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Development of an Enzymatic Method To Quantify Methyl Ketones from Bacterial Origin

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Methyl ketones are detected in dry fermented sausages in which they contribute to the cured aroma. They have been associated with the inoculation of *Staphylococcus carnosus* used as starter culture. To evaluate the ability of bacterial starters to produce methyl ketones it was necessary to develop a rapid method. The method consists of a reaction catalyzed by a commercial NADPH-dependent alcohol dehydrogenase that reduces the 2-pentanone to its secondary alcohol. The linearity, the specificity, and the robustness were studied. Its accuracy was confirmed by comparison with the gas chromatography technique. Finally, the method was validated on biological samples such as the 2-pentanone produced by *Staphylococcus carnosus*. The enzymatic method offers some advantages over the gas chromatography, as it is faster, simpler, and inexpensive, guaranteeing an effective way to assess bacterial ketone production.

KEYWORDS: Methyl ketones; aroma; 2-pentanone; *Staphylococcus carnosus*; fermented sausage; NADPH-dependent alcohol dehydrogenase

INTRODUCTION

Aliphatic methyl ketones are ubiquitous in nature and can be found in diverse sources such as plants, dairy products, and the odorous secretions of insects (*1*). It was early recognized that methyl ketones of intermediate chain length were metabolic products of certain fungi and made an important contribution to the flavor characteristics of mold-ripened cheese (*2*, *3*). Later, Berdagué et al. (*4*) and Montel et al. (*5*) showed that inoculation of *Staphylococcus carnosus* or *Staphylococcus xylosus* into sausage mixture increased the level of some volatile compounds, including ketones, which contributed to the cured aroma. In filamentous fungi, it is often mentioned that methyl ketones are formed from an incomplete β-oxidation of medium chain fatty acids (*6*). This pathway includes the release of β-ketoacyl CoA esters that are deacylated into β-ketoacids and finally decarboxylated into methyl ketones. Although the β-oxidative pathway in *Staphylococcus carnosus* has already been described (*7*), the final step of β-decarboxylation leading to CO_2 and methyl ketone has not yet been identified.

According this pathway, the decarboxylation can be measured by either CO_2 or methyl ketone quantification. As the former could be produced by other metabolic reactions, it is necessary to use radioactive substrates to measure specifically marked CO_2 (*3*, *8*).

The methods most commonly employed to evaluate volatile carbonyl groups in biological materials are physicochemical techniques, such as liquid−liquid column paper chromatography (*9*), thin-layer chromatography (*10*, *11*) and some spectrometric methods such ultraviolet (*12*) or infrared absorption (*13*). More recently, gas−liquid chromatography associated with mass-spectrometry and direct sampling techniques (dynamic or static headspace) have been developed and are the most sensitive and specific methods available for providing information about the composition of food volatile compounds (*4*, *14*−*16*). However, all these methods have some inconveniences, such as lengthy analysis times, complicated sample preparation (derivative formation, i.e., DNP hydrazones), solvent extractions, or lack of sensitivity for quantification assays.

With the objective to study the metabolic pathway involved in methyl ketone production by *Staphylococcus*, we were first interested in finding a fast and practical method to carry out enzymology research. Then, we developed a simple enzymatic method with detailed validation obtaining an accurate and sensitive evaluation of 2-pentanone production from bacterial origin. The procedure developed employs a thermostable NADPH-dependent alcohol dehydrogenase (EC 1.1.1.2) with a broad substrate range (*17*). This enzyme reduces the methyl ketone to its secondary alcohol and the rate of NADPH oxidation is directly related to the ketone content.

EXPERIMENTAL PROCEDURES

Materials. Alcohol dehydrogenase from *Thermoanaerobium brockii* (5−15 units/mg protein), β-NADPH, and esterase from porcine liver (250 units/mg protein) were obtained from Sigma-Aldrich Co. (St. Louis, MO). The 2-pentanone (99% redistilled) and buffer salts were analytical reagent grade products from Aldrich Co., Inc. (Milwaukee, WI).

