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Measurement of alcohol acetyltransferase and ester hydrolase activities in yeast extracts

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Abstract

A method has been developed for measurement in vitro of the simultaneous activities alcohol acetyltransferase (AATase) and ester hydrolase (EHase) in yeast extracts taking into account the possibility of interaction between the two opposite activities or the rapid inactivation of the AATase activity. A mathematical model, including as parameters a first order kinetic constant corresponding to the EHase activity and the inactivation constant of AATase, is proposed for the evaluation of AATase activity. To determine ester concentrations, the Headspace-SPME-GC technique has been used. The method has been successfully applied to three yeast strains belonging to the species *Pichia anomala, Pichia heedii* and *Saccharomyces cerevisiae* and the corresponding parameters for AATase and EHase have been calculated.

Keywords: Alcohol acetyltransferase; Ester hydrolase; Headspace-SPME

1. Introduction

Esters, which are produced during food fermentation processes, have long been recognized as important flavor determinants in wine, beer and other alcoholic beverages [1,2]. In *Saccharomyces cerevisiae* and other yeasts, the synthesis of esters occurs via an intracellular process catalyzed by an alcohol acyltransferase (EC 2.3.1) using energy provided by the thioester linkages of acyl coenzyme A compounds [3]. With respect to acetate esters, these are believed to be synthesized from alcohols and acetyl-CoA by the alcohol acetyltransferase (AATase, EC 2.3.1.84), a membrane associated enzyme firstly purified by gel filtration and chromatography on DEAE [4]. Later, three alcohol acetyltransferases which catalyze the synthesis of ethyl acetate, ethyl n-hexanoate and isoamyl acetate were resolved by affinity chromatography from a cell extract of *Saccharomyces cerevisiae* [5]. In contrast, the relevance attributed to the ester synthase (ESase, a reverse esterase) as an ester-synthesizing activity is rather limited: two esters, ethyl caprylate and ethyl acetate have been reported as been produced respectively by breadmaking [6] and beer [7] yeast strains of *S. cerevisiae* from ethanol and the respective acids. Yeasts are also able to hydrolyse esters via various intracellular ester-hydrolases (EHase) [8]. Thus, the production of esters is widely believed to be dependent on the balance of ester synthesis by AATase and ester hydrolysis by EHase [9,10]. Hence it is of considerable interest to have good methods for the determination of such activities in cellular extracts.

The methods described in the literature to measure AATase activities are based on the usual assumption of proportionality between the amount of product formed and incubation time without taking into account that activities acting antagonistically, AATase and EHase, are present simultaneously. It is common practice for a reaction mixture consisting of an enzyme source, alcohol and acetyl-CoA in buffer phosphate pH 7.5 and some enzymatic protective agents (glycerol, DTT, PMSF, etc.) to be incubated for 1 h and the ester formed taken as a measure of the AATase activity [3,4,11–13]. Malcorps and Dufour [5] used similar methods to measure activities during the purification of an AATase from *S. cerevisiae* and Inoue et al. [9] applied the

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procedures reported by Yoshioka and Hashimoto [4] to quantify the distribution of AATase and esterase activities (either synthesizing or hydrolyzing) between different cell fractions in *Hansenula mrakii* (syn. *Williopsis saturnus*). Though no comment is made on the possible interference between the two opposite activities in any of the above references dealing with extracts from yeasts, in cell free extracts from fruits such as banana [14] and strawberry [15], the addition of pectinase to the extraction buffer has been reported to eliminate the esterase activity allowing the AATase to be measured.

In practice, any methodology aiming to measure AATase activities in vitro must take two facts into account: i) cell disintegration yields extracts in which the two opposite activities are almost always present and ii) as reported previously [16], in contrast to ester hydrolases, AATase is a very labile enzyme loosing as much as 50% activity in periods as short as 30 min. Hence AATase activity determination, based on the assumption of proportionality between product (or reagent) concentration and time, very frequently leads to false results even though measurements are carried out over the very short times demanded by the initial velocity technique. An approach to indirectly evaluate the two antagonistic activities is to use a mathematical model including rate expressions for each of the simultaneous reactions. The best fit of the data obtained from a 'double opposite activity assay' to this model should yield the AATase activities.

