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Spectrophotometric assay of α-acetolactate decarboxylase

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Abstract

The usual colorimetric method to determine α-acetolactate decarboxylase activity is long, is of poor accuracy (35%), and does not suit brewing fermentation conditions. By simple modification in the procedure, it was possible to improve the measurement to an accuracy of 11%, to allow more samples to be analyzed in a shorter period, and to reduce the cost per analysis. The recommended changes are easy to set up: the temperature of work should be decreased, the number of buffers should be reduced from two to one, and the chosen buffer should be maleate instead of 4-morpholineethanesulfonic acid. We also mention a way to measure both diacetyl and acetoin contents in the same set of measurement, based on absorbance readings of the reaction medium at two different times. These improvements are in accordance with brewing fermentation conditions.

Keywords: α-Acetolactate decarboxylase; Spectrophotometric determination; Activity

1. Introduction

During beer production process, diacetyl is formed by spontaneous but slow oxidative decarboxylation of α-acetolactate, a product of yeast metabolism. Diacetyl at very low level gives a strong off-flavor to beer [1,2]. The conventional way of making beer requires a maturation period of 2–12 weeks, allowing diacetyl to be formed from α-acetolactate and then to be consumed by the yeast [3]. In 1982, Godtfredsen and Ottesen proposed to use α-acetolactate decarboxylase to accelerate the brewing process [4], as it shunts the formation of diacetyl (Fig. 1).

α-Acetolactate decarboxylase (EC 4.1.1.5, also called (*S*)-hydroxy-2-methyl-2 3-oxobutanoate carboxy-lyase) is an enzyme produced by a modified strain of *Bacillus subtilis*. α-Acetolactate decarboxylase commonly is assayed according to an analytical procedure described by Novo-Nordisk (Copenhagen, Denmark) [5]. This procedure consists of measuring the enzymatically formed acetoin from α-acetolactate by colorimetric assay [6]. Although satisfactory for enzyme activity calibration, this method is not suitable to evaluate the impact of α-acetolactate decarboxylase during fermentation, essentially because it is time-consuming.

During beer production, the medium contains α-acetolactate, diacetyl, and acetoin (Fig. 1). These compounds are chemically quite similar, so they are difficult to analyze separately. To evaluate the impact of α-acetolactate decarboxylase during fermentation, a large set of samples (up to 50) must be analyzed in a short time period (a few hours). With respect to the application field, determination procedure has to be carried out in conditions close to the brewing process. Moreover, the present study is part of a larger research project dealing with encapsulation of α-acetolactate decarboxylase [7]. Consequently, the assay has to be possible with immobilized enzyme. Describing in detail the usual procedure of α-acetolactate decarboxylase is not the aim of this paper, because it has already been published. But, some remarks lead us to study the determination procedure itself:

- Usual procedure consists of an enzymatic reaction, followed by a colorimetric measurement of the acetoin formed. The enzymatic reaction is conducted at 30°C in a medium containing synthetic activators (nonionic detergent) and in the absence of stirring. These conditions are far from brewing conditions: primary fermentation occurs at 8–15°C, CO_2 bubbling induces a strong mixing, and there are no detergents in beer.
- Enzymatic reaction of the usual procedure is not

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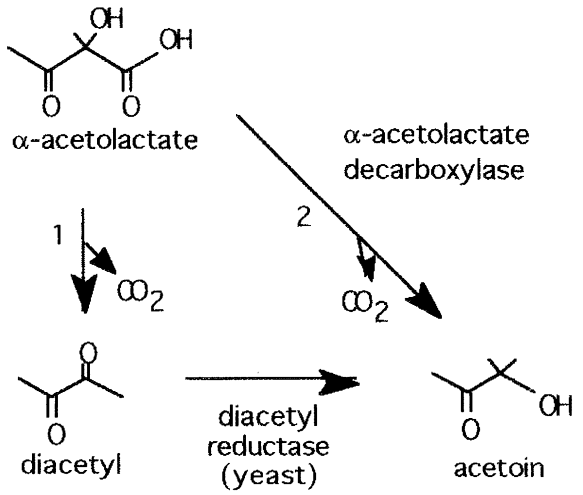

Fig. 1. Reaction catalyzed by α-acetolactate decarboxylase. 1: oxidative decarboxylation; 2: enzymatic nonoxidative decarboxylation.

properly stopped, so colorimetric assay must be done *instantaneously* to obtain accurate results.

