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The Influence of Acetic and Other Weak Carboxylic Acids on Growth and Cellular Death of the Yeast *Yarrowia lipolytica*

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Summary

Acetic, lactic, propionic, malic, succinic, citric and oleic acids were used by *Yarrowia lipolytica* as the sole carbon and energy source, this capacity being, in most cases, independent of the pH of the culture media. When the yeast was grown in a mixed medium with glucose 0.1 % (w/v) and a volume ratio of acetic, succinic, propionic or malic acid 0,1 %, pH= 3.0 and 5.0, the acid was used simultaneously with the glucose. Diauxic growth was observed when the yeast was grown in glucose and citric or lactic acid. These results suggest that the utilisation of these two acids, is subjected to glucose repression. Presence of acetic acid in the extracellular medium decreased the specific growth rate of the yeast grown in glucose medium at 26 °C, pH=3.0. Propionic, butyric and sorbic acids also had inhibitory effects on yeast growth. The effects of acetic, propionic, butyric and sorbic acids on the kinetics of cell death in glucose-grown cells, were studied as well. For each one of these compounds, and under isothermic conditions, cell death was exponentially stimulated in the presence of increasing extracellular acid concentrations; the toxic effects induced by the acids were higher for sorbic and butyric acids. In terms of acetic acid resistance this strain seems to be qualitatively more resistant than *Saccharomyces cerevisiae* and is closer to *Zygosaccharomyces bailli*, one of the most important food spoilage yeasts.

Key words: Yarrowia lipolytica, carboxylic acids, weak acids toxicity, cell death

Introduction

In the past few years *Yarrowia lipolytica* is a yeast species which interest has been increased due to its numerous biotechnological applications. Although it cannot be considered among the dangerous food spoilage yeasts *(1)* it has been often reported from food environments and, in some cases, associated with severe damage *(2)*. It is usually isolated from lipid-rich environments, such as mayonnaise and salad dressing, but it can also be found in dairy products – mainly butter, yogurt and cheese *(3–6)*. The presence of this species in such environments could be related to their resistance/tolerance towards stress environments, such as weak carboxylic acids, which are generally used as chemical preservatives in the food industry. Attempts to elucidate the mechanisms which underlie this behaviour are of considerable value for improving the quality control of different food products. In this context *Y. lipolytica* has been poorly understood, particularly in terms of its responses to the presence of weak acids.

In this paper, a strain of *Y. lipolytica* isolated from spoiled liquid yogurt was studied. Besides the evaluation of the yeast ability to use weak carboxylic acids

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alone or in mixture with glucose, the effects of the acids on growth and cell viability were investigated. Comparison of *Y. lipolytica* behaviour with other yeast species, described as sensitive or strongly resistant to weak acids, such as *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii*, respectively, is also presented.

Material and Methods

Microorganism and growth conditions

A strain of *Yarrowia lipolytica* (*Y. lipolytica* ISA 1834) isolated from spoiled liquid yogurt was used. The cultures were maintained on slants with a medium containing w/v of: glucose (2 %), peptone (1 %), yeast extract (0.5 %) and agar (2 %).

Cells were grown in 500 or 1000 mL Erlenmeyer flasks with mineral medium containing vitamins *(7)* supplemented with the different carbon and energy sources as indicated in the results section, at pH=3.0 and 5.0. The cultures were carried out at 26 °C, with mechanical shaking (180 rpm). Growth was monitored through the increase of absorbance measured at 640 nm. Dry weight of cells in culture samples (100 mL) was determined using acetate cellulose filters (pore size 0.45 μm). After removal of the medium by filtration, the filters were washed with ice-cold distilled water and dried overnight at 80 °C. Yield coefficient (*Y*) was based on dry weight determinations (see results).

Estimation of glucose and acid concentrations

Glucose and weak carboxylic acid (acetic, citric, malic, propionic, succinic and lactic) concentrations in the media were measured by high performance liquid chromatography (HPLC), using a refractive index detector and a Polyspher OAKC (Merck) column. Arabinose was used as an internal standard.