The aim of the present study was to develop a methodology for measuring AATase activities in vitro when esterhydrolase activities are also present in the cellular extract. To this end, two kinds of assays were carried out to obtain the kinetic data required: reactions performed in the absence of acetyl-CoA (i.e. no ester synthesis) yielding a progress curve strictly corresponding to EHase activities, and reactions in the presence of acetyl-CoA yielding a progress curve corresponding to the effect of simultaneous AATase and EHase activities. By introducing the appropriate parameter describing the EHase activity, obtained from the acetyl-CoA-free assay, this model permits the evaluation of AATase activity and its inactivation constant k_i.

2. Mathematical model

In a reaction mixture containing substrates (isoamyl alcohol and acetyl-CoA) and a cellular extract in which the activities AATase and EHase are present, the reactions are:

$$Alcohol + ACoA \xrightarrow{AATase} Ester$$

$$Alcohol + Acetate \xleftarrow[EHase]{} Ester$$

Since the formation of the enzyme-substrate complex is a very rapid reaction and the reactions of ester synthesis and hydrolysis are both irreversible under the assay conditions employed, a material balance leads to the following expression:

$$\left(\frac{dE}{dt}\right)_{overall} = \left(\frac{dE}{dt}\right)_{AATase} - \left(\frac{dE}{dt}\right)_{EHase} \quad (1)$$

where E is the ester concentration and t is time. Bearing in mind that $(dE/dt)_{AATase}$ is the AATase activity of interest and that, as will be shown below, hydrolase activity fits first order kinetics, equation (1) can be written:

$$\left(\frac{dE}{dt}\right)_{overall} = AATase - kE \quad (2)$$

where k is the rate constant for the ester hydrolysis. However, AATase is a very labile enzyme [9] a fact which can be duly accounted for by introducing the corresponding exponential factor [17] into the AATase term:

$$\left(\frac{dE}{dt}\right)_{overall} = (AATase)_0 e^{-k_i t} - kE \quad (3)$$

where k_i is the inactivation constant for the AATase activity. Integration between 0 and E and between 0 and t for ester concentration and time respectively, leads to the following expression

$$E = \left(\frac{A_0}{k - k_i}\right)(e^{-k_i t} - e^{-kt}) \quad (4)$$

which describes the variation of the ester concentration during incubation of the reaction mixture, commonly known as the progress curve.

Analysis of Equation (4) shows that the progress curves can present different shapes depending on the relative values of the constants k_i and k. If either of the constants is zero, the progress curve shows no maximum. If the two constants have finite values, the progress curve presents a maximum whose position can be calculated by deriving equation (4) and equaling it to zero to obtain:

$$t_m = \frac{\ln\left(\frac{k}{k_i}\right)}{k - k_i} \quad (5)$$

where t_m, the time at which the ester concentration reaches its maximum, depends only on the relative values of the constants k and k_i.

If equation (4) fits the experimental data obtained from incubation of a reaction mixture containing the AATase substrates, it is evident that from knowledge of the hydrolase rate constant (k) (which can be determined by a straightforward parallel assay), this model can yield values for AATase activity and its inactivation constant k_i. The possibility of simultaneous evaluation of the three parameters without any previous determination of k (the EHase rate constant) was discarded because of the mathematical characteristics of equation (4).

3. Materials and methods

3.1. Chemicals

Acetyl-CoA (trilithium salt) was purchased from Boehringer-Mannheim (Mannheim, Germany). Isoamyl alcohol, isoamyl acetate and dithiothreitol (DTT) were obtained from Fluka Chemie AG, Buchs (Switzerland). Phenyl methyl sulphonyl fluoride (PMSF) was purchased from Sigma (St. Louis, Mo, USA). Potassium phosphates and glycerol were of analytical grade.

