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Research Article

New insights into the toxicity mechanism of octanoic and decanoic acids on *Saccharomyces cerevisiae*

Anna Borrull, Gema López-Martínez, Montse Poblet, Ricardo Cordero-Otero and Nicolas Rozès*
Dpt. Bioquímica i Biotecnología, Facultat d'Enologia, Universitat Rovira i Virgili, Campus Sescelades, Tarragona, Spain

**Correspondence to:*
N. Rozès, Dpt. Bioquímica i Biotecnología, Facultat d'Enologia, Universitat Rovira i Virgili, Campus Sescelades, c/Marcel·lí Domingo s/n, Tarragona 43007, Spain.
E-mail: nicolasrozes@urv.cat

Abstract

Octanoic (C8) and decanoic (C10) acids are produced in hypoxic conditions by the yeast *Saccharomyces cerevisiae* as by-products of its metabolism and are considered fermentation inhibitors in the presence of ethanol at acidic pH. This study aims to broaden our understanding of the physiological limits between toxicity and ester production in yeast cells. To this end, the non-inhibitory concentration (NIC) and maximum inhibitory concentration (MIC) values were first established for C8 and C10 at physiological pH (5.8) without ethanol. The results showed that when these acids were added to culture medium at these values, they tended to accumulate in different cellular fractions of the yeast. While C8 was almost entirely located in the cell wall fraction, C10 was found in the endocellular fraction. Cell fatty acid detoxification was also different; while the esterification of fatty acids was more efficient in the case of C10, the peroxisome was activated regardless of which fatty acid was added. Furthermore, the study of the Pdr12 and Tpo1 transporters that evolved during the detoxification process revealed that C8 was mostly expelled by the Pdr12 carrier, which was related to higher β-oxidative damage in the presence of endocellular C10. C10 is more toxic at lower concentrations than C8. Although they are produced by yeast, the resulting intracellular medium-chain fatty acids (MCFAs) caused a level of toxicity which promoted cell death. However, MCFAs are involved in the production of beverage flavours.

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Keywords: **medium-chain fatty acid; NIC; MIC; detoxification mechanism; ROS; Pdr12; Tpo1**

Introduction

Yeasts are subjected to very stressful conditions when fermenting grape must. The medium undergoes changes; levels of both ethanol and other secondary subtracts, such as medium-chain fatty acids (MCFAs), increase, while the nutrient content decreases and nitrogen levels drop to below sufficient (Alexandre and Charpentier, 1998). The main MCFAs, octanoic (C8) and decanoic (C10) acids, are produced by yeasts during alcoholic fermentation under hypoxic conditions as by-products of lipid synthesis (Taylor and Kirsop, 1977) and are toxic by themselves (Lafon-Lafourcade *et al.,* 1984). Overcoming this stress is more difficult than may be expected, due to interaction between the various stress factors.

The undissociated form of MCFAs and weak acids in general, which can be found in a free form in acidic medium (e.g. wine), can pass across the plasma membrane and dissociate in the neutral cytoplasm by means of passive diffusion, causing a decrease in intracellular pH (Viegas and Sá-Correia, 1997) and possibly leading to cell death. Meanwhile, at physiological pH, fatty acids dissociate and require a carrier to cross the yeast membrane. It is not yet known which carriers make fatty acids enter cells.

The toxicity of MCFAs is also related to their lipophilic properties, which may also affect the cell

membrane (Tenreiro *et al.,* 2002). In order to maintain membrane stability, yeast can change its composition when exposed to stressful conditions, such as increased levels of MCFAs in the medium. The liposolubility of MCFAs is an important factor in understanding their toxicity. For instance, C10, which is more toxic than C8 (Viegas *et al.,* 1989), can more easily enter the cell because it is more liposoluble than C8, causing an increase in cell permeability (Alexandre *et al.,* 1996). However, Cabral *et al.* (2001) observed that rapid exposure of yeast cells to sublethal concentrations of C8 provokes an adaptive response, allowing the cells to resist higher amounts of inhibitor.

The characterization of MCFA toxicity is unclear and often related to adaptation (Cabral *et al.,* 2001) or detoxification (Peddie, 1990) mechanisms. More recently, an analysis of the transcriptome response to exposure to C8 and C10 revealed that two partial mechanisms seem to occur (Legras *et al.,* 2010), but these two fatty acids also have a detoxification response, which both of them use. Most responses to C8 stress involve the transporter Pdr12p, which mainly effluxes weak acids under the control of the transcription factor War1p (Kren *et al.,* 2003), and the major facilitator superfamily transporter Tpo1p, a plasma membrane-bound exporter involved in the detoxification of excess spermidine in yeast (Albertsen *et al.,* 2003). Resistance to C10 also requires Tpo1p, several genes in the β-oxidation pathway and ethyl ester synthesis (Legras *et al.,* 2010). Therefore, there are two different detoxification mechanisms for these two molecules, even though they only differ by two carbons.

