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Inhibition of Yeast Growth by Octanoic and Decanoic Acids Produced during Ethanolic Fermentation

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The inhibition of growth by octanoic or decanoic acids, two subproducts of ethanolic fermentation, was evaluated in *Saccharomyces cerevisiae* and *Kluyveromyces marxianus* in association with ethanol, the main product of fermentation. In both strains, octanoic and decanoic acids, at concentrations up to 16 and 8 mg/liter, respectively, decreased the maximum specific growth rate and the biomass yield at 30°C as an exponential function of the fatty acid concentration and increased the duration of growth latency. These toxic effects increased with a decrease in pH in the range of 5.4 to 3.0, indicating that the undissociated form is the toxic molecule. Decanoic acid was more toxic than octanoic acid. The concentrations of octanoic and decanoic acids were determined during the ethanolic fermentation (30°C) of two laboratory media (mineral and complex) by *S. cerevisiae* and of Jerusalem artichoke juice by *K. marxianus*. Based on the concentrations detected (0.7 to 23 mg/liter) and the kinetics of growth inhibition, the presence of octanoic and decanoic acids cannot be ignored in the evaluation of the overall inhibition of ethanolic fermentation.

During ethanolic fermentation by yeasts and as a result of their metabolism, a wide variety of compounds is produced besides ethanol, the main product. Some of these subproducts, which are present in fermented media in low concentrations (some in the range of 0.5 to 10 mg/liter), are of great importance, considering the organoleptic properties of the final product (25, 31, 37). They are mostly fatty acids of medium chain length (C_6 to C_{12}) and their respective ethyl esters, aldehydes, and ketones, as well as longer-chain alcohols and organic acids. The last group also contributes to the acidity of the fermented product (25, 31). Glycerol and succinate are two other secondary products that are usually produced at higher concentrations (25, 37). It has been reported that some of these subproducts inhibit the growth of *Saccharomyces cerevisiae*, therefore contributing to a decrease in the ethanol productivity of alcoholic fermentation (33). It has also been suggested that the toxic actions of the hexanoic, octanoic, and decanoic acids are partly responsible for the premature stoppage of wine fermentation by *S. cerevisiae* (14, 26). Several pieces of evidence indicate that the medium-chain fatty acids excreted to the wort during alcoholic fermentation by *S. cerevisiae* are intermediate compounds in the biosynthesis of long-chain fatty acids and lipids rather than the result of the catabolism of the long-chain fatty acids that are present in the wort or in the yeast cells (52). The amount of medium-chain fatty acids that is produced during the alcoholic fermentation and that is released into the fermentation medium is strongly dependent on the yeast strain, the medium composition, and the fermentation conditions (such as temperature, pH, and aeration) (1, 20, 22, 25, 31, 37).

When octanoic and decanoic acids were added to growth media with a sublethal concentration of ethanol that was sufficient to solubilize them when in concentrations up to 16 mg/liter for octanoic acid and 8 mg/liter for decanoic acid (the range present in wines [25]), the specific growth rate of *Saccharomyces bayanus* (now *S. cerevisiae* [24]) decreased as an exponential function of the toxic concentration (55). When octanoic and decanoic acids acted together with lethal concentrations of ethanol, they were found to stimulate the specific death rate exponentially (41). On a molar basis, the order of toxicity found was decanoic acid > octanoic acid ≫ ethanol, corresponding to their liposolubilities (41, 55). Therefore, it has been suggested that even when present at low concentrations, the toxic action of octanoic and decanoic acids could explain why the ethanol produced during alcoholic fermentation had a higher apparent toxicity than ethanol added to the growth medium (41, 55).

Lipophilic acids are an important class of antimicrobial food additives (3, 7, 11, 13, 23, 46, 56). Their action shows a clear pH dependency, being less effective as the pH increases (7, 11). They enter the microbial cell as undissociated molecules, because those forms are readily soluble in membrane phospholipids (13, 16). Although many metabolic reactions have been reported to be inhibited by benzoic, sorbic, and short-chain fatty acids, the most significant effect in yeasts is probably by the uncoupling of transmembrane proton gradients from the energy-requiring processes, which are linked to the interactions of these toxins with cell membranes (3, 7, 8, 23, 46, 56).