Effects of weak carboxylic acids on growth

In order to study the effects of the addition of acetic and other weak carboxylic acids on the kinetics of growth of *Y. lipolytica*, experiments were performed with cells previously grown on mineral medium with glucose 2 % (w/v). The yeasts were collected at mid-exponential phase and transferred to 250 mL Erlenmeyer flasks containing 100 mL of the same medium (pH=3.0) to which the different acid concentrations were added. For each situation specific growth rate (μ) was calculated as well as the growth inhibition constants for the different acids expressed in the following equation:

$$\ln \mu^x = \ln \mu^0 - k_i (x - x_{min}) \qquad /1/$$

where μ^x and μ^0 represent the specific growth rates at x acid concentration and in the absence of acid, respectively, k_i the inhibition growth constant characteristic for each of the acids and x_{min} the minimum acid concentration below which no significant inhibition was detected.

Death experiments and calculation of death parameters

Loss of cell viability of *Y. lipolytica* ISA 1834 in the absence and presence of weak carboxylic acids was assessed by methods previously described *(8)*. Glucose-grown cells were harvested at mid-exponential growth phase and transferred to 500 mL Erlenmeyer flasks containing 100 mL of mineral medium with or without the desired amount of acid at pH=3.0. The flasks were closed with rubber stoppers to avoid the evaporation of the acid from the culture media and placed in constant temperature baths, at 26 °C. The contents were kept homogenous by magnetic stirring. At established time intervals 0.1 mL samples were taken in triplicate and spread on the surface of agar plates with w/v of: glucose (2 %), peptone (1 %), yeast extract (0.5 %) and agar (2 %). After incubation at 25 °C until visible growth, the colonies were counted and the average count for each time interval was used for estimating the number of colony forming units (cfu). These values were expressed as percentage of cfu and used for drawing the semilog survival plots. The specific death rates (k_d) were calculated by least-square fitting to the linear parts of the semilog survival plots according to:

$$\ln N_t/N_0 = -k_d t \qquad /2/$$

where N_0 and N_t represent the average of colony forming units on the linear parts at time zero and time t, respectively.

Reproducibility of the results

All the experiments were repeated at least three times and the data reported here represent the average values.

Results and Discussion

Utilisation of carboxylic acids alone or in mixture with glucose

Growth experiments using several carboxylic acids as the sole carbon and energy sources were performed. Results, obtained with 0.1 % (w/v) of acid, at pH=3.0 and 5.0, are summarised in Table 1. As it can be seen, *Y. lipolytica* ISA 1834 was able to use acetic, lactic, propionic, succinic, malic, citric and oleic acids but not sorbic or palmitic acids. In all the cases a lag phase was observed whose duration was not strongly affected by the culture pH except for propionic acid, where it increased from about 16 to 100 hours at pH=5.0 and 3.0, respectively. Also, in most acids the values of both the biomass and the specific growth rates (μ) were not significantly affected by the culture pH. The strongest dependence was noticed in malic and lactic acid utilisation, in which the highest values for μ were observed at pH=5.0. It should be emphasised that in the case of growth on propionic acid, despite the significant difference found in the lag phase duration at pH=3.0 and 5.0, either μ or the final biomass values were about the same at both pH. When the acid volume ratio in the culture medium was higher (0.5 %) the acid utilisation pattern was similar to that described above for 0.1 %, at pH=5.0. However, under the same experimental conditions but at pH=3.0, the yeast was not able to grow on most of the tested acids, keeping only the capacity to utilise succinic, citric and oleic acids (results not shown).

Table 1. Growth parameters of the yeast *Yarrowia lipolytica* ISA 1834 in mineral medium with different carboxylic acids (0.1 % w/v) at 26 °C and pH=3.0 and 5.0