β-Oxidation is known to be the primary process by which fatty acids are degraded, producing, among other molecules, H_2O_2, which is converted by peroxisomal catalase into water and oxygen. It is a very important enzyme in terms of protecting the cell from oxidative damage by reactive oxygen species (ROS). Genes related to β-oxidation seem to be overexpressed for C10 but not for C8, which has a very efficient main transporter that is also used to overcome toxicity caused by other weak acids (Legras *et al.,* 2010). It was hypothesized that both C8 and C10 activate certain genes related to the weak acid response, because they belong to an acidic group. However, C10 is identified by the cell as a detergent, which might activate a detergent-like stress response to prevent C10 toxicity (Legras *et al.,* 2010).

In this experiment, octanoic and decanoic acids were added to a synthetic medium in order to characterize the tolerance limits of the BY4742 laboratory strain and the yeast changes in order to adapt to the supplemented medium. The study was conducted at physiological pH (5.8) to ensure that no other stresses affected the cell apart from exposure to MCFAs.

This study focuses on the differences between the detoxification processes for C8 and C10. An analysis of specific carriers and the β-oxidation process provided the key to understanding this difficult issue, in which not all of the detoxification mechanisms of C8 and C10 are activated in the same manner.

Materials and methods

Strain and culture medium

The *Saccharomyces cerevisiae* strain used in this study was the haploid laboratory strain BY4742 (*MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*), obtained from EUROSCARF (Winzeler *et al.,* 1999). The haploid gene deletion mutants in the BY4742 background were YLL028W (*Tpo1*, polyamine transporter that recognizes spermine, putrescine and spermidine) and YPL058C [*Pdr12*, plasma membrane ATP-binding cassette (ABC) transporter]. Strains were precultured in YPD [1% w/v yeast extract (Cultimed, Barcelona, Spain); 2% w/v bacto peptone (Panreac, Barcelona, Spain) and 2% w/v glucose (Panreac)] in aerobiosis at 28 °C. The cell yeasts were harvested and washed once with sterile water before being inoculated into SC medium (0.64% w/v yeast nitrogen base without amino acids; Difco, Madrid, Spain) with 2% w/v glucose (Panreac) and 0.08% w/v auxotrophic mixture consisting of histidine, leucine, lysine and uracil (1:1:1:1, w/w). All experiments were conducted without agitation (semi-anaerobic conditions) at 28 °C.

Determination of non-inhibitory and maximum inhibitory concentrations

Growth was monitored at 600 nm using a Biogen analyser with a 96-well plate (BMG LabTech, Wertheim, Germany) at 28 °C. A stock solution of each fatty acid (50 g/l) diluted in ethanol was

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used to achieve concentrations in SC medium in the range 10–250 mg/l (0.07–1.73 mM for C8; 0.06–1.45 mM for C10) in sterile tubes. Measurements were taken every 30 min over 4 days after preshaking for 20 s. All the experiments were carried out under aerobic conditions. The wells of the microplate (96-well plate) were filled with 0.01 ml inoculum and 0.25 ml SC medium, reaching an initial OD $\approx$ 0.2, corresponding to an initial cell number of 2×10^6 cells/ml. For each condition, triplicate growths were monitored for 2 days at 28 °C. For each experimental series, non-inoculated wells were also included in the microplate to subtract the noise signal.

Non-inhibitory concentration (NIC) and maximum inhibitory concentration (MIC) parameters were estimated by comparing the area under the OD–time curve of a positive control (absence of MCFAs, optimal conditions) with the areas of the tests (presence of MCFAs, increasingly inhibitory conditions). The areas under the OD–time curve were calculated by integration, using GraphPad Prism software (GraphPad Software Inc., v. 4, 1992–2003). In keeping with the method published by Arroyo-López *et al.* (2010), the relative amount of growth for each MCFA concentration, denoted as the fractional area (f_a), was obtained using the ratios of the test area (areatest) to that of the positive control of the yeast (areacont), according to the following formula: f_a = (areatest)/(areacont). With the modified Gompertz function for decay (Lambert and Pearson, 2000), the sigmoid curve generated by plotting f_a vs $\log_{10}$ MCFA concentration was fitted. The expression of the modified Gompertz function is: $y = A + C \times \exp\{-\exp[B(x - M)]\}$, where A is the lowest asymptote of f_a (ca. 0), B is a slope parameter, C is the distance between the upper and lower asymptote (ca. 1) and M is the $\log_{10}$ MCFA concentration of the inflexion point. These different parameters were obtained by a non-linear regression procedure, using GraphPad Prism software. The NIC and MIC parameters were estimated using the equations of Lambert and Pearson [9] as follows: $\mathrm{NIC} = 10^{(c\ -\ 1.718/b)}$ and $\mathrm{MIC} = 10^{(c\ +\ 1/b)}$.