- Usual procedure requires two different buffers, one for α-acetolactate (substrate) and another one for α-acetolactate decarboxylase (enzyme). Consequently, several blanks are needed, limiting the number of samples analyzed per unit of time.
- During the summertime, the number of sets of measurements out of the range strongly increases: up to one out of three. In the case of α-acetolactate decarboxylase activity determination, the major reason for a set of measurements to be out of the range is a too strong degradation of α-acetolactate (substrate of reaction) leading to a narrower range.

The aim of this study is to set an α-acetolactate decarboxylase assay more appropriate for the application, which means to get a quicker and more accurate test. Moreover, the cost per analysis has been reduced.

2. Materials and methods

2.1. Chemicals

Ethyl 2-acetoxy-2-methylacetoacetate, α-naphtol, and creatine were purchased from Aldrich (Saint Quentin Fallavier, France), acetoin from Fluka (Saint Quentin Fallavier, France), morpholinethane sulfonic acid (MES) from Sigma (Saint Quentin Fallavier, France), Brij 35, diacetyl, and sulphuric acid from Merck (Nogent-sur-Marne, France), and maleic acid, sodium sulphite, and cobalt sulphate from Prolabo (Paris, France). α-Acetolactate decarboxylase was used either purified (Fluka) or as an enzymatic preparation called Maturex® L (Novo-Nordisk).

2.2. Materials

Automatic titrations were performed on a Titroline α (Schott, Hofheim, Germany), and spectrophotometric measurements on a Shimadzu UV-120-02 (Kyoto, Japan).

2.3. Synthesis of α-acetolactate (final procedure adopted)

Ethyl 2-acetoxy-2-methylacetoacetate (diester of α-acetolactate) is transformed into α-acetolactate, ethanol, and acetate by saponification. One hundred microliters of ethyl 2-acetoxy-2-methylacetoacetate is added to 10 ml of 180 mM sodium hydroxide at 10°C for 20 min in a stirred closed vessel. Then, 20 ml of 100 mM maleate buffer pH 6.0 ± 0.05 and 3.5 ml of 100 mM maleic acid are added. The obtained solution corresponds to 15.4 mM α-acetolactate in maleate buffer at pH 6.0. It has to be prepared just before use and to be used within the following 3 h. Efficiency of the procedure is checked by α-acetolactate determination.

2.4. Colorimetric determination of acetoin and diacetyl

The colorimetric determination of acetoin concentration (more precisely the sum of acetoin and diacetyl concentration; see section 3 for discrimination between acetoin and diacetyl) is carried out by colorimetric method [5]. Acetoin and diacetyl react with guanido groups of creatine in alkaline medium to give a pink color (Voges and Proskauer reaction [6]. A color-reagent is prepared by mixing an equal volume of 0.2% creatine solution in distilled water and 1% α-naphtol freshly dissolved in 2.5 M sodium hydroxide. This color-reagent must be protected from light and used within the following 4 h. Color-reagent (2.5 ml) is introduced into spectrophotometric disposable cuvettes. Two hundred microliters of the sample medium is added, mixed, and left at 20°C. After exactly 40 min of color development, absorbances at 525 nm are read. Blanks are made with 200 μl of buffer in the same conditions. Standards of acetoin (200 μl from 0 to 800 μM in water) are analyzed at the beginning and at the end of each set of measurement. The mean values between the two standards are used for the calibration (correlation coefficient >0.99).

