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Citation: Chin. Phys. B, 2021, 30 (**5**): 054203. DOI: 10.1088/1674-1056/abda32
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A pressure-calibration method of wavelength modulation spectroscopy in sealed microbial growth environment*

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(Received 20 December 2020; revised manuscript received 7 January 2021; accepted manuscript online 11 January 2021)

A new pressure-calibration method for calibrating the reduction of second harmonic ($2f$) amplitude caused by pressure broadening effect in sealed microbial growth environment is present. The new method combines with linewidth compensation and modulation depth compensation and makes the $2f$ amplitude accurately retrieve metabolic CO_2 in microbial growth. In order to verify the method, a simulation experiment is developed, in which the increasing CO_2 concentration leads to the increasing pressure. Comparing with the relation between the traditional $2f$ amplitude and gas concentration, there is a monotonous relation between the calibrated $2f$ amplitude and CO_2 concentration, particularly, a linear relation is present when the CO_2 concentration is replaced with the CO_2 particle number. In terms of microbial measurement, the growth of *Escherichia coli* is measured, and the culture bottle is sealed during the microbial growth process. The experimental results show that, comparing to the microbial growth retrieved by traditional $2f$ amplitude, the calibrated $2f$ amplitude can accurately retrieve microbial growth in sealed environment.

Keywords: second harmonic amplitude, pressure-calibration, microbial growth, sealed environment, CO_2

PACS: 42.55.Px, 42.62.Fi, 33.20.Ea **DOI:** 10.1088/1674-1056/abda32

1. Introduction

Monitoring microbial growth plays an important role in a wide range of fields spanning from medical diagnosis to food microbiology.[1–4] Microbial growth occurs in a sealed-environment bottle (or medium) with a limited supply of nutrients, it goes through by four distinct phases:[5] the lag phase, the exponential growth phase (or log growth phase), the stationary phase, and the death phase (or decline phase). The lag phase is the initial period of slow growth, during which time microorganisms adapt to the medium and synthesize critical proteins. The lag phase is followed by the exponential growth phase. With the number of cells doubling every unit of time, rapid microbial growth presents exponential growth. During the stationary phase, the growth rate of microorganisms is nearly equal to the death rate of microorganisms because nutrients are depleted in the medium, and the conditions become unfavorable for survival. During the death phase, the growth rate of microorganisms decreases and finally reaches zero.[2,6] The modified Gompertz model is widely used to describe these phases.[5] Microorganisms using respiration as a metabolic pathway release carbon dioxide (CO_2). Thus CO_2 as a reliable indirect indicator, the amount of CO_2 produced in this period accurately reflects the above microbial growth process. BacT/ALERT blood culture bottles based on reflectance spectrometry have been applied to measure the color variation of CO_2 sensitive receptors in microbial growth.[7] However, such detection is susceptible to contamination and each data point needs to take half an hour.[8]

Wavelength modulation spectroscopy (WMS) with second harmonic ($2f$-WMS) as a real time, non-invasive, and high-accuracy detection technology is especially suited for gas detection.[9–12] Though detecting the CO_2 metabolized by microorganisms, this technology can quantify microbial growth. Thus in recent years, WMS has been used to study metabolic CO_2 produced above culture medium, for example, our group used WMS to swiftly assess CO_2 produced by microbial growth with a Hz resolution.[13] This implies that WMS can rapidly and accurately obtain the information of microbial growth. Brueckner *et al.* used to determine CO_2 changes in headspaces of the bottles that induced by metabolically active microorganisms.[14] However, it is worth noting that the above traditional WMS is affected by pressure broadening effect when detecting metabolic CO_2 in sealed microbial growth environment. Because, in traditional WMS, the $2f$ amplitude is often employed for gas concentration retrieval under constant pressure condition,[15–17] but for microbial growth, produced CO_2 will continue to increase and cause the pressure continuously increase in the sealed-environment bottle. It affects the line shape of the $2f$ signal and leads to the reduction of the $2f$ amplitude.[18] This effect makes $2f$-WMS

*Project supported by the National Key Research and Development Program of China (Grant Nos. 2017YFC0209700 and 2016YFC0303900) and the National Natural Science Foundation of China (Grant No. 41730103).

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unable to accurately detect metabolic CO_2 in sealed microbial growth environment, thus the pressure calibration should be performed in this detection.