Viability of yeast cells

Viability was evaluated for cells exposed to NIC and MIC concentrations in SC culture medium and plated on YPD agar, using a Whitley Automatic Spiral Plater (DW Scientific, West Yorkshire, UK). YPD plates were incubated for 48 h at 28 °C. ProtoCOL SR/HR software (v. 1.27.1664; Synoptics Ltd, Cambridge, UK) was used to count colonies of spirally diluted plates. All experiments were conducted in triplicate.

Vitality of yeast cells

The same sampling point used for lipid analysis was also used to measure cell vitality, using a Bac-Trac® 4300 microbiological analyser (SY-LAB Instruments, Austria). This device measures the electric impedance generated by a 0.02% w/v KOH solution. This impedance decreases due to the reaction between KOH and the CO_2 produced by the yeast. SC medium (5 ml) was inoculated with 10^7 cells/ml YPD–overnight yeast culture in an open vial that allowed CO_2 to come into contact with the KOH solution. The impedance level was monitored every 10 min by the Bac-Trac and a curve was drawn, expressing the percentage decrease in impedance over time. Cell vitality was expressed as the time needed (in h) to reach the maximum fermentation rate at 28 °C, considered to be the point at which total impedance had decreased by 10%. The M 10% discrepancy between control and the MCFA-supplemented conditions was calculated using the formula: $dM\ 10\% = (M_{\mathrm{Control}}10\% - M_n10\%)/M_{\mathrm{Control}}10\%$, where n is the tested condition.

Determination of fatty acids

Free fatty acids in cell walls

Sedimented cells (5×10^8 cells/ml) were placed in Eppendorf microtubes (T-2795-1000EA) and 10 ml internal standard (heptanoic acid 0.5 mg/ml, heptadecanoic acid 2 mg/ml; Sigma, Spain) and 200 ml diethyl ether (Panreac) were added. After soft vortexing, the pellet was centrifuged at 5000 rpm for 30 s and the organic upper phase was removed. This extract was analysed by means of gas chromatography.

Free fatty acids in the endocellular fraction

Glass beads were added to the same pellet analysed above. After vortexing for 30 s (3×) at a speed of 10 000 rpm, 10 ml internal standard (heptanoic acid 0.5 mg/ml, heptadecanoic acid 2 mg/ml) and

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200 ml diethyl ether were added. After vortexing at speed, the pellet was centrifuged at 5000 rpm for 30 s and the organic upper phase was removed. This extract was analysed by means of gas chromatography.

Total fatty acids of cells

The pellet analysed above was placed in sealed tubes with a Teflon-lined screw cap and saponified using 1 ml 5% w/v NaOH in 50% methanol:water (1:1), as described by Rozès *et al.* (1992); 10 µl solution of heptanoic acid (1 mg/ml) and heptadecanoic acid (4 mg/ml) was added before saponification. The extraction process was then performed with 300 µl hexane:MTBE (1:1).

Gas chromatography conditions

All the extracts were analysed on a Hewlett-Packard 6850 gas chromatograph (Agilent Technologies), using the protocol described by Redón *et al.* (2009).

Determination of catalase activity in peroxisomes of cells exposed to MCFAs

After exposing the yeasts to MCFAs, the protein fraction was extracted according to the method described by Blomberg (2002). Proteins were separated on 10% SDS–PAGE and analysed by western blot, in line with *Current Protocols in Protein Science* (Coligan *et al.,* 2000). For the immunodetection process, the non-specific binding sites on the membrane were blocked using 0.1% v/v Tween 20, 5% w/v dried skimmed milk in phosphate-buffered saline (PBS) for 1 h. Excess volume was removed by rinsing in PBS-T (0.1% v/v Tween 20 in PBS) for 5 min. Incubation was then allowed to take place overnight with the primary antibody (rabbit polyclonal to catalase–peroxisome marker, ab1887; Abcam, Cambridge, UK). After washing with PBS-T, a second incubation with the antibody (actine, peroxisome-labelled anti-rabbit antibody made in goat, ab8227; Abcam) was performed for 1 h. Finally, the membranes were viewed using an ECL Advanced Western Blotting Detection Kit (Amersham–GE Healthcare, UK), in accordance with the manufacturer's instructions. Images were captured using a FluorChem FC2 Imaging System (Cell Biosciences, CA, USA) and were quantified using ImageJ v.1.41.