The levels of octanoic and decanoic acids in wine and beer have already been reported on account of their important contribution to the organoleptic properties of alcoholic beverages (19, 21, 22, 25, 32, 47). However, as far as we know, under the conditions used in industrial ethanolic fermentations, information on their levels is not available. In this study we measured the concentrations of octanoic and decanoic acids produced during alcoholic fermentation by *S. cerevisiae* IGC 3507 III of two laboratory media and during the fermentation of Jerusalem artichoke juice by *Kluyveromyces marxianus* IGC 2671. In the latter case, we intended to evaluate the importance of the toxicity of these acids in the production of ethanol using a substrate with industrial interest (39, 40). The inhibition of the specific growth rate of *S. cerevisiae* IGC 3507 III and *K. marxianus* IGC 2671 by the action of octanoic acids, decanoic acids, or both in combination with ethanol were also evaluated in media with pHs in the range of 3 to 5.4. Based on the inhibition kinetics and on

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the concentrations of octanoic and decanoic acids produced by both yeast strains, the role of these two toxic subproducts in the global inhibition of alcoholic fermentation was determined.

MATERIALS AND METHODS

Microorganisms. *S. cerevisiae* IGC 3507 III, a respiratory mutant and the main strain used by van Uden (54) in studies on ethanol toxicity, and *K. marxianus* IGC 2671, an inulin-fermenting strain selected for Jerusalem artichoke juice fermentation, were used (39, 40, 42, 43).

Growth medium and conditions. The liquid media used in growth experiments were composed of glucose (30 g/liter in yeast nitrogen base [YNB] [Difco Laboratories, Detroit, Mich.] for *S. cerevisiae*) or sucrose (30 g/liter in YNB for *K. marxianus*). Base media for growth were prepared on citrate-phosphate buffer at different pHs (3, 4, and 5.4); the buffers were previously sterilized at 120°C for 15 min. The YNB solution (10× concentrated) was filter sterilized. Alcoholic solutions of octanoic or decanoic acids were added to these media in order to obtain 6% (vol/vol) ethanol plus octanoic acid (1, 2, 4, 8, and 16 mg/liter) or decanoic acid (0.5, 1, 2, 4, and 8 mg/liter) or 0.6% (vol/vol) ethanol plus octanoic acid (2, 4, 8, 16, and 60 mg/liter) or decanoic acid (1, 2, 4, 8, and 30 mg/liter). Growth experiments were carried out at 30°C in shake flasks with 50 ml of growth medium. The shake flasks were closed with a rubber bung that was perforated with a needle, to avoid the evaporation of volatile toxic compounds.

To inoculate the growth media, cells were pregrown in shake flasks closed with a cotton plug for 14 h at 30°C in a liquid medium [containing the following, per liter: 100 g of glucose, 5 g of yeast extract, 5 g of $(NH_4)_2SO_4$, 5 g of KH_2PO_4, and 1 g of $MgSO_4 \cdot 7H_2O$]. Cells were centrifuged and washed twice with the growth medium and suspended in a suitable volume to start growth experiments. The inoculum consisted of 0.5 ml of cell suspension, which resulted in an initial dry weight of 140 mg/liter. The cellular growth was followed by turbidimetric measurement. The specific growth rate was calculated by least-squares fitting to the linear parts of semilogaritumic (optical density at 640 nm) plots versus time. The contants of growth inhibition (K_i) by octanoic or decanoic acids were calculated based on the following equation (equation 1), which was previously used with success for *S. bayanus* (55):

$$\ln \mu^x = \ln \mu^{x_m} - K_i (X - X_m) \quad (1)$$

where μ^x and μ^{x_m} were the maximum specific growth rates in the presence of 6% (vol/vol) ethanol plus, respectively, the concentration X of the toxic acid and their MICs, X_m. K_i was the constant of the exponential inhibition of growth for each toxic acid.

Analysis of ethanol, unfermented sugar, and free fatty acids during alcoholic fermentations. Alcoholic fermentations by *S. cerevisiae* were carried out in two different base media with 250 g of glucose per liter. F_1 was a rich medium containing the following, per liter: 5 g of yeast extract, 5 g of $(NH_4)_2SO_4$, 5 g of KH_2PO_4, 1 g of $MgSO_4 \cdot 7H_2O$, and 220 mg of $CaCl_2 \cdot 2H_2O$ in distilled water. F_2 was mineral medium composed of 6.7 g of YNB (Difco) per liter and 5 g of KH_2PO_4 per liter in distilled water. Alcoholic fermentation of Jerusalem artichoke juice (250 g of total sugars per liter) by *K. marxianus* was also undertaken.

The base medium F_1 was heat sterilized together with glucose at 120°C for 15 min. The base medium F_2 (10× concentrated) was filter sterilized and added to the glucose solution that was previously sterilized at 120°C for 15 min. Jerusalem artichoke juice was used without additives or sterilization, after preheating at 55°C for 15 min. The initial pH of the medium was adjusted to 4.5. Fermentations were carried out at 30°C in shake flasks. The inoculum used in these experiments was similar to that described above for growth experiments.