In this paper, we propose a new pressure-calibration method to calibrate the reduction of the $2f$ amplitude which is caused by pressure broadening effect in sealed microbial growth environment. The new method differs from traditional $2f$ amplitude in a significant way: it combines with linewidth compensation and modulation depth compensation, and makes the $2f$ amplitude accurately retrieve metabolic CO_2 without being affected by pressure broadening effect. Furthermore, in order to verify the method, a simulation experiment was developed, in which the increasing CO_2 concentration leaded to the increasing pressure. Comparing with the relation between the traditional $2f$ amplitude and CO_2 concentration, there was a monotonous relation between the calibrated $2f$ amplitude and CO_2 concentration, particularly, a linear relation was developed when CO_2 concentration was replaced with the CO_2 particle number. In terms of microbial measurement, the growth of *Escherichia coli* was measured, and the culture bottle was sealed during the microbial growth process. The traditional $2f$ amplitude and calibrated $2f$ amplitude were used to detect CO_2 in the microbial growth. The experimental result showed that comparing to the traditional $2f$ amplitude, the calibrated $2f$ amplitude can accurately retrieve microbial growth in sealed environment.

2. Methods and system details

2.1. Theory

According to the article mentioned above, as microorganisms growing and metabolizing CO_2, the pressure P and the particle number N in the sealed culture bottle environment satisfy the state equation of gas

$$PV = nRT = \frac{N}{N_A}RT, \tag{1}$$

where N_A [mol^{-1}] is the Avogadro constant, V is the gas volume, n is the gas amount of substance, R [J/(mol·K)] is the gas constant, and T [K] is the thermodynamic temperature. The air particle number in the sealed-environment culture bottle is N_0, the initial pressure P_0 is normal pressure (1 atm); at a certain time t ($t > 0$), the CO_2 particle number produced by microbial growth is N_t, and the pressure in the sealed-environment culture bottle is P_t [atm]. According to the above equation, the pressure P_t at time t can be obtained as

$$P_t = \frac{N_0 + N_t}{N_0}P_0. \tag{2}$$

Here $N_t + N_0$ is the total particle number in the sealed-environment culture bottle at time t. Thus, the CO_2 concentration X [%] at time t can be obtained as

$$X = \frac{N_t}{N_0 + N_t}. \tag{3}$$

From Eqs. (2) and (3), the relation between pressure P_t and concentration X in the sealed-environment culture bottle can be given by

$$P_t = \frac{P_0}{1 - X}. \tag{4}$$

It can be seen from Fig. 1 that the relation between P_t and X in the sealed-environment culture bottle is a nonlinear curve, and the increasing CO_2 concentration causes the increasing pressure.

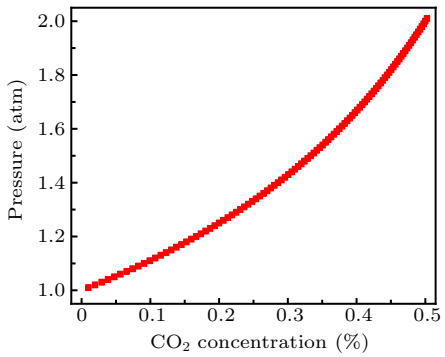


Fig. 1. The relation between pressure P_t and concentration X in the sealed-environment culture bottle.

The fundamental principle of $2f$-WMS is the Beer–Lambert law, which describes the relation between the incident and the transmitted radiation when the laser beam passes through a uniform gas medium. According to the Beer–Lambert law and the weak gas absorption, the absorption coefficient α_v is defined by

$$\alpha_v = \alpha_0\bar{\phi} = PXSL\left[\frac{1}{\pi}\cdot\frac{(\Delta\nu_c/2)}{(\nu-\nu_0)^2+(\Delta\nu_c/2)^2}\right]. \tag{5}$$

The $\Delta\nu_c$ [cm^{-1}] is the full width at half maximum (FWHM) of the Lorentzian line shape and can be given by

$$\Delta\nu_c = P[X\gamma_{self} + (1-X)\gamma_{air}], \tag{6}$$

where α_v is the absorption coefficient at frequency v; L [cm] is the path length; S [cm^{-2}·atm^{-1}] is the line strength of the transition; ν_0 [cm^{-1}] is the central frequency; γ_{self} and γ_{air} are the self-broadening and air-broadening coefficients, respectively. It can be seen from the above formula that the fraction of the bracketed terms is peak-normalized Lorentzian line shape function $\bar{\phi}$, and α_0 is the absorption coefficient given by $PXSL$ and proportional to the concentration under constant pressure.