Determination of reactive oxygen species (ROS)

ROS determination was performed by DHE-staining the cells. Cells (5×10^8 cells/ml) treated with MCFAs were resuspended in 250 µl DHE/PBS (1:1000 dilution of 2.5 mg/ml stock DHE in PBS 1×, pH 7.0). The suspension was kept incubated for 5–10 min in the dark. Finally, the stained cells were washed with 250 µl 1× PBS, pH 7.0. The stained ROS cells were examined and counted using a fluorescence microscope (Leica DM4000 B) equipped with a digital camera (Leica DFC300FX); Leica IM50 software was used for image acquisition. Positive cells containing ROS compounds emitted red fluorescence. The total number of cells evaluated was approximately 1500 for each condition. The evaluation was conducted in triplicate for each experimental condition.

Statistical analysis

The statistical analysis was performed using SPSS 19 statistical software (SPSS Inc., Chicago, IL, USA).

Results

Estimation of NIC and MIC values

The growth of BY4742 in SC medium at physiological pH (5.8) with increasing concentrations of C8 or C10 was monitored at 600 nm, using a microplate reader. The maximum ethanol content was 0.5% v/v in all wells. The estimations of NIC and MIC values for both acids were calculated from the OD–time curves. Table 1 shows that the NIC value found for C10 was around half that found for C8; 0.15 mM (25 mg/l) and 0.24 mM (35 mg/l), respectively. The MIC for C10 also was around half the MIC value of C8: 0.46 mM (80 mg/l) and 0.87 mM (125 mg/l), respectively. The progressive inhibitory region (PIR), which represents the difference between MIC and NIC values, was also found to be lower for C10 than for C8, at 0.31 mM (55 mg/l) and 0.63 mM (90 mg/l), respectively. It can therefore be concluded that C10 is more toxic than C8.

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Table 1. Determination of non-inhibitory concentration, progressive inhibitory region and maximum inhibitory concentration parameters for BY4742, Pdr12 and Tpo1 transporter mutants for octanoic and decanoic acids

	BY4742		*Δtpo1*		*Δpdr12*	
	C8	C10	C8	C10	C8	C10
NIC	0.24	0.15	0.14	0.15	0.14	0.20
PIR	0.63	0.31	0.09	0.28	0.09	0.22
MIC	0.87	0.46	0.23	0.43	0.23	0.42

NIC, non-inhibitory concentration; PIR, progressive inhibitory region; MIC, maximum inhibitory concentration; C8, octanoic acid; C10, decanoic acid. Concentrations are expressed in mM.

Changes in the viability and vitality of cells exposed to MCFAs

Because high concentrations of MCFAs can inhibit cell growth, defined above by means of the estimated NIC and MIC values for C8 and C10, the viability of BY4742 was evaluated under our experimental conditions. At NIC, cell viability was reduced from 100% to 54.9% for C8 and 42.3% for C10 compared to the control cell, while at MIC the viabilities were 14.7% for C8 and 6.5% for C10 (data not shown).

It was also possible to determine vitality, which tells us whether the cells in contact with C8 or C10 are metabolically active (Figure 1). The vitality of yeast cells in contact with increasing concentrations of C8 (range 0.14–0.42 mM) was higher than that of the control (BY4742 without the addition of MCFAs) (Figure 1A). Nevertheless, vitality decreased as the content of C8 increased and at concentrations of C8 around MIC values of 0.55–0.87 mM, where viability had drastically decreased. The same phenomenon was observed at lower concentrations for the vitality of cells in contact with C10 (Figure 1B). The positive concentration values (growth-improved) were 0.12 and 0.23 mM, and the negative values were 0.35 and 0.46 mM, the latter being the MIC value for C10.

One interesting finding resulted from combining both C8 and C10 at NIC and MIC values (Figure 1C). From calculations based on the individual results of impedance changes for both acids (Figure 1A, B), it is clear that the real combination of these acids never provoked the effects of the theoretical sum of responses. It was found that the combination of the two acids increased impedance at their MIC values. However, the inverse was observed in the case of C10 at its NIC concentration, confirming that C10 toxicity is higher than that of C8.