3.2. Micro-organisms and cultures

Saccharomyces cerevisiae CECT 1894 (T_{73}), *Pichia anomala* CECT 10590 and *Pichia heedii* CECT 11452 were obtained from the Spanish Type Culture Collection (CECT, Burjassot, Spain). Nutrient medium (pH 6) for all three yeasts contained 4 *g* glucose, 0.5 *g* yeast extract, 0.5 *g* malt extract, 0.5 *g* peptone and 0.1 *g* $MgSO_4 \cdot 7H_2O$ in 100 ml of distilled water. Cultures consisted of 0.8 L of medium in 2L Erlenmeyer flasks.

S. cerevisiae was grown without shaking while *P. anomala* and *P. heedii* were grown in an orbital shaker at 200 rpm [12,18]. In all cases yeast cells were grown at 30°C up to late log phase taking as reference optical density at 600 nm.

3.3. Buffer solutions

Disruption buffer: 10 mM potassium phosphate, pH 7.5, containing 10% (w/v) glycerol, 0.8 mM $MgCl_2$, 5 mM DTT and 100 mM PMSF in 1% (v/v) dimethyl sulfoxide solution. **Glycerol buffer**: 50 mM potassium phosphate, pH 7.5, containing 10% (w/v) glycerol. **Acetate buffer**: 136 mM sodium acetate buffer, pH 5.0 containing 10% (w/v) glycerol.

3.4. Protein determination

Protein content was estimated by the Coomassie dye-binding assay [19] using bovine serum albumin fraction V (Boehringer-Mannheim) as standard.

3.5. Preparation of yeast cell extracts

Yeast cells (4–8 g) were collected by centrifugation at 4500× g for 5 min, washed twice with 0.85% NaCl, resuspended in ice-chilled disruption buffer and disrupted with 15 ml of zirconium beads (0.4–0.5 mm diameter) in a 30 ml volume Bead-Beater Cell Disrupter (Biospec Products, Bartlesville, OK, USA). Disruption was achieved by subjecting the cells to five bursts of 45 s with resting periods of 5 min. In all cases this treatment was carried out with the chamber (air-free) placed in ice. The resulting homogenate was centrifuged at 15000× g for 30 min at 4°C and the supernatant used as the cell extract under study.

3.6. Enzyme reactor

A 20 ml graduated Jena glass syringe provided with a stop-cock and containing a magnetic bar was used as the enzyme reactor. Reaction mixtures were prepared by pipetting into the syringe appropriate aliquots of glycerol buffer containing the substrate (alcohol or ester plus alcohol, depending on the assay), glycerol buffer containing acetyl-CoA (if required) and finally the cell extract, in that order. After replacing the plunger and removing any entrapped air, agitation was immediately started by putting the syringe on a magnetic stirrer. At scheduled times 1.5 ml samples of the reaction mixture were removed from the syringe and transferred to 9 ml vials containing a tiny magnetic stirrer and 60 μl of a saturated KSCN solution to stop enzyme activity and 0.3 g NaCl in order to enhance the amount of analyte (isoamyl acetate) available for subsequent headspace-SPME [20]. Vials were immediately closed with a Teflon lined rubber cap and placed on a magnetic stirrer to assure rapid mixing.

3.7. Enzyme assays

i) **Double antagonistic activity assays (AATase + EHase)**. To obtain the corresponding progress curve (due to the action of AATase and EHase activities), the starting reaction mixture in the reactor consisted of glycerol buffer containing isoamyl alcohol, glycerol buffer containing acetyl-CoA, and cell extract present in the volume ratio 1:0.1:0.4, the final volume being determined by the number of samples to be taken during the reaction. The final concentrations for isoamyl alcohol and acetyl-CoA were 46 mM and 0.80 mM, respectively [9]. During the incubation time (60–120 min), 8–10 samples of 1.5 ml were taken for ester quantification.