Multiplying the numerator and denominator of Eq. (5) by $\Delta\nu_c/2$, the new formula can be expressed as

$$\begin{aligned}\alpha_v &= \alpha_0\bar{\phi} = \frac{PXSL}{\Delta\nu_c/2}\left[\frac{1}{\pi}\cdot\frac{(\Delta\nu_c/2)^2}{(\nu-\nu_0)^2+(\Delta\nu_c/2)^2}\right] \\ &= \frac{2PXSL}{\pi\Delta\nu_c}\left[\frac{(\Delta\nu_c/2)^2}{(\nu-\nu_0)^2+(\Delta\nu_c/2)^2}\right].\end{aligned} \tag{7}$$

According to Eq. (7), the amplitude absorption coefficient of the Lorentzian line shape function[19] α_0' can be written as $2PXSL/\pi\Delta\nu_c$. In addition, for the theory of the $2f$-WMS, the result of Pawel and Ove.[20] is adopted, so the $2f$ Fourier coefficient of signal $S_{2f}(\bar{\nu}_d, \bar{\nu}_a)$ can be written as

$$S_{2f}(\bar{\nu}_d, \bar{\nu}_a) = -\eta I_D \alpha_0 \bar{\chi}_{L,2}(\bar{\nu}_d, \bar{\nu}_a), \tag{8}$$

where $\bar{\nu}_a$ is the modulation depth (given by $2\nu_a/\Delta\nu_c$, ν_a is the modulation amplitude), $\bar{\nu}_d$ is the width-normalized center detuning (given by $2(\nu-\nu_0)/\Delta\nu_c$ for the Lorentzian case), η is the instrumentation factor, I_D is the detected laser intensity in the absence of gas absorption, and $\bar{\chi}_{L,2}$ is the $2f$ signal of the Lorentzian line shape function. In order to obtain the $2f$ amplitude, we substitute α_0' into Eq. (8) and obtain the $2f$ amplitude function

$$\begin{aligned} S_{2f}(\bar{\nu}_d = 0, \bar{\nu}_a) &= -\eta I_D \alpha_0' \bar{\chi}_{L,2}(0, \bar{\nu}_a) \\ &= -\eta I_D \frac{2PXSL}{\pi\Delta\nu_c} \bar{\chi}_{L,2}(0, \bar{\nu}_a). \end{aligned} \tag{9}$$

Then the $2f$ amplitude of the Lorentzian line shape function can be easily derived as[20]

$$\bar{\chi}_{L,2}(0, \bar{\nu}_a) = -\frac{2}{\sqrt{1+\bar{\nu}_a}} \left(\frac{\sqrt{1+\bar{\nu}_a}-1}{\sqrt{1+\bar{\nu}_a}+1} \right). \tag{10}$$

Combining Eqs. (6) and (9), we can find the principle of pressure broadening effect: Increasing pressure P can broaden linewidth $\Delta\nu_c$ and affect the modulation depth $\bar{\nu}_a$, so that reduce the $2f$ amplitude. Thus, we need to build a calibrated $2f$ amplitude function which is independence of linewidth $\Delta\nu_c$ and modulation depth $\bar{\nu}_a$, while, dependent on the gas concentration or the particle number. In this way, the pressure P cannot affect the line width and modulation depth, so that the $2f$ amplitude will not be reduced. In order to build this equation, the linewidth compensation and modulation depth compensation are introduced: According to Eq. (9), multiplying the equation by linewidth $\Delta\nu_c$ and the reciprocal of equation (10) $1/\bar{\chi}_{L,2}$, the $2f$ amplitude S_{2f} becomes an equation that satisfies the above conditions. Thus, the pressure-calibration coefficient (or the linewidth compensation and modulation depth compensation coefficient) κ can be written as

$$\kappa = \Delta\nu_c \frac{1}{\bar{\chi}_{L,2}(\bar{\nu}_a)} = \Delta\nu_c \left(\frac{1+(\bar{\nu}_a)^2+\sqrt{1+(\bar{\nu}_a)^2}}{1-\sqrt{1+(\bar{\nu}_a)^2}} \right). \tag{11}$$