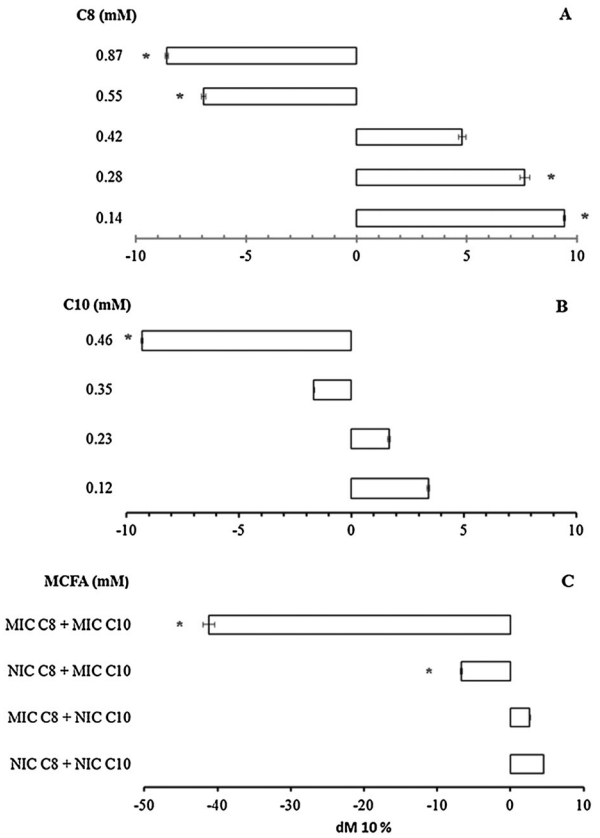

Figure 1. Effect of medium-chain fatty acids on cell vitality. BY4742 cells were grown in YPD overnight. The yeasts were incubated semi-anaerobically at 28 °C in SC medium at pH 5.8, in the presence of C8 (A), C10 (B) and a combination of both MCFAs (C), at different concentrations ranging between NIC and MIC values. Time 0 represents the time taken by the cells to reach a 10% decrease in total impedance in the SC condition. All experiments were carried out in quadruplicate and SDs are indicated. *Results with statistically significant differences ($p < 0.05$)

Changes in medium-chain fatty acid composition in different cellular fractions of cells exposed to MCFAs, as measured by CG–FID

It was taken in consideration that MCFA uptake (C8 and C10) may depend on: (a) the solubility; (b) the hydrophobicity; (c) the pH; and (d) the concentration of each fatty acid. To characterize which of these variables have a significant impact on the uptake and cell localization of both MCFAs, free fatty acids and their corresponding

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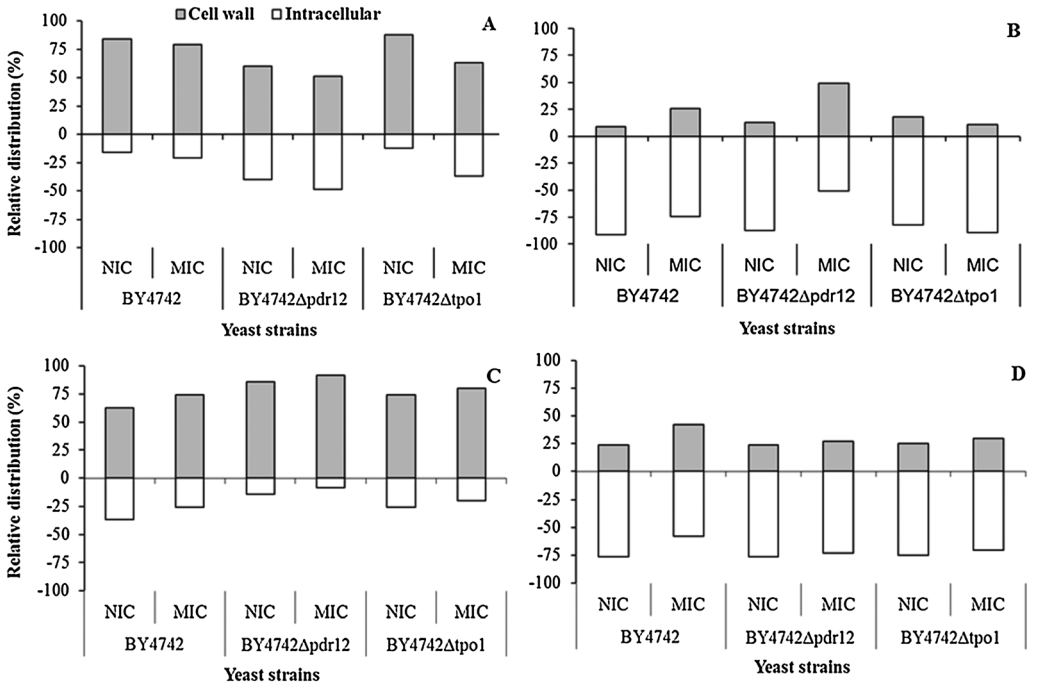

Figure 2. Free MCFA distribution in cell wall and intracellular fractions. BY4742 and Tpo1 and Pdr12 transporter mutant cells were exposed to C8 (A, C) or C10 (B, D) for 3 and 12 h, respectively, at NIC or MIC. Free fatty acids located in the cell wall were removed with an initial wash with organic solvent and analysed by means of GC. After cell disruption, another wash was carried out with organic solvent, but this time the recovered extract corresponded to the intracellular fraction

ethyl esters for the same cell pellet, obtained under different physiological conditions, were quantified in: (a) the cell wall fraction washed with organic solvent (OSW); (b) cell breaking + OSW for the intracellular fraction; and (c) saponification of intracellular fraction to determine the esterified fractions of C8 and C10.