ii) **Single hydrolase activity assays (EHase)**. To study the kinetics governing the hydrolysis reaction, the starting reaction mixture consisted (per 1.5 ml of total volume) of 1.1 ml of glycerol buffer containing appropriate amounts of isoamyl acetate and isoamyl alcohol, and 0.4 ml of cell extract. The concentrations in the reaction mixture were 46 mM for isoamyl alcohol and from 5 to 10 mg/liter of isoamyl acetate. During the incubation time (40–60 min), 6–8 samples of 1.5 ml were taken and the residual ester analyzed.

iii) **Ester-synthase (ESase) assays.** Isoamyl acetate-synthesizing activity from the sustrates isoamyl alcohol and acetic acid was measured according to Fukuda et al. [10] with some modifications. The starting reaction mixture (1.5 ml) consisted of 1.1 ml of 136 mM acetate buffer containing the alcohol at

20 mM and 0.4 ml of cell extract. After 2 h the reaction was stopped and the vial closed as indicated above for further analysis.

3.8. Headspace-SPME and gas chromatography

The sample-containing vials were maintained at 25°C for 2 h to establish a liquid/gas equilibrium prior to SPME analysis [20]. An SPME fibre (Supelco, Sigma-Aldrich, Spain) coated with poly(dimethylsiloxane) of 1 cm in length and thickness 100 μm (bonded phase) was used for sampling isoamyl acetate. The fibre was inserted into the headspace and held for 7 min at 25°C during stirring of the liquid phase. The fibre was then inserted into the GC injector port at 220°C and exposed to helium flow for 4 min to desorb the analytes. A Hewlet-Packard HP5890 Series II gas chromatograph (Waldbronn, Germany) equipped with a Restek Corporation MXT-1 capillary column (length, 30 m; internal diameter, 0.28 mm; film thickness, 0.10 μm) and a flame ionization detector was used. The detector and oven temperatures were kept constant at 250°C and 90°C, respectively.

3.9. Statistical methods

Linear and non-linear regression analyses and graphics were performed with SigmaPlot for Windows version 4.01 (SPSS Inc., Chicago, IL, USA).

4. Results and discussion

4.1. Ester hydrolase

S. cerevisiae strain T_{73}, *P. anomala* and *P. heedii* cellular extracts were selected to demonstrate the applicability of the model for the in vitro determination of AATase activity in the presence of antagonistic hydrolase activities. Firstly, the rate constants for the EHase activities were measured in acetyl-CoA-free 'single hydrolase activity assays' with and without the addition of isoamyl alcohol to 46 mM (the same alcohol concentration as in the 'double antagonistic activity assays'). Data plotted as the natural logarithm of ester concentration vs. time (Figs. 1a, 2a and 3a) showed that i) hydrolysis of isoamyl acetate followed a first order kinetics (i.e. the slopes of the linear plots yield the corresponding rate constants), thus permitting the use of the first order kinetic expression in equation 2 (see section 2) and ii) the reaction was strongly inhibited by isoamyl alcohol. The possibility of inhibition by acetate, the other reaction product, was also studied. Results showed that at 1 mM, a concentration 10-fold greater than that attainable in our assays, inhibition was not observed.

Cellular extracts from *P. heedii* exhibited the greatest hydrolase activity. The rate constant for this reaction (the isoamyl alcohol-free assay) reached a value of 4.3 h^{-1} mg^{-1}, 28-fold higher than that exhibited by the *P. anomala* cellular extracts. No EHase activity was detected in the case of *S. cerevisiae*. When the hydrolysis reaction was carried out in the presence of 46 mM isoamyl alcohol, the rate constants decreased by 79% and 65% for *P. anomala* and *P. heedii* extracts, respectively.