At suitable times, samples were centrifuged and analyzed for ethanol (gas chromatography) and glucose (dinitrosalicylic acid method) (34). In the case of Jerusalem artichoke juice fermentation, total sugars were determined by the same dinitrosalicylic acid method after total hydrolysis of fructans (1 h of heating at 120°C and pH 2).

For each analysis of octanoic and decanoic acids, we used 50 ml of supernatant to which 0.5 ml of an alcoholic solution of heptanoic acid (500 mg/liter in absolute ethanol) was added. Heptanoic acid was used as a reference, since it is not produced in the alcoholic fermentation by yeasts (31, 52). The pHs of the solutions were adjusted to 8.2 to 8.4 with 3 M NaOH. Then, 50 ml of pentane was added to separate the neutral compounds from fatty acids and the pentane layer was discarded (51). The remaining liquid was concentrated in a rotary vacuum evaporator at 30°C until it was dry, to eliminate alcohols and esters which interfere in the chromatograph dosage of the fatty acids. The residue was then dissolved in 5 ml of an ethanolic solution at 40% (vol/vol). The pH was adjusted to 1 to 2 with H_2SO_4 (1:5). The fatty acids in the solution were extracted 3 times with 5 ml of ethyl ether. The extracts were concentrated in a rotary vacuum evaporator until there was a final volume of 1 ml.

The fatty acids were analyzed (three replicates) in a gas chromatograph (5840; Hewlett-Packard Co., Palo Alto, Calif.) equipped with a flame ionization detector. A stainless steel column (3 mm [inner diameter] by 4 m) packed with FFAP (Carbowax 20M/Nitroterephtalic acid ester) (8% [wt/vol]) + H_3PO_4 (0.2% [vol/vol]) on Chromosorb W (Supelco) was operated isothermally at 220°C, the injector was operated at 240°C, and the detector was operated at 310°C. The carrier gas was nitrogen (flow rate, 15 ml/min), and the volume of injection was 3 μl.

Calibration was obtained with ether solutions composed of heptanoic, octanoic, and decanoic acids (50 mg/liter each), using nonaoic acid as the internal standard. Another sample with 5 mg of heptanoic, octanoic, and decanoic acids per liter was treated identically to the fermentation samples. The values obtained after gas chromatographic analysis did not deviate more than 10% from the initial concentration of the three fatty acids. Therefore, it was accepted that the standard treatment led to a quite good and similar extraction of the three acids.

RESULTS

Inhibition of growth by octanoic and decanoic acids. Octanoic (0 to 16 mg/liter) and decanoic acids (0 to 8 mg/liter) decreased the maximum specific growth rate of *S. cerevisiae* in the presence of 6% (vol/vol) ethanol (Fig. 1). Growth inhibition was more drastic for lower pHs, suggesting that the undissociated form is the toxic form (Fig. 1). Moreover, for the range of concentrations used, the two toxic fatty acids presented no toxicity if they were unsolubilized (growth medium present on two phases), as occurred with low concentrations of ethanol at low pHs (for example, pH 3.0 and 0.6% [vol/vol] ethanol).

On a major basis and in the order of their lipossolubilities

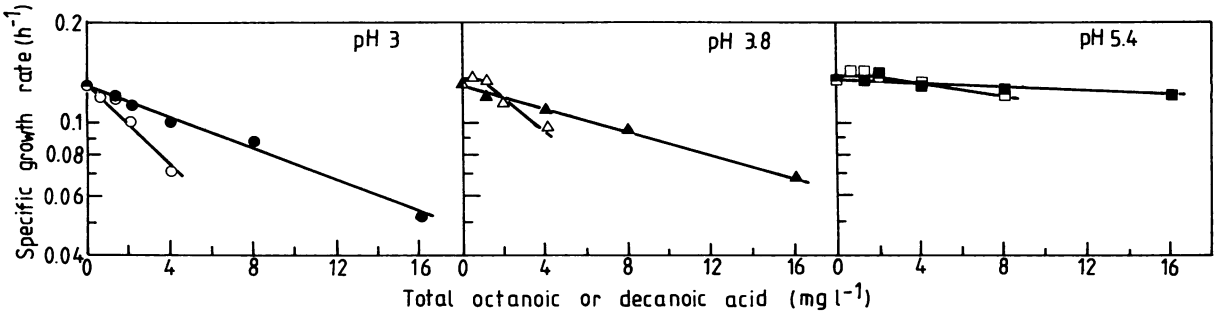

FIG. 1. Inhibition of the maximum specific growth rate of *S. cerevisiae* IGC 3507 III at 30°C and in the presence of 6% (vol/vol) added ethanol at pH 3, 3.8, and 5.4 by the addition of octanoic (●, ▲, ■) and decanoic (○, △, □) acids.