In the fatty acid-free medium (control): (a) free C8 and C10 were rapidly synthesized by the cells in the first hour of fermentation and were localized both in the cells and on the cell wall fraction; (ii) more C10 was produced than C8; and (c) 86% of total C10 was found after saponification, which means that decanoic acid is esterified (results not shown). As expected, the addition of C8 and C10 in the culture medium induced an increase in these fatty acids in the different fractions of the yeast cells. However, octanoic acid seemed to remain on the cell wall, as nearly 80% of the total content was recovered from this fraction, regardless of the concentration added and the fermentation time analysed (Figure 2A). The proportion of C10 found in the different fractions was 70% inside the cell and 30% in the cell wall (Figure 2B). For instance, when NIC or MIC values of C8 were used, the total cellular C10 content decreased but the C8 concentration increased. The same trend was observed at the NIC and MIC values of C10 with respect to C8 content (data not shown). Definitively, the increase of one MCFA by exposure produced a decrease in the synthesis of the other.

As for the effect of the addition of MCFAs on ethyl ester formation, it was found that C10 seemed to use esterification as one of the main metabolic pathways to detoxify the cell, especially in the early hours of contact (Figure 3C). C8 also synthesized ethyl esters, although to a lesser degree (Figure 3A, B). This was surely related to the higher concentration of C10 than C8 found in the intracellular fraction. Another aspect of the effect of adding MCFAs was that more ethyl ester of C8 was excreted from the cells at 12 h (Figure 3B) than the corresponding ester of C10 (Figure 3D). In other respects, the control cells rapidly produced ethyl hexanoate, which was localized in both the cell wall and the endocellular fractions, and its level increased over

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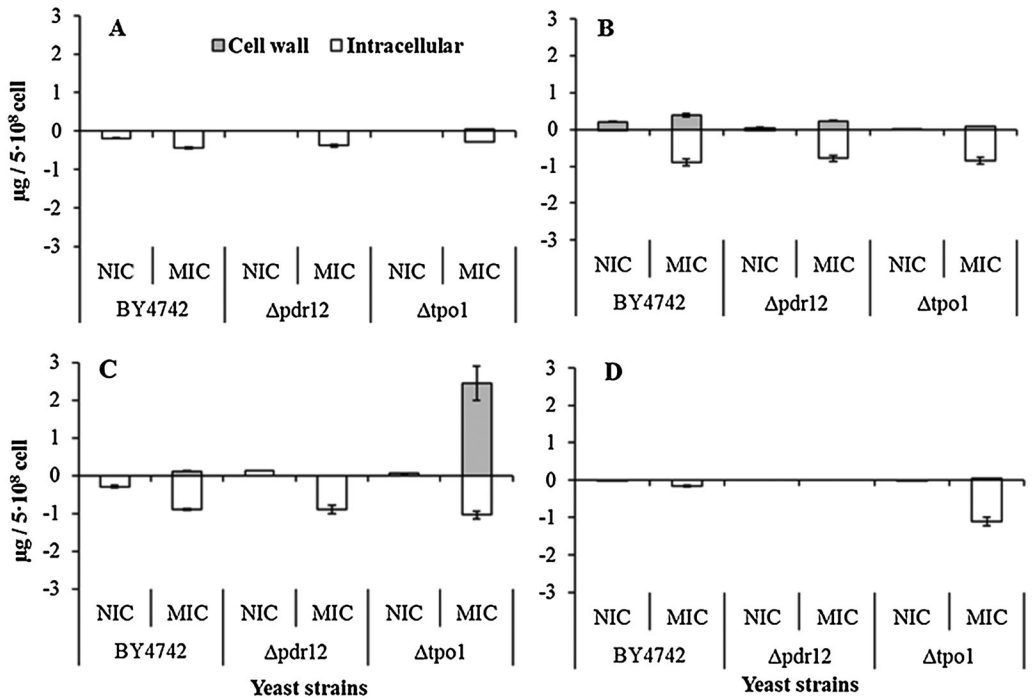

Figure 3. Ethyl esters of MCFA (µg/5 × 10^8 cells) distribution in cell wall and intracellular fractions. BY4742 and Tpo1 and Pdr12 transporter mutant cells were exposed to C8 (A, B) or C10 (C, D) for 3 and 12 h at NIC or MIC. Ethyl esters of MCFA located in the cell wall were removed with a first wash with organic solvent and analysed by means of GC. After cell disruption, another wash was carried out with organic solvent, but this time the recovered extract corresponded to the endocellular fraction

time (data not shown). After 3 h, ethyl decanoate was detected inside the cells but, interestingly, it was not found after 12 h.