2.5. Determination of α-acetolactate concentration

One volume of α-acetolactate-containing sample is added to 1 vol of 2% sulphuric acid. The mixture is brought to 60°C for 15 min in a tightly closed tube. Samples are brought back to room temperature under running water, analyzed for diacetyl and acetoin total concentration (see above), and converted into initial acetolactate concentration. Saponification of ethyl 2-acetoxy-2-methylacetoacetate into α-acetolactate also was conducted under pH-stat [8], and consumption of sodium hydroxide was followed as an estimation of the saponification degree.

2.6. α-Acetolactate stability evaluation

Effect of oxygen and temperature was tested on α-acetolactate decarboxylation. The sum of acetoin and diacetyl formed by spontaneous decarboxylation of α-acetolactate was studied as a function of time. The different conditions tested were:

1. All solutions kept under nitrogen.
2. All solutions containing an oxygen sink, namely 1 mM sodium sulphite and 1 mM cobalt sulphate [9].
3. Blank (no particular care).
4. Each condition being repeated for 0°C, 10°C, and 25°C.

2.7. Assay of α-acetolactate decarboxylase activity (final procedure adopted)

The assay is performed at 10°C in a controlled temperature room. Enzyme solutions are prepared in 100 mM maleate buffer, pH 6.0. Activity can be evaluated in the range 0–200 μg/ml for purified enzyme and 0–6 mg/ml for Maturex L.

Two hundred microliters of α-acetolactate decarboxylase solution are poured into capped tubes disposed on a shaking device (IKA Labortechnics KS 125, Staufen, Germany) at 10°C. Reaction is allowed to occur by addition of 2 ml of 15 mM α-acetolactate solution, under stirring. After 20 min, the enzymatic reaction is stopped by addition of equivalent volume (2.2 ml) of 2.5 M sodium hydroxide and vigorous mixing. Acetoin production is determined by the method described above, and activity is expressed as molar concentration of acetoin formed per minute.

3. Results and discussion

3.1. Synthesis of α-acetolactate

Acetolactate is an unstable β-keto-acid. It cannot be stocked and has to be synthesized just before use. Synthesis of α-acetolactate is achieved by saponification of ethyl 2-acetoxy-2-methylacetoacetate in alkaline medium. In the usual procedure [5], synthesis of α-acetolactate is long because it requires volume and pH adjustments. It leads to a significant degradation of α-acetolactate into diacetyl and acetoin before its use, allowing low accuracy in the α-acetolactate decarboxylase activity determination.

To define the best conditions of α-acetolactate synthesis, saponification of ethyl 2-acetoxy-2-methylacetoacetate into α-acetolactate was conducted under pH-stat [8]. Two examples are given in Fig. 2 for pH 11.5 and 13.2. A 95% yield of synthesis was achieved in both cases. Saponification was faster when operating at pH 13.2 (molar ratio NaOH/ethyl 2-acetoxy-2-methylacetoacetate: 3.4), this initial sodium hydroxide level being then kept for further experiments.

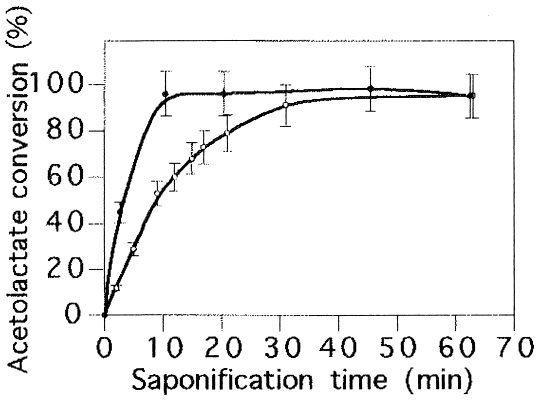

Fig. 2. Conversion of ethyl 2-acetoxy-2-methylacetoacetate into α-acetolactate at 20°C. ○, initial pH 11.5; ●, initial pH 13.2.