(Table 1), decanoic acid was more inhibitory than octanoic acid (Fig. 1).

Above a critical concentration, octanoic or decanoic acid increased the duration of growth latency (Fig. 2). This critical concentration was lower and the duration of the latency was higher for lower pHs and for decanoic acid when compared with those for octanoic acid (Fig. 2).

The presence of ethanol at different concentrations, which still allowed both yeast growth and the solubilization of the fatty acids (0.6% [vol/vol] or 6% [vol/vol] at pH 5.4), appeared to have no effect on the fatty acid growth inhibition constants. However, it remains to be seen whether this result can be generalized for lower pHs, at which octanoic and decanoic acids are more potent.

The exponential constant of growth inhibition, K_i, which was calculated based on equation 1 and on the concentration of the undissociated forms, was also pH dependent (Fig. 3b) but less significantly so than those based on total concentrations (Fig. 3a). For the calculation of the concentrations of the undissociated forms, pK_a was considered to be independent of the alcohol concentration and equal to 4.9 (13) for both fatty acids in alcoholic solutions, which, up to 6% (vol/vol) ethanol, seems to be reasonable (15). The pH dependence of the growth inhibition constants of the undissociated octanoic and decanoic acids (Fig. 3b) cannot be attributed solely to this estimation of pK_a, as evidenced by the more drastic pH dependence detected for decanoic acid (Fig. 3b).

Exponential kinetics were also found for the growth inhibition of *K. marxianus* by octanoic and decanoic acids (Fig. 4a). Since *K. marxianus* IGC 2671 is less ethanol tolerant than *S. cerevisiae* IGC 3507 III (41, 42), a lower concentration of ethanol (0.6% [vol/vol] at pH 4.0) was used. These conditions allowed the solubilization of the fatty acids in the range of concentrations used, but did not induce an inconveniently strong ethanol inhibition.

Besides the effect that octanoic and decanoic acids had on the specific growth rate of fermenting yeasts (Fig. 4a), we also detected an impressive decrease in the biomass yield, given by the optical density at 640 nm attained in the stationary phase after the total consumption of glucose (Fig. 4b). The decrease of biomass yield was also an exponential function of the total acid concentration, with the slope being close to that observed for the inhibition of the specific growth rate (compare panels a and b in Fig. 4).

Under the experimental conditions used, the addition of

TABLE 1. Constants for the exponential inhibition of growth by octanoic and decanoic acids and the relation to their lipossolubilities

Toxic compound	Constant values for the following microorganisms:			
	S. cerevisiae IGC 3507 III[a]		*K. marxianus* IGC 2671[b]	
	K_i (liter/mol)[c]	K_i/P (liter/mol)[d]	K_i (liter/mol)[c]	K_i/P (liter/mol)[d]
Octanoic acid	5,910	5.3	4,480	4
Decanoic acid	19,040	1.5	9,587	0.8

[a] Growth medium with 6% (vol/vol) ethanol plus toxic acid (pH 3.8).
[b] Growth medium with 0.6% (vol/vol) ethanol plus toxic acid (pH 4.0).
[c] K_i, Calculation based on equation 1 and on the concentration of the undissociated forms.
[d] Partition coefficient (octanol/water) of octanoic acid (P = 1,120) and decanoic acid (P = 12,300) (16, 46).

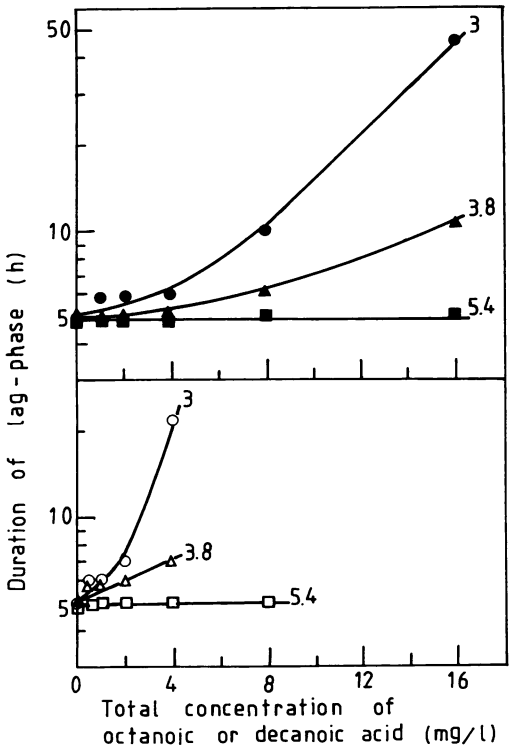

FIG. 2. Effect of the addition of octanoic (●, ▲, ■) or decanoic (○, △, □) acids on the duration of growth latency of *S. cerevisiae* IGC 3507 III growth at 30°C in a growth medium with 6% (vol/vol) ethanol and different pHs.