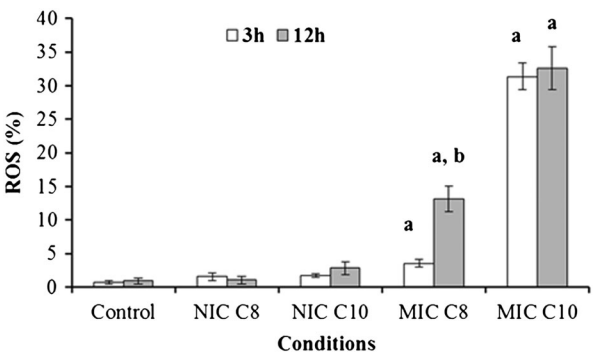

Figure 4. Yeast cells undergo ROS accumulation during stress imposition. BY4742 cells were grown overnight in YPD medium. The yeast cells were incubated for 3 and 12 h semi-anaerobically in SC medium at pH 5.8 in the presence of C8 and C10. DHE staining was used to quantify ROS accumulation. Values are means of $n = 2$ determinations ± SD; in each experiment, 5 × 10^4 cells/ml were evaluated. (a), Values significantly different ($p \leq 0.05$) from control values; (b), significant differences between 3 and 12 h for any condition

Changes in ROS accumulation in yeast during exposure to C8 and C10

The accumulation of intracellular reactive oxygen species (ROS) was evaluated in order to assess cell damage caused by MCFA toxicity (See Figure S1). The accumulation of ROS was demonstrated using the reduced form of ethidium bromide (dihydroethidium, DHE) to visualize the accumulation of ROS when cell yeasts were exposed to C8 or C10 after 3 and 12 h of incubation (Figure 4). A total of 15% of cells were found to display ROS accumulation in MIC values of C8 at 12 h, due to the damaged cells caused by the β-oxidation process. For C10 at the NIC and MIC values, the number of stained cells rose to 30%. In conclusion, C10 induced earlier and higher accumulation of ROS than C8.

Changes in catalase activity in peroxisomes caused by MCFAs

β-Oxidation is the most logical pathway whereby the cell might suppress the toxicity of MCFAs.

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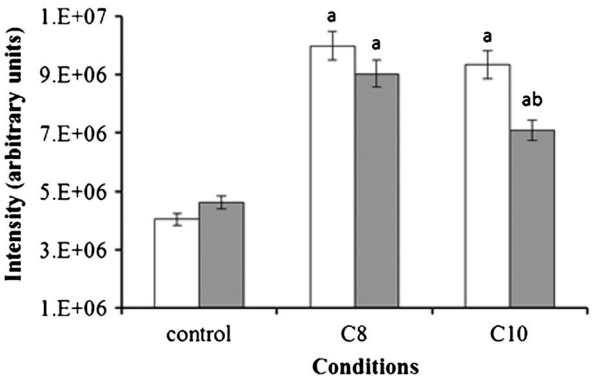

Figure 5. Peroxisome activity in BY4742 cells treated with MCFAs. Yeasts were grown in YPD overnight. Cells were inoculated at 2×10^6 cells/ml in SC medium containing C8 and C10 at pH 5.8. Pellets were collected after 3 and 12 h. The protein extract was analysed to determine catalase protein expression after 3 h (light bars) and 12 h (dark bars). The experiments were conducted in duplicate and the SD is indicated. Values significantly different from (a) control values and (b) other over-expressed conditions are labelled

The catalase is the main peroxisomal enzyme and it enables the degradation of the peroxide produced during β-oxidation. The catalase activity in the cells exposed to NIC concentrations of both C8 and C10, regardless of exposure time, showed double the catalase activity compared to the reference condition (Figure 5). However, cells in the presence of C10 after 12 h showed 30% less catalase content than at the other time points for C8 and C10.

Carrier experiments with mutants

Based on our results, it was suspected that C8 had a higher affinity transporter than C10, because it is not accumulated in proportions as high as those of C10. The evaluation of BY4742 mutants in fatty acid carriers was the next point that was studied. According to Legras *et al.* (2010), the main carriers capable of detoxifying C8 and C10 are Tpo1p and Pdr12p. Therefore, the knockout mutants for these expected fatty acid carriers were used to see what effects they had on the MCFA partition through the cells, in order to determine what roles they play in the actual detoxification process.

In the evaluation of free fatty acids in the cell wall and the intracellular fractions, Pdr12p and Tpo1p seemed to have a higher affinity for the detoxification of C8 (Figure 2A, C). Our experiments showed that C8 was accumulated in the cell wall fraction as a function of time, ca. 50% for 3 h and ca. 75% for 12 h of exposure, indicating that Pdr12p takes part in C8 detoxification. The same results were observed for Tpo1p. Meanwhile, our results suggest that Pdr12p and Tpo1p have little or no effect on C10 detoxification (Figure 2B, D). Esterification also had a different affinity for these two fatty acids in the mutant tested (Figure 3A–D).