The $2f$ amplitude calibration equation H_{2f} is defined, which is proportional to the $2f$ amplitude S_{2f} and the pressure-calibration coefficient κ. The equation H_{2f} can be expressed as

$$H_{2f}(\bar{\nu}_a) = S_{2f}(\bar{\nu}_a) \cdot \kappa. \tag{12}$$

Combining Eqs. (2) and (3), the $2f$ amplitude calibration equation H_{2f} has a linear relation with the CO_2 particle number, as described by Eq. (12). Combining Eqs. (3) and (4) the equation H_{2f} has a monotonically increasing relation with the CO_2 concentration, as described by Eq. (14):

$$H_{2f}(\bar{\nu}_a) = S_{2f}(\bar{\nu}_a) \cdot \kappa = G I_D \frac{N_t}{N_0}, \tag{13}$$

$$H_{2f}(\bar{\nu}_a) = S_{2f}(\bar{\nu}_a) \cdot \kappa = G I_D \frac{1}{1-X}, \tag{14}$$

where G is the proportionality coefficient including the constant parameters.

2.2. Simulation

In order to verify the validity of the pressure-calibration method, the absorption line located at 4991.25 cm^{-1} for high-precision detection of CO_2 was selected from the HITRAN 2016 database, whose strength was 1.26×10^{-21} cm/molecule at the temperature of 296 K. The optical length was 3 cm, which was the same as the width of the culture bottle, the initial pressure was 1 atm. As the CO_2 concentration increased from 0 to 50%, the pressure also increased from 1 atm to 2 atm (8 groups, each group increasing 0.125 atm). Figure 2 shows the traditional $2f$ signal at 0.08 cm^{-1} modulation amplitude under different pressure. It can be seen that, with the CO_2 concentration and pressure increasing, the $2f$ signal increased until reaching 33% CO_2 concentration condition (1.5 atm pressure condition), after that the $2f$ signal decreased due to the pressure broadening effect.

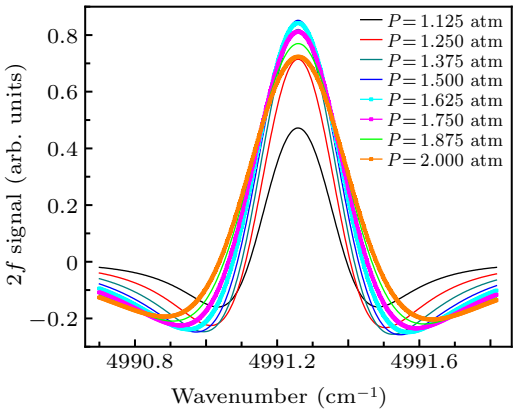


Fig. 2. The simulated $2f$ signal under 8 groups of concentration and pressure at 0.08 cm^{-1} modulation amplitude.

Figure 3 depicts the relation between the traditional $2f$ amplitude and CO_2 concentration. The other different modulation amplitudes (including low modulation amplitude 0.04 cm^{-1} and large modulation amplitude 0.16 cm^{-1}) were plotted in the figure. It can be seen from the simulation experiments that there are non-linear and non-monotonic curves between the traditional $2f$ amplitude and CO_2 concentration. The traditional $2f$ amplitude is proportional to CO_2 concentration in the 0–20% concentration range (1–1.25 atm pressure), and the pressure broadening effect becomes the main factor affecting the $2f$ amplitude in 20%–50% concentration range

(1.25–2 atm pressure range). In addition, although increasing modulation amplitude can increase the traditional $2f$ amplitude and optimize the monotonicity of the curves, it cannot change the non-monotonic characteristics of the curves.

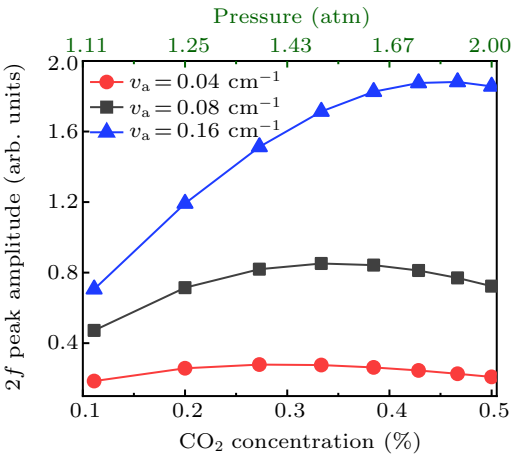


Fig. 3. The relation between the traditional $2f$ amplitude and CO_2 concentration at three different modulation amplitudes.