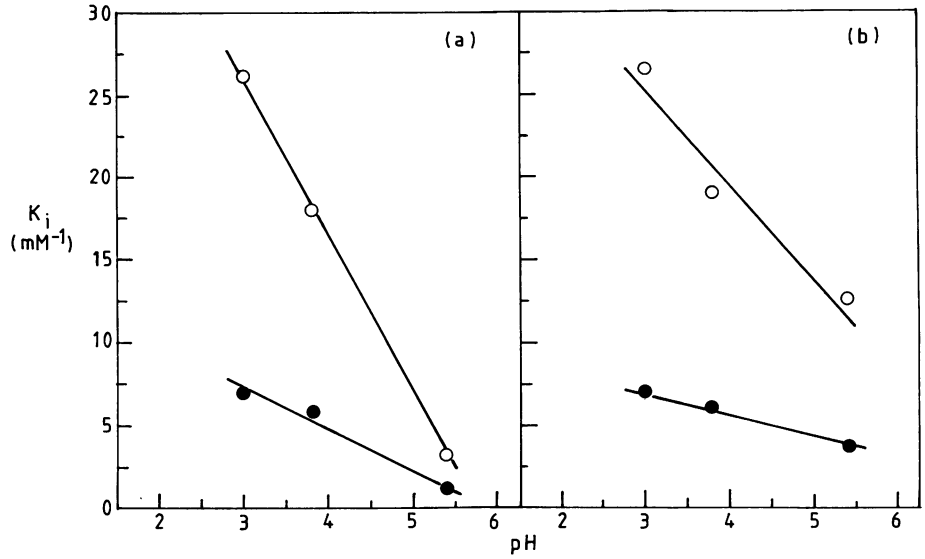

FIG. 3. Influence of pH on the exponential inhibition constant (equation 1) of the growth in the presence of 6% (vol/vol) of ethanol by the addition of octanoic (●) and decanoic (○) acids, calculated with base on the total concentration of added acids (a) or on the concentration of the undissociated form (b).

6% (vol/vol) ethanol to the growth medium reduced 50% of the maximum specific growth rate of *S. cerevisiae* at 30°C. The simultaneous addition of 8 mg of octanoic acid per liter led to an additional growth inhibition of 26.2% at pH 3.8 or 33.1% at pH 3.0 (Fig. 1). In the same way, the addition of only 4 mg of decanoic acid per liter to the growth medium with 6% (vol/vol) ethanol led to an additional inhibition of 24.6% at pH 3.8 or 45.3% at pH 3.0 (Fig. 1). In the case of *K. marxianus*, the presence of 0.6% (vol/vol) ethanol only inhibited the specific growth rate by 4% at 30°C and pH 4.0. The presence of 8 mg of octanoic acid per liter additionally inhibited the specific growth rate by 18.5% and the presence of 4 mg of decanoic acid per liter inhibited the specific growth rate by 20% (Fig. 4a).

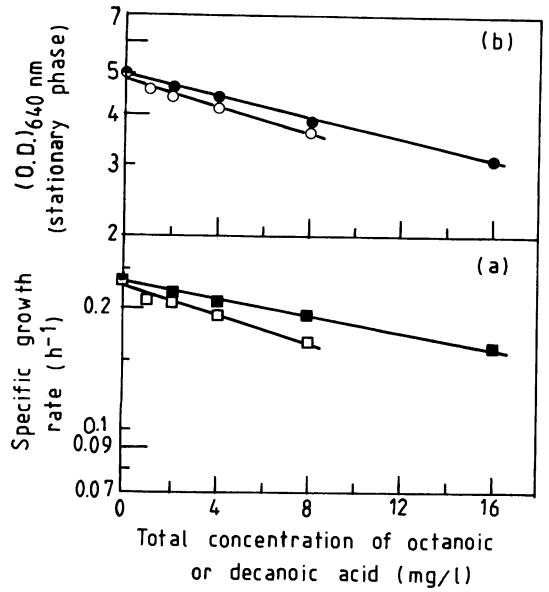

FIG. 4. Inhibition of the maximum specific growth rate (a) and decrease of the biomass yield (b) of *K. marxianus* IGC 2671 in the presence of 0.6% (vol/vol) ethanol at 30°C and pH 4.0 by the addition of octanoic (■, ●) and decanoic (□, ○) acids. OD, Optical density.