Enzymatic Reaction. The enzymatic reduction of 2-pentanone by the alcohol dehydrogenase (ADH) was monitored spectrophotometrically with a Shimadzu UV−Visible 160A spectrophotometer in kinetic mode. The reaction mixture, in Tris−HCl buffer (100 mM, pH 7.8)

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Table 1. Effect of Factor Slight Variations on 2-Pentanone Measurement[a]

factor	normalized values −1	normalized values +1	prob.	Δ*x*	Δ*y*
temperature (°C)	39	41	*	1 °C	3%
pH	7.7	7.9	NS	0.1	4%
wavelength (nm)	333	335	NS	1 nm	<1%
enzyme units	0.019	0.021	***	0.001	6%
final volume (μL)	990	1010	NS	10 μL	1%
incubation time (min)	9	11	***	1 min	11%

[a] Δ*y*, variation of ketone measurement; Δ*x*, factor variation; probability - *** $p < 0.1\%$; ** $0.1\% < p < 1\%$; * $1\% < p < 5\%$; NS, not significant.

and made up to a final volume of 1 mL, contained the following reagents: 200 μL of 2-pentanone (0−500 μM), 100 μL of β-NADPH (100 mM), 20.8 μL of ADH solution (0.94 units/mL). The kinetic of β-NADPH oxidation was followed continuously at 334 nm and 40 °C during 10 min. The β-NADPH and enzyme solutions were kept in ice.

Development of the Enzymatic Method. Each enzymatic ketone assay was made in quadruplicate. The linearity, the specificity, and the robustness tests were made following the statistical data analysis proposed by Feinberg (*18*).

Linearity of the Method. To determine the limits of the linear domain, five independent solutions of 2-pentanone (100 mM) were prepared and then diluted to establish a calibration curve in the 0 to 500 μM range. A linear regression was performed and a Fisher test was used to verify the linear hypothesis.

Specificity of the Method. To evaluate possible interference with the matrix of biological samples four assays were undertaken in duplicate: (1) blank, only reactives and solvents; (2) a standard of a known concentration of 2-pentanone; (3) a biological sample (described below) with an unknown concentration of 2-pentanone; (4) the biological sample (3) with addition of the standard (2). Two linear regressions were made combining (a) the results of the assays (1) and (2) and (b) the results of the assays (3) and (4). One hypothesis is tested: the slopes of the two lines are equal.

Robustness of the Method. Robustness is a measure of the capacity of the method to remain unaffected by slight operating modifications. An experimental protocol was set up as a factorial fractional design (2^{6-3}). A lower and an upper level for each variable were determined as shown in **Table 1**. A multiple linear regression analysis was performed on the normalized data. The parameters of the regression were studied in order to determine the influence of each variable on the response.

Determination of 2-Pentanone by Gas Chromatography (GC). The GC analyses were carried out to assess the accuracy of the enzymatic method. Three stock solutions of 2-pentanone (100 mM) were prepared and diluted in dichloromethane in a range from 0 to 400 μM. The 2-pentanone solution was injected directly into a gas chromatograph (Agilent HP 8890A) coupled to a flame ionization detector (FID). The chromatographic conditions were as follows: an apolar capillary SPB5 column (length 60 m, internal diameter 0.32 mm), an injection temperature of 240 °C, a GC oven temperature from 40 to 150 °C with a slope of 3 °C/min, mode splitless, and helium vector 1 mL/min. The elution of 2-pentanone was identified by its retention time.

Biological Samples. *Bacterial Strain and Growth Conditions. Staphylococcus carnosus* 833 strain was grown in MC (*19*) or chemically defined (*20*) media for 24 h at 30 °C with stirring (170 rpm). The cultures were centrifuged at 1000*g* at 4 °C. The harvested cells were washed, resuspended in phosphate buffer (pH 7.0), and used as a β-decarboxylase source for the 2-pentanone production.

Production of 2-Pentanone. The incubation mixture contained 400 μL of resting cells (0.2 g wet weight cells), ethylbutyryl acetate (4 mM) as substrate, esterase (2.7 units), and K_2HPO_4/KH_2PO_4 buffer (100 mM, pH 7.0) to 1 mL final volume. Two controls were used: one without resting cells and the other without substrate. The reaction mixture was incubated in a 2-mL Eppendorf tube in shaking condition at 30 °C for 1 h. The enzymatic reaction was stopped by removal of the cells by centrifugation (15000*g*). The supernatants containing 2-pentanone were kept at −20 °C until enzymatic quantification as described above. Three independent assays were carried out. The results were expressed as μmol of 2-pentanone/h/0.2 g of wet weight cells.