After saponification, α-acetolactate solution had to be brought to pH 6 and diluted quickly in buffer to avoid substrate degradation. Titration of freshly prepared alkaline solution of α-acetolactate with maleic acid is shown in Fig. 3. From this curve, it appears that 176 μmol (±6, based on four experiments) is needed to bring to pH 6.0 this saponification medium containing 885 μmol of sodium hydroxide and 258 μmol of α-acetolactate, ethanol, and acetate. To simplify and accelerate the preparation of α-acetolactate solution, we propose to follow the procedure described in Section 2. The usual whole procedure for preparing α-acetolactate solution requires ≈50 min whereas this present procedure is performed in <15 min. The resulting blank absorbance by colorimetric method is reduced from 0.4–0.5 to 0.25. Because the calibration curve is linear up to absorbance of 1.2, this decrease in the blank value allows the range to be widened by 35%.

3.2. Colorimetric acetoin determination

As shown in Fig. 1, α-acetolactate decarboxylase catalyzes the conversion of α-acetolactate into acetoin. Assay of the enzyme can be followed by measurement of α-aceto-

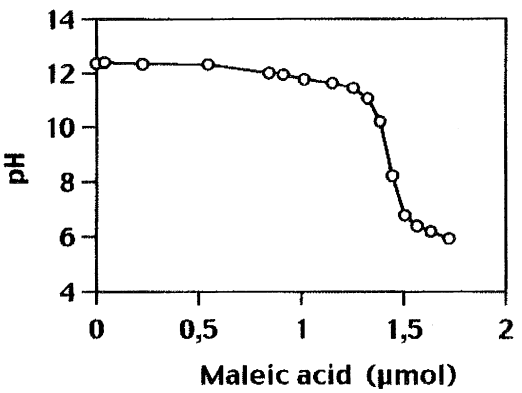

Fig. 3. Titration curve of saponification medium containing α-acetolactate.

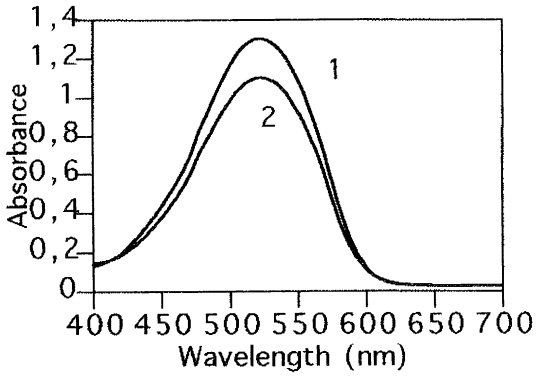

Fig. 4. Spectra of colorimetric reaction medium by Westerfeld method (10 min of color development, 20°C). 1: 280 μmol diacetyl; 2: 272 μmol acetoin.

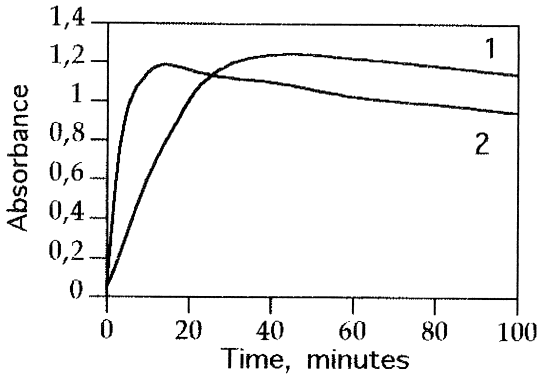

Fig. 5. Behavior on time of color developed by 1: 272 μmol acetoin and 2: 280 μmol diacetyl.

lactate or acetoin. However, α-acetolactate is unstable. It degrades itself spontaneously to diacetyl and acetoin. Concentration of α-acetolactate is estimated through its degradation products, after hydrolysis in acidic medium and heating. The better way to measure α-acetolactate decarboxylase activity is to make it through a direct determination of acetoin in the enzymatic reaction medium, such as a colorimetric determination.