Production of octanoic and decanoic acids during alcoholic fermentation. The levels of octanoic and decanoic acids detected during the fermentative process by *S. cerevisiae* depended significantly on the composition of the fermentation media (Fig. 5a and b). In the mineral medium, with salts and vitamins besides glucose (Fig. 5a), the production of decanoic acid was higher than that of octanoic acid and increased until the middle of the fermentation process, with inhibitory concentrations of both acids being achieved (23 and 7.5 mg/liter for decanoic and octanoic acids, respectively). The levels detected then decreased drastically. During the fermentation the medium pH also decreased significantly, from 4.5 to 2.4 (Fig. 5a), which could contribute to its premature stoppage (residual glucose).

By the fermentation of the medium which contained yeast extract (Fig. 5b), a higher final concentration of ethanol was produced and glucose consumption was nearly complete. During the entire fermentation the levels of decanoic acid were very low and the concentrations of octanoic acid achieved a maximum (10 mg/liter) near the end of the fermentation; they then decreased until the end of the fermentation. This occurred after the production of 14% (vol/vol) ethanol, with 4 mg of octanoic acid per liter and 2 mg of decanoic acid per liter remaining. The decrease in the concentration of those fatty acids could probably be due to their esterification with the ethanol produced (35, 36, 48). However, after 3 weeks the concentrations of octanoic and decanoic acids were 27.4 and 7.8 mg/liter, respectively (data not shown). The increase in their levels, after a prolonged contact of the yeast cells with the fermentation medium, could be due to cell leakage. During this fermentation, the medium pH also suffered an important decrease (Fig. 5b).

During the fermentation by *K. marxianus* IGC 2671 of a complex medium, like Jerusalem artichoke juice, which is rich in proteins, amino acids, vitamins, and mineral salts, besides total sugars (≈250 g/liter), the production profiles of octanoic and decanoic acids were also different (Fig. 5c).