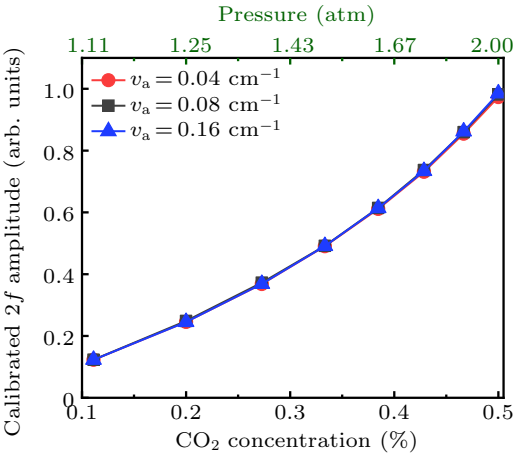


Fig. 4. The relation between the calibrated $2f$ amplitude and CO_2 concentration.

Figure 4 depicts the relation between the calibrated $2f$ amplitude and CO_2 concentration, according to Eq. (14). Comparing with the traditional $2f$ amplitude, there are monotonic curves between the calibrated $2f$ amplitude and CO_2 concentration. At the same time, these curves were not only unaffected by pressure broadening effect, but also independent of modulation amplitudes, so the similar concentration results can be obtained by using the calibrated $2f$ amplitude. Through these simulation experiments we can find that the calibrated $2f$ amplitude can achieve CO_2 concentration detection in the sealed environment with increasing pressure, and effectively eliminate the effects of pressure broadening effect. Furthermore, the linear relation between the calibrated $2f$ amplitude and the CO_2 particle number is plotted in Fig. 5, according to Eq. (13), and the linear relation can better retrieve microbial growth by using the calibrated $2f$ amplitude. Thus, this pressure-calibration method provides theoretical support for $2f$-WMS in the sealed microbial growth measurement.

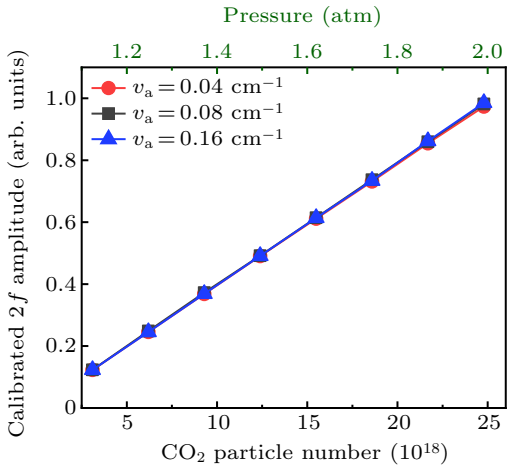


Fig. 5. The relation between the calibrated $2f$ amplitude and the CO_2 particle number, the air particle number N_0 is 2.48×10^{19} and the initial pressure P_0 is 1 atm.

3. Experiment

A schematic of experimental system was designed for verifying the pressure-calibration method, as exhibited in Fig. 6. The 2004 nm distributed feedback (DFB) tunable diode laser was used as a light source. The DFB laser was controlled by a laser driver (Stanford Research System, LDC501) and scanned at 1 Hz by a function generator (RIGOL, DG4162). The laser beam was collimated by an antireflection lens (Thorlabs, F260APC-1550) and introduced into the culture bottle (3 cm path length and 100 mL volume). An InGaAs photodiode (Thorlabs, DET10D/M) was used to detect the laser beam transmitted from the headspaces of the culture bottle. A digital lock-in amplifier (Stanford Research Systems SR830) was used to generate sinusoidal modulation signal at 19 kHz and receive the electrical signal from the photodiode detector. The $2f$ signal produced by the lock-in amplifier was acquired by a NI card (National Instruments, NI 6351) and fitted by a computer.