The colorimetric method allows the determination of acetoin but also of diacetyl [6]. As shown in Fig. 4, the peak of absorbance of the colored complex obtained in the Westerfeld method gives a similar maximum at 522 nm for both acetoin and diacetyl. Acetoin and diacetyl calibration curves, measured after 40 min of color development, are linear up to absorbance 1.2 [7]. But, as can be seen from the regression equations, the curves are superposed:

$$Abs\ (40\ \text{min}) = 0.001^{*}\ [\text{diacetyl}, \mu\text{M}] - 0.014,\ r^2 = 0.989;$$

$$Abs\ (40\ \text{min}) = 0.001^{*}\ [\text{acetoin}, \mu\text{M}] - 0.021,\ r^2 = 0.994,$$

where *Abs* (40 min) is absorbance at 525 nm after 40 min of color development.

3.3. Discrimination between acetoin and diacetyl

Discrimination between acetoin and diacetyl would provide an interesting approach. Fig. 5 shows that acetoin and diacetyl differ in the rate of color development: maximum color intensity is obtained after 5 min for diacetyl and 40 min for acetoin. Based on that observation, calibration curves of mixtures of acetoin and diacetyl were made after 5 min of color development, giving the following regression curves:

$$Abs\ (5\ \text{min}) = 0.888^{*}\ [\text{diacetyl, mM}] + 0.011,\ r^2 = 0.99;$$

$$Abs\ (5\ \text{min}) = 0.564^{*}\ [\text{50/50 acetoin/diacetyl, mM}] + 0.003,\quad r^2 = 0.99;$$

$$Abs\ (5\ \text{min}) = 0.225^{*}\ [\text{acetoin, mM}] - 0.002,\ r^2 = 0.99.$$

Discrimination between acetoin and diacetyl can be achieved on the basis of absorbance measurements after 5 and 40 min of color development with the following hypothesis:

$$Abs\ (5\ \text{min}) = a[\text{acetoin, mM}] + b[\text{diacetyl, mM}] \quad (1)$$

$$Abs\ (40\ \text{min}) = a'[\text{acetoin, mM}] + b'[=\text{diacetyl, mM}] \quad (2)$$

where *Abs* (5 min) and *Abs* (40 min) are absorbances at 525 nm of the same reaction medium after 5 and 40 min of color development and a, b, a′, and b′ are constants. Both acetoin and diacetyl concentrations can be extracted from absorbance measurements (after 5 and 40 min). However, this would require double calibration curves (at 5 and 40 min) and a mathematical treatment. Accuracy of such a "double measurement" is related to the amount of each component (diacetyl and acetoin). The major one is measured more precisely than the other one. In the case of α-acetolactate decarboxylase activity determination, measurement of absorbances at two different times was not carried out in routine. Nevertheless, the double time determination may be useful for discrimination between acetoin and diacetyl in a medium.

3.4. Accuracy of the spectrophotometric determination of α-acetolactate decarboxylase

The Novo-Nordisk procedure requires four measurements (four tubes) for one activity determination (d): one for enzyme activity ($H1$), one blank as a correction for degradation of substrate ($H2$), and two blanks used for interferences of the buffers ($B1$ and $B2$). Each tube is submitted to the color-reaction and absorbance at 525 nm is read after exactly 40 min of color development.

Determination (d) is given by:

$$d = (H1 - B1) - (H2 - B2) \quad (3)$$

the relative error is:

$$\frac{\Delta d}{d} = \frac{\Delta H1}{H1} + \frac{\Delta B1}{B1} + \frac{\Delta H2}{H2} + \frac{\Delta B2}{B2} \quad (4)$$