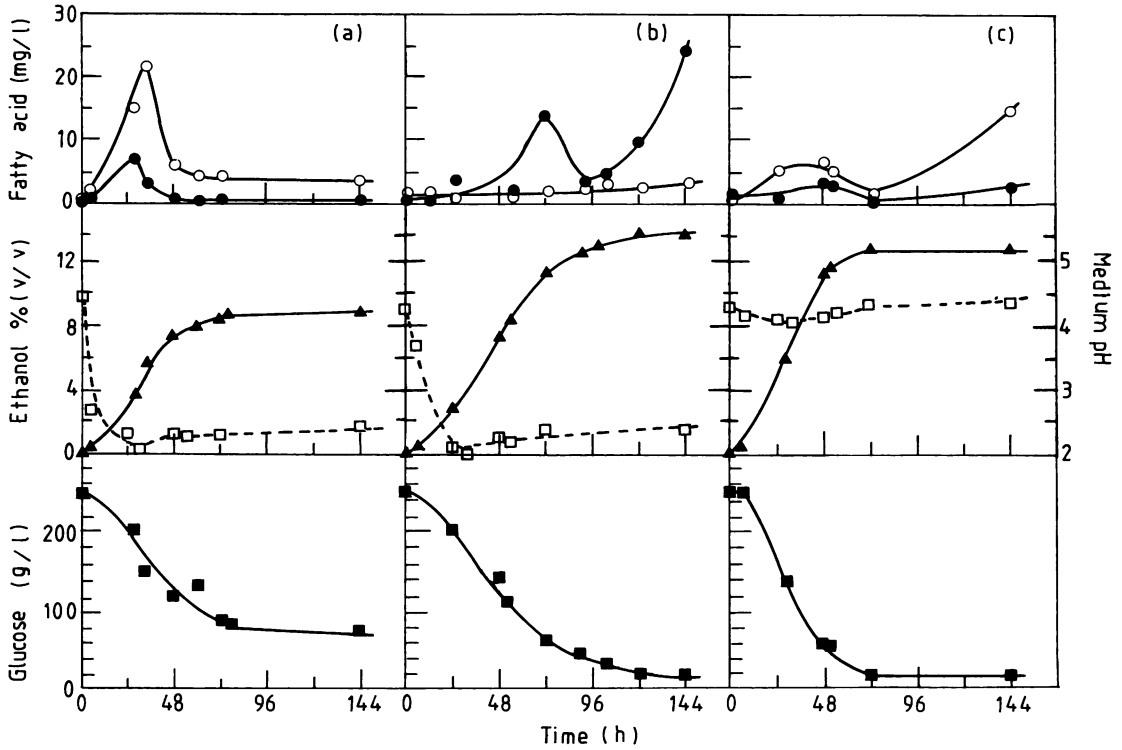

FIG. 5. Production of ethanol (▲) and octanoic (●) and decanoic (○) acids, consumption of glucose (■), and pH variation (□) during the alcoholic fermentation of a mineral medium (F_2) (a) and a rich medium (F_1) (b) by *S. cerevisiae* IGC 3507 III at 30°C and of Jerusalem artichoke juice by *K. marxianus* IGC 2671 at 30°C (c).

The concentration of decanoic acid in the fermentation medium was higher than that of octanoic acid. Both fatty acids achieved a peak value by the middle of the fermentation (5.5 and 2.4 mg/liter for decanoic and octanoic acids, respectively), decreasing after the middle of fermentation. Nevertheless, after 72 h of fermentation, their concentrations increased and after 144 h they reached 1.68 and 13.3 mg/liter, respectively, for octanoic and decanoic acids. During Jerusalem artichoke juice fermentation the pH value did not change much (Fig. 5c) because of the high buffering power of the juice.

DISCUSSION

The inhibitory effects of octanoic and decanoic acids on the growth of *S. cerevisiae* were evaluated when these two acids were associated with ethanol. The presence of ethanol allowed the solubilization, in the pH range of 3 to 5.4, of both fatty acids (up to 16 mg of octanoic acid per liter and 8 mg of decanoic acid per liter, which is in the range of concentrations present in wines [25]). Moreover, these conditions simulated the environment to which yeasts were subjected during alcoholic fermentation. Low concentrations of ethanol and low pHs (for example, 0.6% [vol/vol] ethanol and pH 3) did not allow the solubilization of octanoic and decanoic acids, and under those conditions, we found that they had no detectable toxic effect.

On a molar basis, decanoic acid was more toxic than octanoic acid, and these two acids were much more toxic than ethanol (Table 1) (55) in the same order of their solubility in lipids. Octanoic and decanoic acids have been proposed to enter the microbial cell across the plasma membrane basically by the passive diffusion of the undissociated molecules, which are readily soluble in membrane phospholipids (49, 50). Their insertion inside the membranes is expected to decrease the hydrophobic lipid-lipid and lipid-protein interactions. Therefore, the spatial organization of membranes decreases, their permeability increases, and the biological function of membrane-bound enzymes such as those involved in the transport systems could be affected indirectly (38, 53). As reported previously for the toxic action of ethanol in *S. cerevisiae* (44), we also found that the presence of octanoic and decanoic acids in association with ethanol led to the decrease of plasma membrane effectiveness in retaining, against a concentration gradient, the intracellular compounds such as amino acids and 260-nm-absorbing and ribose-containing compounds (C. A. Viegas, I. Sá-Correia, and J. M. Novais, manuscript in preparation).

The growth inhibitory action of decanoic acid appeared to be less important than expected based on its liposolubility compared with the liposolubility and the toxic effect of octanoic acid (Table 1). Alcohols with more than 10 carbon atoms have also been found to be relatively inefficient as inhibitors (18, 45). This phenomenon was partially explained by the fact that liposoluble compounds with a hydrophobic chain larger than a critical dimension are not so intrusive in membranes as smaller compounds are (12, 18, 28). Moreover, and even at low concentrations, fatty acids and alkanols with long chains tend to make aggregates (30, 45, 46). To have a toxic effect, lipophilic drugs must be present in free solution in contact with the cells; this idea was also used to explain the inefficiency as anesthetics of compounds with chain lengths longer than those of decane, dodecanol, and octanoic acid (45). The presence of ethanol in the growth

medium allowed the solubilization of fatty acids, but it is still possible that, at the higher concentrations, both toxic compounds might not be completely free in solution. This could also explain the lower growth inhibition constants calculated for *K. marxianus* (in growth media with only 0.6% [vol/vol] ethanol) in the case of decanoic acid (Table 1). Because *K. marxianus* IGC 2671 is a much less ethanol-tolerant strain than *S. cerevisiae* IGC 3507 III (43), higher growth-inhibitory constants for both fatty acids were anticipated; but this was not confirmed and experiments that were undertaken in growth media with 6% (vol/vol) ethanol did not change the reported relationship of tolerances (unpublished data).