Acid	μ / h^{-1}		Lag phase / h		Final biomass / g / L	
	pH=3	pH=5	pH=3	pH=5	pH=3	pH=5
Citric	0.30 ± 0.01	0.25 ± 0.02	13.00 ± 1.00	14.00 ± 0.00	0.30 ± 0.05	0.32 ± 0.07
Acetic	0.19 ± 0.01	0.25 ± 0.04	10.00 ± 2.00	12.00 ± 0.00	0.20 ± 0.03	0.37 ± 0.04
Propionic	0.29 ± 0.00	0.28 ± 0.01	103.50 ± 5.50	16.67 ± 1.70	0.39 ± 0.01	0.39 ± 0.05
Lactic	0.08 ± 0.01	0.19 ± 0.02	9.00 ± 0.50	16.00 ± 3.00	0.21 ± 0.00	0.20 ± 0.02
Malic	0.03 ± 0.01	0.20 ± 0.03	26.00 ± 4.00	22.67 ± 0.94	0.14 ± 0.02	0.29 ± 0.04
Succinic	0.23 ± 0.01	0.39 ± 0.04	11.00 ± 1.00	14.00 ± 0.00	0.32 ± 0.01	0.39 ± 0.06
Sorbic	n.m.	n.m.	–	–	–	–
Oleic	0.24 ± 0.00	0.21 ± 0.01	8.00 ± 0.00	8.00 ± 0.00	0.96 ± 0.04	0.78 ± 0.02
Palmitic	n.m.	n.m.	–	–	–	–

n.m.– not measurable; μ= specific growth rate

The present strain of *Y. lipolytica* was also tested for its capacity to utilise acetic acid and other weak carboxylic acids in the presence of glucose. Experiments were performed in mineral medium with glucose 0.1 % (w/v) and each of the carboxylic acids volume ratio of 0.1 %, at pH=3.0 and 5.0. Fig. 1 shows the results obtained for growth in glucose-acetic acid medium, pH=5.0. In this case only one phase of growth was observed corresponding to the simultaneous disappearance of both glucose and acetic acid from the medium. At pH=3.0, the growth behaviour was identical to what was observed at pH=5.0 but with a lower specific growth rate and a more delayed lag phase. Attempts were made to clarify if, under these growth conditions, both acetic acid and glucose were used as carbon and energy sources by *Y. lipolytica*. For that purpose, the experimental biomass yields in media with glucose and/or acetic acid were estimated at pH=3.0 and 5.0. Results are shown in Table 2.

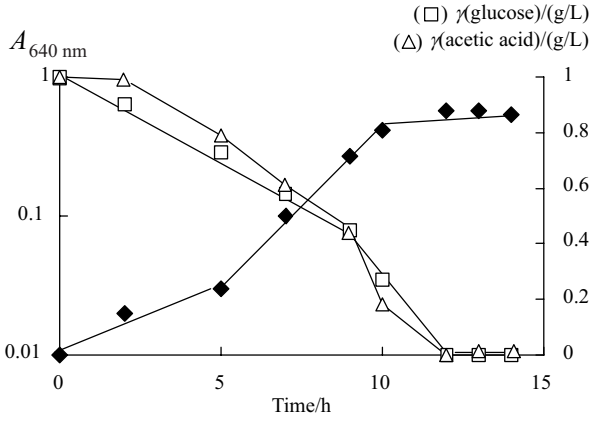

Fig.1. Growth of the yeast *Yarrowia lipolytica* ISA 1834 in mineral medium with 0.1 % (w/v) glucose and 0.1 % (w/v) acetic acid, at 26 °C and pH=3.0

Table 2. Biomass yields of *Y. lipolytica* ISA 1834 ($Y_{S/X}$, g dry wt. (g^{-1} carbon)) in simple and mixed media with 0.1 % (w/v) glucose and 0.1 % (w/v) acetic acid at pH= 3.0 and 5.0

Substrates	$Y_{S/X}$	
	pH=3.0	pH=5.0
Glucose	0.707 ± 0.053	1.047 ± 0.040
Acetic acid	0.370 ± 0.011	0.774 ± 0.004
Acetic acid and glucose	1.238 ± 0.030	1.045 ± 0.030

According to Jong-Gubbels *et al. (9)*, if the simultaneous use of two substrates does not affect growth efficiency, biomass yield for that mixed medium can be calculated from the following equation:

$$Y_{SX}=fY_{S1X} + (1-f)Y_{S2X} \qquad /3/$$

where Y_{SX} represents total biomass yield in a mixed medium and Y_{S1X} and Y_{S2X} represent the biomass yields for substrates *s1* and *s2*, respectively. Parameter *f* stands for the *s1* ponderal fraction of utilisation in relation to the total.