The growth of *Escherichia coli* coming from American Type Culture Collection (ATCC) was detected in the culture bottle and its initial concentration was 10^3 cfu/mL. The culture temperature was 22 °C and kept by a heated incubator. The culture bottle was sealed during the microbial growth process. The growth period was about 70 hours and the measurement interval was 40 min. Finally, the traditional $2f$ amplitude and calibrated $2f$ amplitude were used to detect CO_2 in the microbial growth.

A flow chart for the pressure-calibration method is shown in Fig. 7. The $2f$ absorption signal produced by the lock-in amplifier is obtained, through using the 2nd Fourier Lorentzian lineshape function, a fitted $2f$ absorption signal can be calculated, where A_n, B_n, C_n, D_n are furthermore solely functions of $\bar{v}_a$ and $\bar{v}_b$, for detailed discussion of the Lorentzian

lineshape function, refer to Ref. [21]. Furthermore, the fitted Δv_c and v_a can be deduced when the best-fit parameters between the measured and fitted absorbance signals are obtained. Then according to Eq. (10), $1/\bar{\chi}_{L,2}(\bar{v}_a)$ can be calculated. Finally, according to Eq. (11), the pressure-calibration coefficient κ can be calculated.

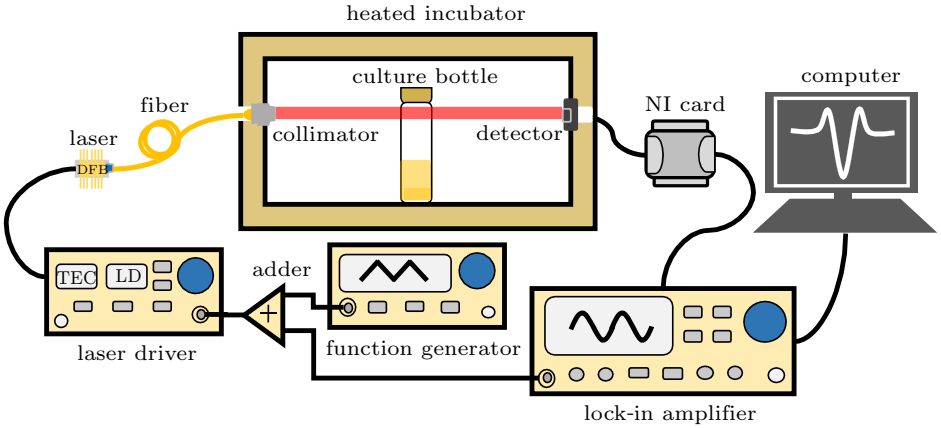


Fig. 6. Schematic of experimental system.

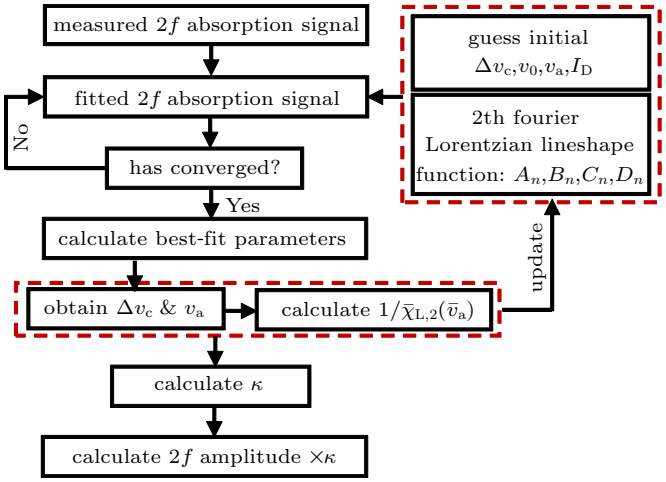


Fig. 7. Flow chart for pressure-calibration method.

4. Results and discussion

As shown in Fig. 8, eight groups of actual fitted $2f$ signals were took with integer time to illustrate the pressure broadening problem. As noted, from the 0th hour to the 30th hour, the $2f$ signals increased with increasing CO_2 concentration and were basically not affected by the pressure broadening effect. With the rapid growth of *Escherichia coli*, the amount of CO_2 increased and caused the pressure in the sealed-environment culture bottle increase rapidly. After the 30th hour, although the amount of CO_2 increased continuously, the pressure made the linewidth of the $2f$ signals broaden and caused the amplitude of the $2f$ signals to decrease. Especially, for the $2f$ signals at the 70th hour (death phase), its amplitude was smaller than the amplitude at 30th hour. Thus, the traditional $2f$ amplitude was unable to accurately detect CO_2 in sealed microbial growth environment and cannot correctly retrieve the growth of microorganisms. Because, according to the modified Gompertz model, the CO_2 concentration should stop increasing at the death phase, rather than decreasing.