Assuming

$$\frac{\Delta H1}{H1} = \frac{\Delta Abs}{Abs} * H1 \quad (5)$$

$$\frac{\Delta B1}{B1} = \frac{\Delta Abs}{Abs} * B1, \quad (6)$$

$$\frac{\Delta H2}{H2} = \frac{\Delta Abs}{Abs} * H2, \quad (7)$$

and

$$\frac{\Delta B2}{B2} = \frac{\Delta Abs}{Abs} * B2 \quad (8)$$

The relative error on determination $\Delta d/d$ is equal to:

$$\frac{\Delta d}{d} = \frac{\Delta Abs}{Abs} * (H1 + B1 + H2 + B2) \quad (9)$$

The relative error $\Delta Abs/Abs$ on absorbance was calculated from 30 values (30 independent experiments conducted at 30°C). It is equal to 23%. Order of magnitude of absorbances are: $H1 \approx 1.0$, $B1 \approx 0.1$, $H2 \approx 0.4$, and $B2 \approx 0.08$, which gives for the relative error on determination: $\Delta d/d = 35\%$.

With the usual analytical method [5], the absorbance obtained for the blank reached values of 0.4 to 0.5 due to both a high initial concentration of diacetyl and a strong degradation of α-acetolactate. The absorbance working range is then 0.4 to 1.2. Several dilutions of the samples need to be tested to get a result in this range. Both range and relative error of measurement can be improved. One solution is to limit the spontaneous degradation of α-acetolactate, i.e. to minimize the value of $H2$.

3.5. Improvement of the determination procedure

Our investigation of the determination procedure consists of:

- setting the physico-chemical conditions (oxygen, temperature, pH) that minimize α-acetolactate degradation, i.e. $H2$,
- simplifying the procedure to get it shorter.

Presence of oxygen does not significantly affect the α-acetolactate decarboxylation rate (data not shown). Reaction temperature is an important parameter for decarboxylation rate, as presumed for most chemical reactions: the higher the temperature, the higher the degradation (Figs. 6 and 7). Fermentation of beer is conducted between 8°C and 15°C. To reduce the degradation rate of α-acetolactate and to set conditions close to reality, activity determination was conducted at 10°C. The pH of the usual reaction medium is 6 although, in fermenting beer, it ranges from ~5.4 to 4.5. Acidic pH favors α-acetolactate degradation, and α-acetolactate decarboxylase activity decreases from 100% at pH 6 to 20% at pH 4 [10]. Thus a pH of 6 is a good compromise for the enzyme assay.

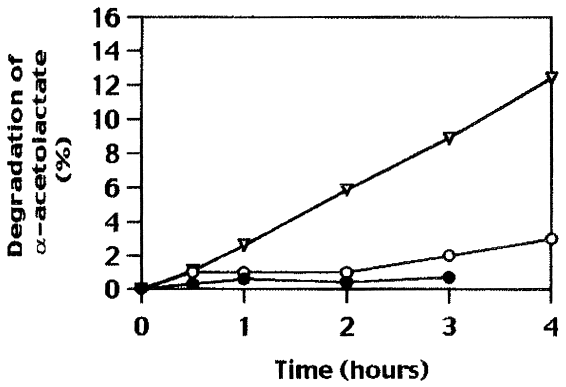

Fig. 6. Effect of temperature on spontaneous decarboxylation of α-acetolactate in 100 mM maleate buffer pH 6.0 at 0°C (●), 10°C (○), and 25°C (▽).

These new parameters of work allowed minimization of $H2$ from ≈0.4 to a value of ~0.25. This decrease had two effects: first, it widened the determination range (absorbances of 0.25 to 1.2 instead of 0.4 to 1.2), and it had an impact on the relative error of measurement (see below).