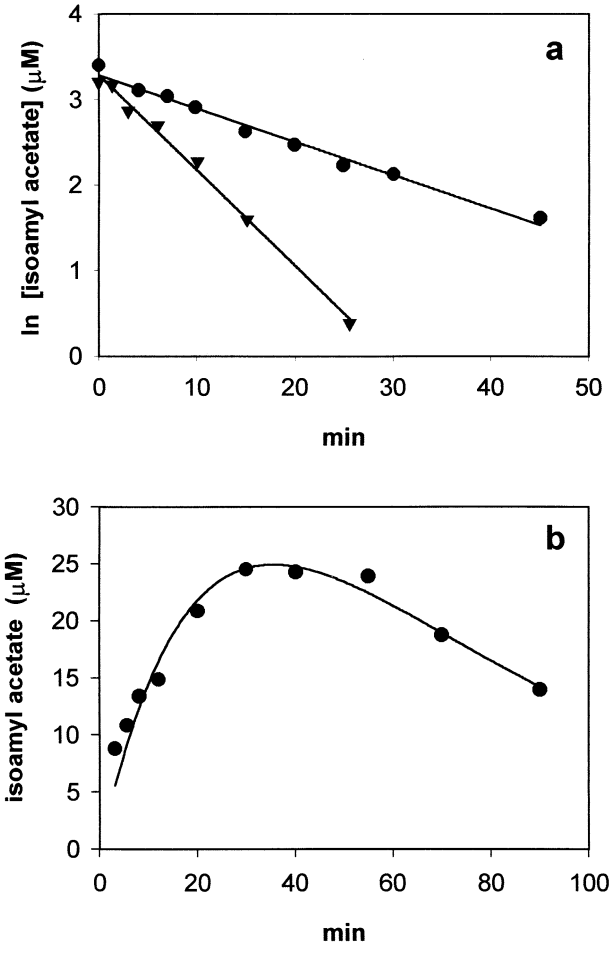

Fig. 1. Time course of isoamyl acetate concentration for the enzyme activity of a *P. heedii* cellular extract. a) Ester disappearance as a result of the hydrolase activity in the absence (▼) or in the presence (●) of 46 mM of isoamyl alcohol. b) Ester variation as a result of the simultaneous action of AATase and EHase.

The absence of EHase activity in the *S. cerevisiae* T_{73} strain is in contrast to other studies in *S. cerevisiae* [21] reporting significant values for isoamyl acetate hydrolyzing activity. This makes the T_{73} strain potentially advantageous in winemaking and other alcoholic beverage industries.

4.2. Alcohol acetyltransferase

Figs. 1b, 2b and 3b show plots of the data corresponding to the acetyl-CoA-added 'double antagonistic activity (AATase and EHase) assays'. From these data and the EHase rate constant values previously obtained, the best non-linear regression fit to the mathematical model yielded