By applying equation /3/ to growth in mixed medium with glucose and acetic acid the following theoretical values for biomass yields could be estimated, at pH=3.0 and 5.0, respectively: 0.847 and 1.049 g dry wt. (g^{-1} carbon). Comparing these values with the experimental ones presented in Table 2, at pH=3.0, it can be seen that both theoretical and experimental values obtained for biomass yields in mixed medium (Y_{SX}) are identical (at pH=5.0 the values are about the same). Therefore, both substrates seem to be used simultaneously as carbon and energy sources.

Regarding the utilization of the other weak carboxylic acids by the yeast in the presence of glucose, it was observed that succinic, propionic and malic acids consumption adhered to the pattern described above for glucose-acetic acid medium under the same experimental conditions. However, in media with glucose and citric acid or lactic acid, growth was biphasic. The first phase of exponential growth corresponded to glucose consumption and the acids were only used after exhaustion of glucose from the medium (not shown). These results suggested that in this latter case and contrary to the other acids, the utilization of the citric/lactic acids is subject to glucose repression.

Effects of acetic acid and other weak carboxylic acids on growth

The effects of acetic, propionic, butyric and sorbic acids on glucose-grown cells of *Y. lipolytica* ISA 1834 were studied at pH=3.0, and at 26 °C. In these experimental conditions the presence of acetic, propionic, butyric and sorbic acids in the extracellular medium decreased the specific growth rates. For each acid the inhibition kinetics obeyed an exponential relation, in the presence of acid concentrations above values corresponding to the respective minimum inhibitory concentrations. Fig. 2 shows representative results of these experiments. The values of the exponential inhibition constant (k_i), the minimum inhibitory concentration (X_{min}) as well as the values necessary to reduce the specific growth rate by 50 % ($C^{inhib}_{50\,\%}$) allowed to express acid toxicity, being characteristic for each of the acids (Table 3). The analysis of the results shows that the acid having the highest toxic effects was sorbic acid, followed by butyric, propionic, and acetic acids.

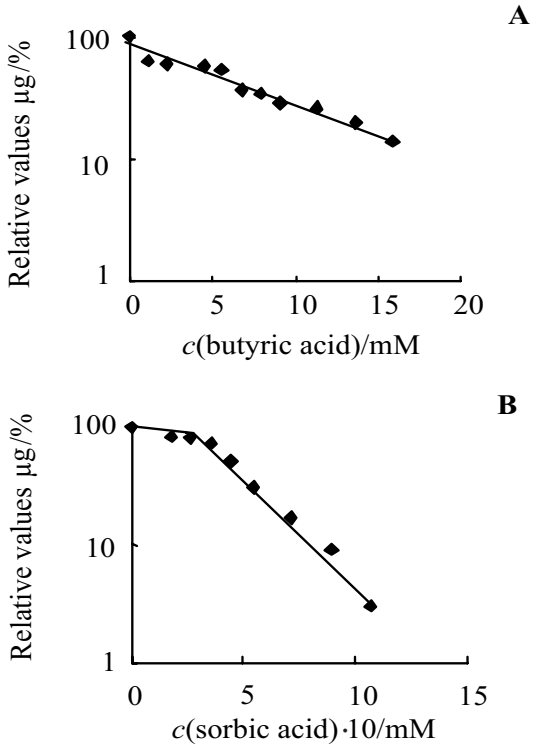

Fig. 2. Variation of the specific growth rate of *Y. lipolytica* ISA 1834, at 26 °C and pH=3.0 with acid concentrations (**A**-butyric acid, **B**-sorbic acid)

Table 3. Values for the exponential inhibition constants (k_i), acid concentrations necessary to reduce the specific growth rate by 50 % ($C^{inhib}_{50\,\%}$) and minimum inhibitory concentrations (x_{min}) for the different carboxylic acids tested at pH=3.0 and 26 °C