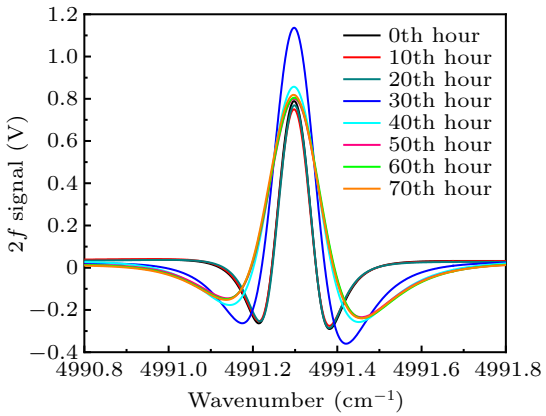


Fig. 8. Fitted $2f$ signals.

Figure 9 depicts the growth of *Escherichia coli* retrieved by the two different $2f$ amplitudes, wherethe blue triangle curve represents the inversion result of traditional $2f$ amplitude and the red square curve represents the inversion result of the calibrated $2f$ amplitude. Before the 20th hour, the growth of *Escherichia coli* was at the lag phase, the growth rate was closed to zero and the amount of CO_2 was almost unchanged. From the 20th hour, *Escherichia coli* entered the growth phase, and the growth rate increased from zero to the maximum at the exponential growth phase. However, the microbial growth retrieved by the traditional $2f$ amplitude reached its maximum at the 24th hour, after that rapidly declined, which cannot accord with the modified Gompertz model. Comparing with the traditional $2f$ amplitude, the calibrated $2f$ amplitude was not affected by the pressure broadening effect and can accurately retrieve the growth of *Escherichia coli*. It can be seen that the amount of CO_2 increased at the exponential phase and the stationary phase and stopped increasing at the

death phase. This result accorded with the modified Gompertz model and proved that the pressure-calibration method has an engineering value for the microbial growth. Except for retrieving microbial growth, the method also can be used to calibrate the $2f$ amplitude in other sealed environments with increasing pressure.

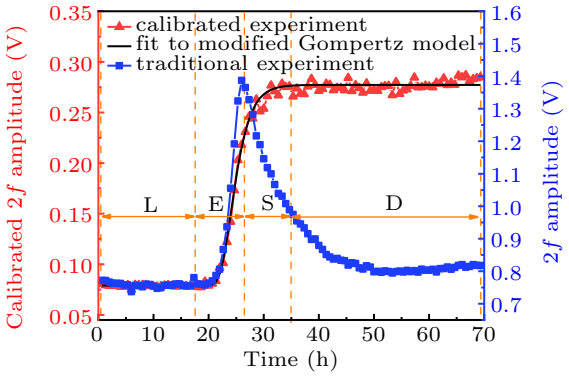


Fig. 9. Two $2f$ amplitudes in the microbial growth, where L: lag phase, E: exponential growth phase, S: stationary phase, D: death phase.

5. Conclusion

A new pressure-calibration method for calibrating the reduction of $2f$ amplitude caused by pressure broadening effect in sealed microbial growth environment was introduced. The new method combined with linewidth compensation and modulation depth compensation and made the $2f$ amplitude accurately retrieve metabolic CO_2 without being affected by pressure broadening effect. A simulation experiment was developed, in which the increasing CO_2 concentration leaded to the increasing pressure. Comparing with the traditional $2f$ amplitude, the simulation results of the calibrated $2f$ amplitude had a monotonous relation with CO_2 concentration, particularly, a linear relation was presented when CO_2 concentration was replaced with the CO_2 particle number. In order to verify the method, the growth of *Escherichia coli* was measured, and the culture bottle was in a sealed environment during the microbial growth process. The experimental results showed that comparing with the traditional $2f$ amplitude, the calibrated $2f$ amplitude can accurately retrieve microbial growth in sealed environment. Except for retrieving microbial growth, the method can also be used to calibrate the $2f$ amplitude in other sealed environments with increasing pressure.

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