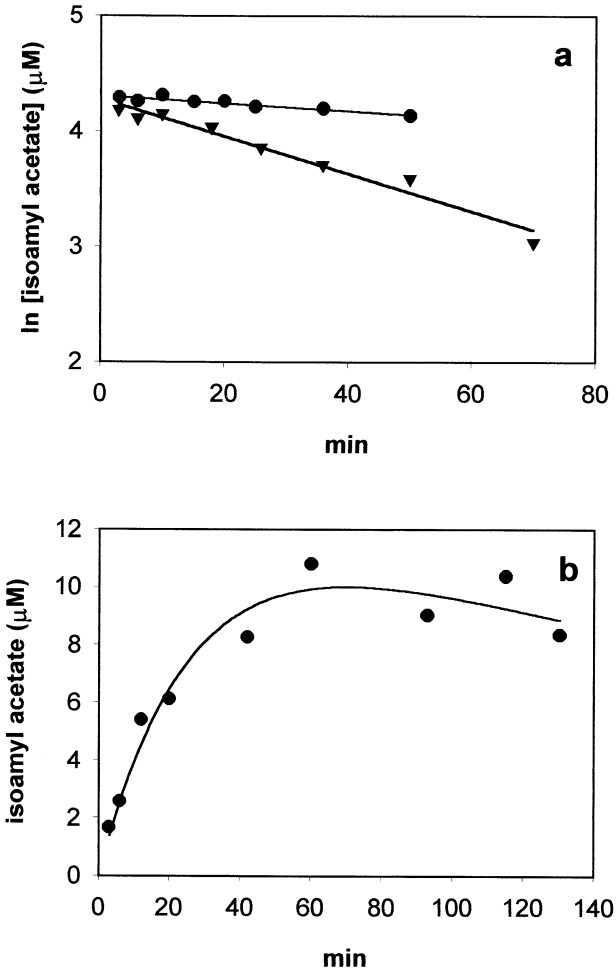

Fig. 2. Time course of isoamyl acetate concentration for the enzyme activity of a *P. anomala* cellular extract. a) Ester disappearance as a result of the hydrolase activity in the absence (▼) or in the presence (●) of 46 mM of isoamyl alcohol. b) Ester variation as a result of the simultaneous action of AATase and EHase.

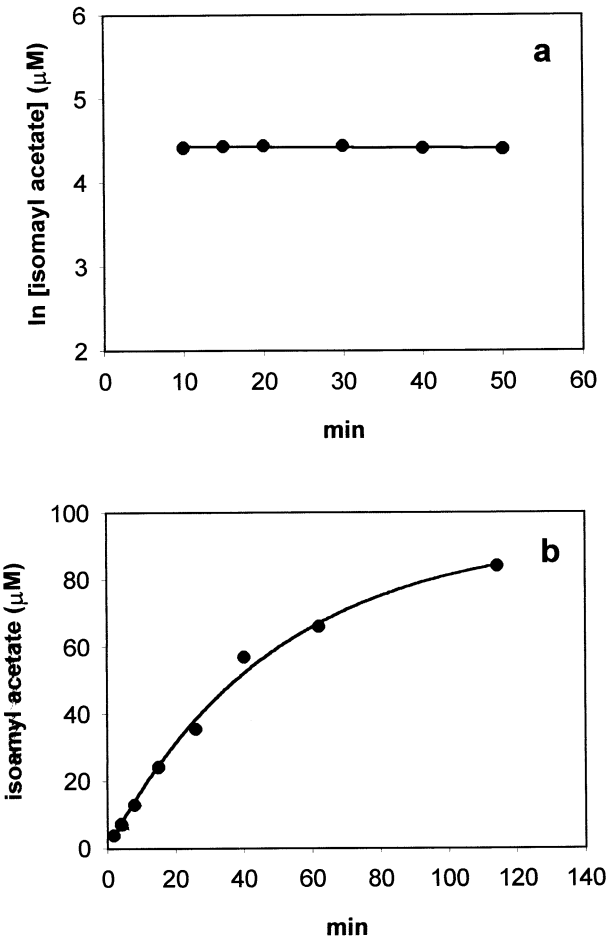

Fig. 3. Time course of isoamyl acetate concentration for the enzyme activity of a *S. cerevisiae* cellular extract. a) Ester disappearance as a result of the hydrolase activity in the presence of 46 mM of isoamyl alcohol. b) Ester variation as a result of the simultaneous action of AATase and EHase.

values for the AATase activity and k_i for each of the cellular extracts assayed (Table 1). The possible occurrence of ester synthase (ESase) activity in the cellular extracts was also studied. It is observed (Table 1) that ESase activities were negligible or not detectable as in the case of *S. cerevisiae.*

Cellular extracts of *P. heedii* exhibited the greatest AATase specific activity, twice and 17-fold those of *S. cerevisiae* T_{73} and *P. anomala*, respectively. The AATase inactivation constants (k_i) obtained (Table 1) clearly correspond to very short half-lives: 34.6, 16.5 and 32.5 min for the cellular extracts of *S. cerevisiae*, *P. anomala* and *P. heedii*, respectively. The half-life value for the *S. cerevisiae* T_{73} extract is in agreement with the observations of Minetoki [16] for the AATase from a sake yeast (55% loss of activity in 30 min at 20°C).