In the usual procedure, enzyme and substrate are dissolved in two different buffers: MES/Brij/NaCl (50 mM/0.05%/600 mM, pH 6) and MES (50 mM, pH 6). The use of two different buffers imposes two different blanks ($B1$ and $B2$) in addition to the "substrate degradation" blank ($H2$). Brij and NaCl are activators of α-acetolactate decarboxylase [13] but are not present in fermenting beer. Both buffers have been replaced by sodium maleate buffer (100 mM, pH 6 ± 0.05) in the further procedures. Sodium maleate buffer allows the same buffering effect as MES (MES: pKa = 6.1, maleic acid: pKa = 6.2). This shift simplifies the determination procedure because two absorbance measurements for one activity determination are needed instead of four (one for enzymatic reaction: H and one for "substrate degrada-

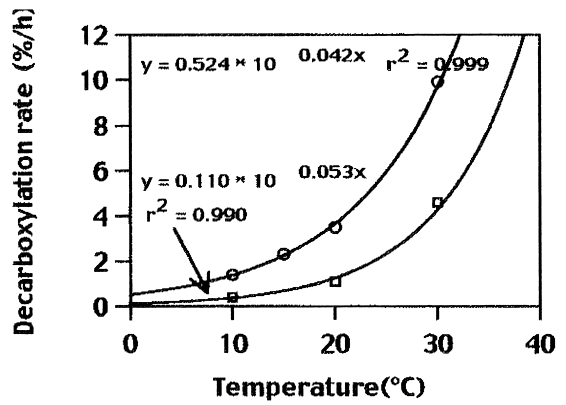

Fig. 7. Decarboxylation rate of α-acetolactate as a function of temperature in degassed beer pH 4.2 (○) and in 100 mM maleate buffer pH 6.0 (□).

Table 1
Characteristics of usual and revisited procedures

	Usual procedure	Final procedure
One set of measurements, min/sample	10 samples, 5 h 30	40 samples, 3 h 5
Cost of reagents, USD for 50 measurements	14	10
USD/sample	0.28	0.2
Range	0.4–1.2	0.25–1.2
Acurracy, %	35	11

tion" blank B) and because only one buffer solution is prepared instead of two.

The determination (d) is given by:

$$d = H - B \tag{10}$$

so the relative error is:

$$\frac{\Delta d}{d} = \frac{\Delta H}{H} + \frac{\Delta B}{B} \tag{11}$$

Assuming:

$$\frac{\Delta H}{H} = \frac{\Delta Abs}{Abs} * H \tag{12}$$

and

$$\frac{\Delta B}{B} = \frac{\Delta Abs}{Abs} * B, \tag{13}$$

$$\frac{\Delta d}{d} = \frac{\Delta Abs}{Abs} * (B + H) \tag{14}$$

The relative error on absorbance $\Delta Abs/Abs$ was calculated from 30 values (from 30 independent experiments conducted at 10°C). It is equal to 9%. Order of magnitude of absorbances are: $H \approx 1.0$, $B \approx 0.25$, so the relative error is equal to: $\Delta d/d = 11\%$.

All of these modifications lead to a better accuracy of the activity measurement. The larger range of the improved method allows determination of activity from one sample dilution whereas the usual method often requires two or three: this is time- and cost-saving. Moreover, with the revisited procedure, 100% of the set of measurements we conducted was in the range of determination. The use of sodium maleate buffer is also time- and cost-saving: time-saving because only one buffer has to be prepared and cost-saving because sodium maleate is cheaper than MES (60 USD/kg for sodium maleate versus 600 USD/kg for MES). The cost per analysis and time needed are presented in Table 1.

Table 2
Summary of recommended changes

Usual procedure	Revisited procedure
Two different buffers (MES and MES/Brij/NaCl)	One buffer (maleate)
Preparation of fragile α-acetolactate (substrate) lasts ≈50 min	Preparation of α-acetolactate lasts <15 min
Temperature of work: 30°C for enzyme reaction, ambiant for coloring reaction	Temperature of work: 10°C

From this table it can be seen that the new determination procedure is quicker, cheaper, and more accurate. A summary of the recommended changes is given in Table 2.

4. Conclusion

By using the optimized method for α-acetolactate decarboxylase assays allows improvement of the accuracy by-two third and enlargement of the range of measurements by 15% to save time (25 min/sample) and to cut down the cost by 28%. With such modifications, it is possible to measure the impact of α-acetolactate decarboxylase upon a fermentation course.

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