NIC and MIC values were also measured for transporter mutants in order to compare the values with the those of the wild strain. For the addition of C10, the NIC and MIC values of the mutant carriers (*Δtpo1* and *Δpdr12*) were the same as for the wild strain (Table 1). Based on these results, C10 does not seem to be directly related to these transporters, or at least Tpo1p and Pdr12p did not significantly change the limit toxicity values. However, C8 was affected by the deletion of both Pdr12 and Tpo1 (Table 1). The NIC value for both mutants was two-fold lower than the control value and the MIC value was nearly four-fold lower. Some authors have reported that, in the case of C8, Pdr12p is more efficient than Tpo1p at allowing fatty acids to exit the cell. However, this last carrier also seems to have a considerable effect on yeast growth, as the same value was found for both the estimated NIC and MIC values. Interestingly, the PIR values for the addition of C8 were very low for both mutant carriers (0.09), compared to the wild yeast value (0.63), which may mean that these transporters are actually involved in the detoxification process.

Discussion

MCFA toxicity

Medium-chain fatty acids are known to be fermentation inhibitors of wine yeast, whose deleterious effects might be enhanced by the presence of ethanol and low pH (Viegas *et al.,* 1989; Viegas and Sá-Correia, 1997). In this study, the toxicity mechanism at pH 5.8 was examined in order to determine which MCFAs alone contribute to yeast toxicity, without considering the combination of other stress factors. The toxicity mechanism of MCFAs is still not clear. However, this and other recent studies may help to clarify and eventually understand this complex situation.

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Differential mechanism

The detoxification mechanism that yeast uses to overcome this stress is complex and, interestingly, distinct for C8 and C10, even though they differ by just two carbons. Our results have led us to agree with the hypothesis presented by Legras *et al.* (2010), who suggest that C8 has a detoxifying response that is similar to a weak acid response, while the C10 response is closer to a detergent response.

It was expected to find different detoxification mechanisms, based solely on the observation of the accumulation of C8 and C10 in the two cell fractions studied. Indeed, C8 was found in the more superficial part of the cell, specifically in the cytoplasmic membrane, while C10 seemed to enter the cell without any difficulty. It is important to note that our working pH was 5.8. As such, our experiments were run at a pH at which C8 and C10 (pKa 5.9) were equally in dissociated and undissociated forms. Both the different location and difference in esterification efficiency have led us to conclude that there may be two different detoxification mechanisms that cells use to prevent the toxic effects of C8 and C10. Therefore, a large part of C8 and C10 was expected either to enter or not enter via a specific transporter. To confirm this, the two carriers described by Legras *et al.* (2010) were evaluated, namely Pdr12p and Tpo1p. The responses were clear, based on the NIC and MIC values and the partition of each fatty acid through the cell: C10 seems to enter the cells by passive diffusion and its intracellular accumulation induces a toxic effect in the cells, while C8 seems to use both carriers to detoxify the cells, based on our studies with mutant strains. However, it is not excluded that part of C8 enters the cells by passive diffusion at pH 5.8 (pKa 5.9 for C8) but the carriers permit cells to transport outside the undissociated form.

In order to evaluate the possible detoxification mechanism in the cells, ROS accumulation and β-oxidation processes were examined in the organelles in which these might take place, the mitochondrion and the peroxisome, respectively. Our results enable us to conclude, first, that ROS accumulation is not caused by the NIC of either of the fatty acids, although it is caused by the MIC. Second, C10, which is considered to be more toxic than C8 based on the limit toxicity values, can not only cause a higher accumulation of ROS than does C8 but also earlier accumulation at MIC exposure. As our results indicated that C8 caused little ROS accumulation, there must be another mechanism responsible for C8 detoxification. This led us to evaluate peroxisome activity and, more specifically, catalase activity, which is the main indicator of peroxisomal activity. The addition of MCFAs was expected to increase catalase content, and this was confirmed by our results (Figure 5), as the cells exposed to MCFAs showed increased accumulation of this enzyme, except in the case of C10 at 12 h, where it decreased. Therefore, it can be concluded that the peroxisome is not primarily responsible for C8 detoxification in the cell and is not the reason for the unexpected distribution of this acid along the different cell fractions. However, it has been demonstrated that: (a) the toxicity of these acids increases peroxisome activity; (b) MCFAs enter the cells; and finally (c) because no differences were observed between the two fatty acids, the peroxisome is probably activated regardless of the fatty acid type. Nevertheless, in the presence of C10 after 12 h, due to lower catalase content, the cells were unable to reduce ROS and, consequently, the viability decreased.

In conclusion, these two MCFAs seem to share a common detoxification process, but each also has a detoxification process that is particular to that MCFA. The main difference between the two is their lipophilicity. While decanoic acid is more lipophilic than octanoic acid and it enters and accumulates in the cells, C8 can be expelled by Pdr12 and Tpo1 transporters. One way that C10 was detoxified was through the esterification process, inducing the production of ethyl decanoate.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's website:

Figure S1. Effect of both C8 and C10 on ROS determination at MIC level at 12 h