Acids	$\frac{x_{min}}{mol/L}$	$\frac{C^{inhib}_{50\,\%}}{mol/L}$	$\frac{k_i}{L/mol}$
Acetic	$9.99 \cdot 10^{-3}$	$13.32 \cdot 10^{-3}$	38.09 194.11
Propionic	$2.70 \cdot 10^{-3}$	$6.75 \cdot 10^{-3}$	117.92
Butyric	$1.13 \cdot 10^{-3}$	$5.57 \cdot 10^{-3}$	104.69
Sorbic	$3.57 \cdot 10^{-4}$	$4.45 \cdot 10^{-4}$	862.68 3378.63

Effects of weak carboxylic acids on kinetics of death at constant temperature

Following the results presented in the previous section and using glucose-grown cells, the effects of the acids on loss of cell viability were also investigated. The specific death rates (k_d) for acetic, propionic, butyric and sorbic acids (pH=3.0) were determined at constant temperature (26 °C), and the results depicted in Fig. 3 show how k_d typically depends on the concentration of the acids. For each of the acids the effect increased with its concentration in the external medium and k_d values were an exponential function of the acid concentration according to the equation:

$$\ln k_d^X = k_d^{Xm} + k_e (X - X_m) \qquad /4/$$

where k_d^X and k_d^{Xm} are the specific death rates in the presence of X and X_m acid concentration, X_m being the minimum concentration above which the enhancement of death was measurable under the experimental conditions used and k_e being the exponential enhancement death constant characteristic for each acid. The values of k_e (L/mol) calculated for each acid according to equation /4/ are as follows: acetic acid, 2.29; propionic acid, 3.89; butyric acid, 17.49; sorbic acid, 45.58. The toxic ef-

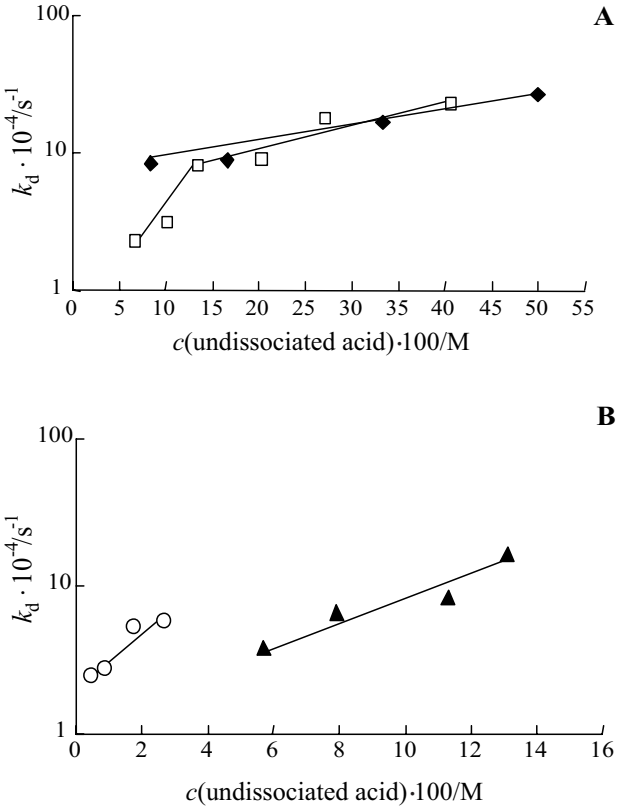

Fig. 3. Dependence of the specific death rates (k_d) at 26 °C and pH=3.0 on the concentration of the extracellular carboxylic acid: (◆) acetic acid; (□) propionic acid; (▲) butyric acid; (○) sorbic acid

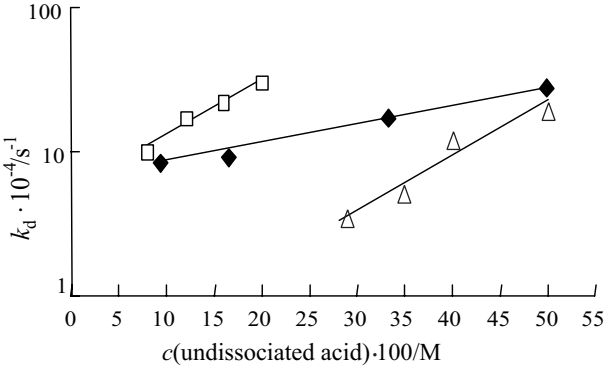

Fig. 4. Comparision of the effects of acetic acid on the cellular death (kd) of (◆) *Y. lipolytica* (present work) with those published for (❑) *Sacch. cerevisiae* (*10*) and (Δ) *Z. bailii* (11)

fects induced by the acids were thus higher for sorbic and butyric acids.