AATase activities cannot be accurately determined in vitro in conventional assays based on the proportionality between the amount of ester formed and reaction time because of the usual presence of accompanying hydrolytic activities and the rapid inactivation undergone by the AATase at assay temperatures. If, as a result, the reliability of performing rather long assays is questionable, then the measurement of initial rates by reducing the incubation time to a minimum (i.e. 1–2 min) also has to be questioned because of the very high hydrolytic activities often encountered and the rapid inactivation of AATase normally observed. We therefore propose the evaluation of the AATase activities present in cell extracts by analyzing the isoamyl acetate progress curves from reactions in which only AATase or both EHase and AATase are involved. This analysis is carried out using a tailored mathematical model that includes the specific kinetic characteristics of each of the simultaneous reactions. This methodology requires two kinds of data: those corresponding to the single ester-hydrolase reaction and those corresponding to both activities. The former permits the evaluation of the hydrolysis rate constant and the latter yields values that fit the integrated model (Section 2, equation 4) and the evaluation of the desired parameters. Since the physicochemical basis used to

Table 1
Summary of the enzymatic parameters obtained

Microorganism	Extract protein (mg/mL)[c]	EHase		AATase		ESase
		Specific rate constant (h^{-1} mg^{-1})[d]		Specific activity (μMh^{-1} mg^{-1})[d]	Inactivation constant (h^{-1})[d]	Specific activity (μMh^{-1} mg^{-1})[d]
		a	b			
S. cerevisiae	8.3 (0.15)	n.d.	n.d.	34.3 (1.8)	1.22 (0.11)	n.d.
P. anomala	16.0 (0.23)	0.15 (0.01)	0.032 (0.005)	4.50 (0.5)	2.26 (0.31)	0.57 (0.03)
P. heedii	3.9 (0.12)	4.29 (0.15)	1.50 (0.06)	75.0 (0.39)	1.18 (0.11)	0.18 (0.01)

[a] No isoamyl alcohol added.
[b] 46 mM isoamyl alcohol.
[c] Mean of three replications and standard error.
[d] Estimated values and standard error.
n.d.: not detected.

develop this model may be valid in other systems in which ester-synthesizing and ester-hydrolyzing activities coexist, broader application of this methodology for the determination of other alcohol acetyltransferase activities seems feasible.

It should be stressed that a multiplicity of AATase and EHase activities are generally present in yeast extracts. Two distinct AATase activities for isoamyl alcohol and other alcohols have been studied in *S. cerevisiae* (encoded by *ATF1* and *ATF2* genes) which exhibit different mechanisms of regulation and, probably, different physiological roles [22]. The existence of at least one additional enzyme with ethyl alcohol acetyltransferase activity has also been suggested [5]. Thus, the model-calculated values for these activities in our cellular extracts are actually values resulting from the contribution of different individual enzymes.

AATase activities and stabilities measured in cell extracts by this methodology are potentially influenced by many factors such as the method used to obtain the extract, the processing of the latter and the nature and concentration of the protective agents used. Nevertheless, the procedure proposed offers the potential to establish comparative measurements when screening for a valuable enzymatic activity has to be made in microorganisms or cells, and/or to carry out physiological or metabolic studies related to AATase activities.

5. Conclusions

A new method has been developed to evaluate the ester-synthesizing activity of alcohol acetyltransferases in microbial cellular extracts which exhibit simultaneous ester-hydrolyzing activities. The use of the headspace-SPME-GC technique to determine the evolution of ester concentration (the progress curve) resulting from both activities has proved to be straightforward and sensitive, providing the data required for mathematical modeling of the alcohol acetyltransferase activity. The results obtained showed that the short half-lives of the AATases and the occurrence of significant hydrolase activities, are important factors to be borne in mind in order to obtain reliable measurements of ester-synthesizing activities in yeast extracts.

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