selection of tolerant strains for the large scale production of lignocellulosic ethanol.

Inhibitors chemical interaction(s) and reaction(s) analysis outside the cells. Chemical analysis was performed to elucidate whether the antagonistic effects detected in this study would be due to chemical interaction(s) between inhibitors molecules. Depending on the reaction conditions glucose can be converted to HMF and/or levulinic acid, formic acid and different phenolics. Correspondingly, xylose can follow different reaction mechanisms resulting in the formation of furfural and/or acetic acid.[12] Furfural and HMF can undergo ring-opening or other chemical reactions accomplished by inorganic or organic acids in specific experimental conditions, such as high temperatures or in presence of inorganic catalysts.[39–43] Although different experimental settings have been employed in this study (25 °C, absence of catalysts), several experiments were performed in order to determine the occurrence of potential reactions between the chemical species outside the cells. Ultraviolet-visible (UV-vis) spectral analysis of furfural and HMF in presence of formic and acetic acids showed an almost complete overlap of the spectra of the molecules in all the spectral range

and at all the concentrations even with the use of a strong acid such as HCl and the increase of acid concentrations until pH = 1 (data not shown). This demonstrated that the presence of these acids did not determine a variation on the aromatic portions of the molecules, therefore their ring openings in our experimental conditions are not likely to occur. These data suggested that the different and lower biocidal and metabolomic effects of the inhibitors mixtures could not be ascribed to reactions between the molecules outside the cells.

Conclusions

To our knowledge, this study reports the first qualitative and quantitative evaluation of the antagonistic effects of inhibitors mixtures on *S. cerevisiae* metabolism. Remarkably, FTIR analysis was able to quantitatively assess the type of interactions among inhibitors, for all strains and RCs, overcoming the saturation effect obtained when the sum of observed mortality values is above 100%. The use of binary and ternary inhibitors mixtures will allow to deeply understand the mechanism sustaining this antagonism. Furthermore, this approach appears particularly promising for eco-toxicological settings, in which complex mixtures rather than single compounds are normally found.

Finally, in terms of strain tolerance characterization, this FTIR-based bioassay proved to be as effective as the measurement of relative growth rate in glucose-containing medium supplemented with inhibitors.[6,28] The ease and rapidity of the FTIR analysis indicate that this method could support future applications to assist the selection of highly inhibitors-resistant strains for the efficient industrial production of lignocellulosic ethanol.

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