Yeasts cells are permeable to undissociated weak acids (49, 50) and have been considered to be impermeable to the respective anions. Studies on the mechanism of the antifungal action of benzoic acid at low external pHs developed by Krebs et al. (23) suggest that its toxic action in cells of *S. cerevisiae* is mainly caused by the dissociation in the cytoplasm of the undissociated form which entered the cell by passive diffusion as a consequence of an internal pH that was appreciably greater than the pK_a of the acid. Therefore, the internal pHs could decrease to values in the range at which phosphofructokinase is sensitive (23) and the subsequent inhibition of glycolysis could cause a fall in the concentration of ATP, which could restrict growth (23). The important intracellular accumulation of benzoate that occurs at external pHs that are significantly lower than the internal pH has also been proposed to underlie the antifungal action of benzoic acid (23). However, recent results indicate that the efflux of anions from the cell must also be taken into consideration (56, 57). Warth (57) has claimed that the major effect of benzoic acid on *S. cerevisiae* (57) and *Zygosaccharomyces baillii* (56, 57) is related to the energy requirements to reduce the concentration of benzoate in the cytoplasm and to maintain the intracellular pH near neutrality (57). The major energy requirement may come from the need to extrude protons (57), probably by the involvement of plasma membrane ATPase. The involvement of plasma membrane ATPase was also proposed by Dombek and Ingram (9, 10) in order to explain the maintenance of the intracellular pH values near neutrality (9) during batch alcoholic fermentations by *S. cerevisiae*, despite the production of toxic concentrations of ethanol (12% [vol/vol]) which induced the passive influx of protons (4, 27). According to this hypothesis, they observed a decrease in the intracellular concentration of ATP and an increase in the AMP/ATP ratio during alcoholic fermentation (10). Considering that octanoic and decanoic acids have been proved to stimulate ethanol-induced leakage (Viegas et al., in preparation) and taking into account the probable additional increase in the intracellular proton concentration because of the dissociation of those acids in the cytoplasm, the increase in the energy requirements for the maintenance of intracellular pH may also be an important factor for their action as growth inhibitors. The drastic decrease in the biomass yield induced by the presence of increasing concentrations of octanoic and decanoic acids in *K. marxianus* (Fig. 4b) and the similarity between their effects in depressing growth rate and biomass yield (Fig. 4a and b) are in accordance with our present hypothesis.

The differences that we detected in the toxicity of ethanol and octanoic or decanoic acids for the two strains of *S. cerevisiae* and for the strain of *K. marxianus* studied may probably be related to the characteristics of their ATPases. Differences in the acid tolerance of plaque bacteria also appear to depend mainly on membrane physiology and are related to proton-translocating ATPases (activity and biochemical characteristics, especially the pH optimum for activity) (2). The increase in the growth inhibition constant by the undissociated acids when the extracellular pH decreases from 5.4 to 3 (Fig. 3) could also be related to the pH sensitivity of *S. cerevisiae* plasma membrane ATPase activity (5) and the pH dependence of plasma membrane permeability (29).

Octanoic and decanoic acids were proved to be produced during alcoholic fermentation by *S. cerevisiae* of two laboratory media and by *K. marxianus* during the alcoholic fermentation of Jerusalem artichoke juice. The concentrations detected in the fermentation media were variable with the yeast strain used and the medium composition. Their levels were expected to depend on the balance between their production and utilization during long-chain fatty acid and lipid biosynthesis and their excretion and chemical modifications. These different contributions may be variable with fermentation conditions and dependent on the phase of fermentation (1, 6, 22).

The increase in the plasma membrane permeability (17, 18, 44), the decrease in the concentration of ATP (10), and the inhibition of ATPase (5) that possible occurs by the end of the fermentation because of the presence of high concentrations of ethanol and other toxic subproducts may explain the increase in the extracellular levels of octanoic and decanoic acids because of extensive cell leakage. Besides enzymatic esterification, the chemical esterification of octanoic and decanoic acids by ethanol, which is catalyzed by the low pHs (48) that are attained during the fermentation by *S. cerevisiae* (Fig. 5a and b), could explain the drastic decrease of fatty acid levels during the last hours of fermentative activity (Fig. 5a and b).

Considering the results reported here, the concentrations of octanoic and decanoic acids produced cannot be ignored in the evaluation of the overall inhibition of alcoholic fermentation. The significance of their contribution, however, depends on medium composition, environmental conditions (pH and temperature), and the tolerance of the fermenting yeast strain.

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LITERATURE CITED

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