Conclusions

Our studies concerning the utilisation and toxicity of weak acids in *Y. lipolytica* ISA1834 indicated that the yeast revealed capacity to utilise, as the sole carbon and energy source, most carboxylic acids tested and often used as preservatives in the food industry. Furthermore, the acids studied, with the exception of citric and lactic, were utilised in the presence of glucose by *Y. lipolytica* ISA1834. This strain exhibited a regulation pattern by glucose which is unusual in the majority of yeast species but similar to that found in *Z. bailii*, one of the most dangerous food spoilage yeasts.

Although the acids could be used by the yeast, alone or in mixture with glucose, at the above specified concentrations they may be toxic either in terms of cell growth or viability. In both cases the relative toxicity, expressed by the respective increase or decrease of exponential constants of the acids was as follows: sorbic > butiric > propionic > acetic acid.

With respect to loss of cell viability, under isothermic conditions, the kinetic profiles of death induced by the acids were not very different from the ones described for *Sacch. cerevisiae* and *Z. bailii (10,11*). Fig. 4 exemplifies, in these three yeast species, the kinetics of cell death induced by acetic acid. From this figure, if both the exponential enhancement death constant and the acid concentration above which the enhancement effects begin to be measurables are taken into account it can be concluded that the strain ISA1834 of *Y. lipolytica* is qualitatively more resistant to the presence of acetic acid than *Sacch. cerevisiae.* Furthermore, the response pattern to acidic media exhibited by *Y. lipolytica* seems to be closer to *Z. bailii* than to *Sacch. cerevisiae*. This is certainly associated with the capacity, shown in this paper, of that species for using most of the acids including acetic acid in the presence of glucose like it has been described for *Z. bailii* in opposition to *Sacch. cerevisiae (12,13)*. Further studies will be needed to deeply understand such behaviour of *Y. lipolytica*, namely, how the transport of the acids across the plasma membrane and its intracellular metabolism could be involved.

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Utjecaj octene i drugih slabih karboksilnih kiselina na rast i preživljavanje kvasca *Yarrowia lipolytica*

Sažetak

Kvasac *Yarrowia lipolytica* koristio je octenu, mliječnu, propionsku, jabučnu, jantarnu, limunsku i oleinsku kiselinu kao jedini izvor ugljika i energije, što najčešće nije ovisilo o pH-vrijednosti podloge. Kada je kvasac uzgajan u miješanoj podlozi s 1 % glukoze i 0,1 % octene, jantarne, propionske ili jabučne kiseline, pri pH=3,0 ili 5,0, kiselina je korištena isto-

dobno s glukozom, Diauksični rast opažen je pri rastu na glukozi i limunskoj ili mliječnoj kiselini, tako da se kiselina koristila tek nakon nestanka glukoze iz podloge. Ovi rezultati pokazuju da uporaba tih kiselina ovisi o stupnju represije glukozom. Prisutnost octene kiseline u podlozi s glukozom pri 26 °C i pH=3, snizuje specifičnu brzinu rasta kvasca. Propionska, maslačna i sorbinska kiselina također su inhibitorski djelovale pri rastu kvasca na glukozi. Ispitan je i utjecaj octene, propionske, maslačne i sorbinske kiseline na kinetiku preživljavanja stanica koje su rasle na glukozi. Za svaki od tih spojeva, pri izotermnim uvjetima, ugibanje stanica bilo je eksponencijalno stimulirano s povećanjem ekstracelularne koncentracije kiseline, a toksično djelovanje inducirano kiselinama bilo je jače izraženo sa sorbinskom i maslačnom kiselinom. Izgleda da je taj soj kvalitativno otporniji prema octenoj kiselini od *Saccharomyces cerevisiae*, a srodniji je kvascu *Zygosaccharomyces bailli*, jednom od najvažnijih uzročnika kvarenja hrane.