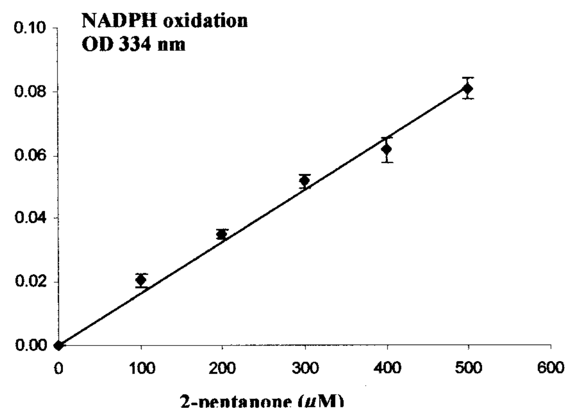

Figure 1. Standard curve of 2-pentanone.

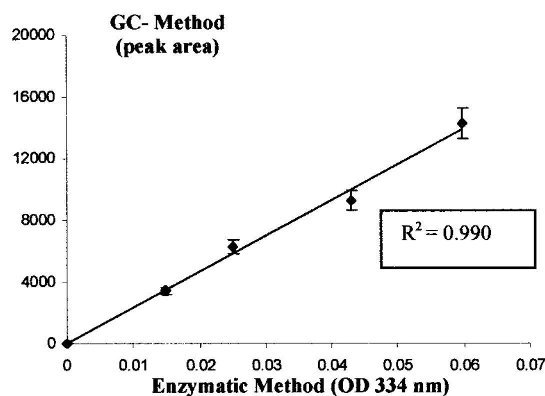

Figure 2. Correlation between the enzymatic and gas chromatography methods for 2-pentanone quantification. Each point is the average of three independent assays.

RESULTS

Development of the Enzymatic Method. The NADP-linked ADH from a thermophilic bacterium was used to catalyze the reduction of 2-pentanone to 2-pentanol. To develop the enzymatic method we have studied the linearity, the accuracy, the robustness, and the specificity.

Linearity Test. Although ADH has a higher affinity for secondary alcohols, it was possible to quantify 2-pentanone contents. The reduction of 2-pentanone by ADH was linear in the range 0−500 μM (**Figure 1**). Least-squares analysis gave a good correlation coefficient ($R^2 = 0.99$) and the Fisher test (3.56; 6.12) was also highly significant. A quantification limit of 100 μM (8.6 ppm) was calculated.

Accuracy. The reliability of the enzymatic method was checked by comparison with the GC reference method. Results were analyzed by plotting 2-pentanone values measured by GC method (*y* axis) against those obtained by enzymatic method (*x* axis). Very significant regression coefficients ($R^2 > 0.99$) were obtained, indicating good correlation between the techniques (**Figure 2**).

Robustness Test. A slight variation in pH, wavelength, or final volume did not significantly modify the enzymatic measure of 2-pentanone (**Table 1**). However, a slight modification in either incubation time or enzyme quantity lead to a considerable variation in the 2-pentanone measurement (11% and 6%, respectively). Given that the incubation time is a factor readily

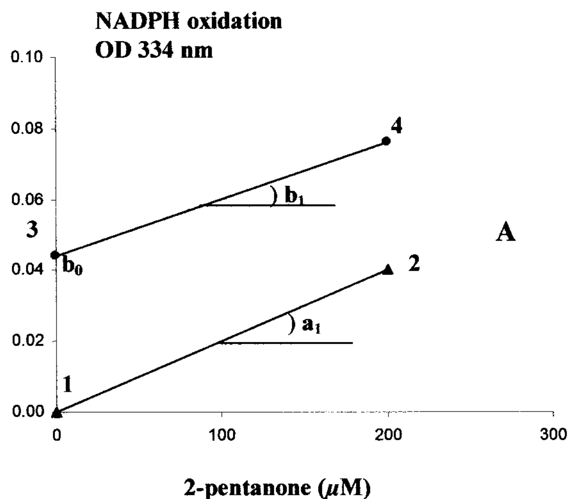

Figure 3. Specificity of the method. Analysis of the constituents of sample in the assay. (▲) **A**, Standard curve; (●) **B**, Dose curve; $\mathbf{a_1}$, slope of curve A; $\mathbf{b_1}$, slope of curve B; **(1)** blank; **(2)** standard; $\mathbf{b_0}$ and **(3)** unknown biological sample; **(4)** standard and unknown biological sample mix.

Table 2. Production of 2-Pentanone by *Staphylococcus carnosus* 833 Grown in Complex or Chemically Defined Media

growth medium	2-pentanone production (μmoles/0.2 g cells/h)
complex	1.80 ± 0.030
chemically defined (1% glucose)	0.20 ± 0.032
chemically defined (0.1% glucose, pH 7.0)	1.75 ± 0.028

controlled by the automatic spectrophotometer setting, enzyme concentration is the main parameter that must be monitored. Therefore, the enzymatic method is robust if careful attention is taken in enzyme preparation.

Specificity. Constituents of the biological matrix could interfere and provoke background noise in the response (up or down estimation). To evaluate this phenomenon the four assays described before (Experimental Procedures section) were carried out (**Figure 3**). The concentration of the unknown biological sample (3) can be calculated either from the standard curves in **Figure 1** or **Figure 3** (curve A: $y = 0.0002x$), or by graphical extrapolation using curve B of **Figure 3**, ($x = -b_0/b_1$). The biological sample with an OD_{334} of 0.044 contained 220 μM of 2-pentanone by these calculation methods; thus, specificity of the method was confirmed.

Applications for Bacterial Production. Production of 2-pentanone by *Staphylococcus carnosus* 833 was compared for the strain grown in chemically defined or in complex media (**Table 2**). The highest ketone production was observed when cells were grown in the complex medium or in chemically defined medium buffered at pH 7.0 with a low concentration of glucose. The low production of 2-pentanone obtained when cells were grown in chemically defined medium with 1% of glucose can be attributed to either glucose inhibition or the pH drop from 7.0 to 5.3.

When resting cells of *S. carnosus* were incubated for 4 h, the production of 2-pentanone increased during the first 2 h and then stayed at the same level (**Figure 4**).

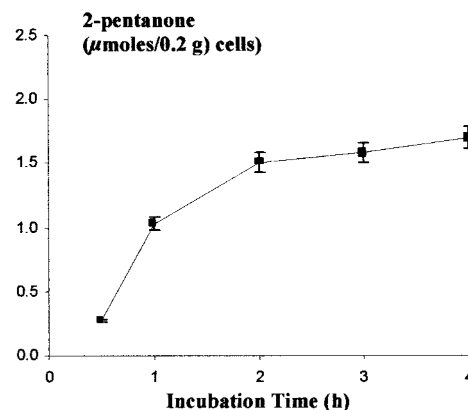

Figure 4. Kinetic of 2-pentanone production by *Staphylococcus carnosus* 833.

DISCUSSION

Alcohol dehydrogenase enzymes are well described in the literature, most having a narrow substrate specificity (*21*) and only a few with broad activity (*22*−*24*). Among the latter, an intracellular alcohol dehydrogenase from *Rhodococcus* sp GK1 was shown capable of reduction of monoketones and diketones and oxidation of secondary alcohols (*25*). Likewise, the NADP-linked alcohol−aldehyde/ketone oxidoreductase from *Thermoanaerobium brockii*, was purified and characterized. Its broad substrate specificity includes primary alcohols (ethanol, propan-1-ol, and butan-1-ol), linear and cyclic secondary alcohols (propan-2-ol, butan-2-ol, and cyclohexanol) and linear and cyclic ketones (propanone, 2-butanone, 2-pentanone, and cyclopentanone) (*17*). These authors also reported its potential uses including production of chiral R(+) 2-pentanol, synthesis of NADPH, regeneration of NADP−NADPH coenzyme, and analytical solvent detection with an immobilized enzyme electrode (*26*). The use of these alcohol dehydrogenases for ketone quantification was already suggested, but the development of the method had not yet been reported. So in this study, we selected the commercially available alcohol−aldehyde/ketone oxidoreductase from *Thermoanaerobium brockii* to detect and quantify 2-pentanone from bacterial origin. The 2-pentanone was chosen because it is a characteristic methyl ketone found in sausages. With this ADH enzyme, methyl ketones from C2 to C5 could be assayed (*17*).

To develop the method, we studied the linearity, the robustness, the accuracy, and the specificity. The enzymatic method offers noticeable advantages in time, cost, and simplicity of analyses, in comparison with the classic procedures such as gas chromatography associated with mass spectrometry, but its sensibility is lower (ppb for gas chromatography and ppm for the enzymatic method).

The method was then validated on bacterial samples. This is the first report on the production of methyl ketones by bacteria from β-ketoacids. The result highlights the existence of a β-decarboxylase activity. This pathway was already suggested for fungi (*3*, *6*, *8*). Work is currently being undertaken on this topic in *Staphylococcus carnosus* in our laboratory.

In conclusion, we have developed an enzymatic method to quantify 2-pentanone for routine usage, in particular for screening bacterial strains or to carry out enzymatic research.

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