APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1989, p. 1801–1807
0099-2240/89/071801-07$02.00/0

Vol. 55, No. 7

Determination of Biomass by Ultrasonic Measurements

A. ZIPS AND U. FAUST*

Institut für Biomedizinische Technik, University of Stuttgart, Seidenstrasse 36, D-7000 Stuttgart, Federal Republic of Germany

Received 23 June 1988/Accepted 7 March 1989

The quantitative determination of biomass in a suspension by means of ultrasound velocity is a simple and on-line-applicable method. Such an ultrasonic sensor offers the advantage of being long-term stable, reliable, and sterilizable. In this paper we present sound velocity measurements made with different microorganisms. The experimental results which we have obtained with an impulse-echo method will be compared with theoretical predictions and discussed with respect to previous findings (Y. Ishimori, I. Karube, and S. Suzuki, Appl. Environ. Microbiol. 42:632–637, 1981).

When considering the possibility and quality of automatic control and monitoring of biological processes, the availability and reliability of experiment measurements are decisive factors. In the area of biotechnology, biomass represents an important parameter and makes possible the calculation of a number of criteria of quality, such as specific growth rate and rate of production. The techniques available today to determine biomass make it necessary to compromise between methods which are either precise or time-consuming. On-line measurements generally tend to produce less exact results than off-line measurements. Sensors which ascertain biomass are based on chemical and physical procedures. Physical methods appear to be suitable for on-line measurements, as they are able to detect differences in physical substance between the solvent and the biomass and generally do not require sampling pretreatment (5). Of the physical methods available, the photometric method has proved to be by far the most reliable. However, this technique cannot be employed in many areas due to the small range of concentrations it can measure.

In this paper we present a physical measuring procedure to determine biomass by using ultrasound (U. Faust and K. M. Irion, patent OS DE 3429367A1, Federal Republic of Germany, 1986). This method of determining concentrations has already proved successful in nonbiological (3, 4) and biological (8, 11) media.

THEORY

Nomenclature. The following terms are used in this paper: c, sound velocity (meters per second); D, path (meters); f, frequency (1/seconds); R, cell radius (meters); t, time (seconds); T, temperature (Kelvin); V, cell volume (cubic meters); α, attenuation (decibels per meter); β, compressibility (seconds squared · meters per kilogram); ρ, density (kilograms per cubic meter); μ, dynamic viscosity (Pascals · seconds); υ, cinematic viscosity (meters squared · seconds). Indices used are L, solvent; S, suspension; and P, particle (i.e., biomass).

Ultrasonic velocity (c) in a homogeneous liquid can be represented by the following equation:

$$c = \sqrt{1/(\rho \cdot \beta)} \tag{1}$$

ρ represents the density of the solution and β its compressibility. Equation 1 can also be applied to nonassociated liquid composites for a small range of concentrations. The density and compressibility of the mixtures can be calculated with the densities and compressibilities of the individual components, which are weighted according to their volume fractions (see equations 2 and 3). This model can also be applied to particle suspensions on the condition that the diameter of the particles is small in comparison to the wavelength (10). This condition is met if the suspended biomass can be regarded as a two-phase system.

$$\rho_S = [\rho_L(1 - x) + \rho_P \cdot x] \tag{2}$$

$$\beta_S = [\beta_L(1 - x) + \beta_P \cdot x] \tag{3}$$

By using equations 2 and 3 in equation 1, the following results:

$$c_S = \{[\beta_L (1 - x) + \beta_P \cdot x] [\rho_L(1 - x) + \rho_P \cdot x]\}^{-0.5} \tag{4}$$

where S stands for suspension, L for cell-free solution, P for biomass, and x for the volume fraction of biomass. Equation 4 reveals a relationship between the volume fraction of biomass and the sound velocity, c_S, in a biosuspension, which depends on the values of density and compressibility in each phase. When these physical properties are known and remain constant during the biological process, then it is easy to measure the biomass content from the sound velocity measurements (c_S) received. It is possible that such constant physical properties are not to be found in a batch reactor. Those physical properties which change during the process can be determined with the aid of a reference measurement taken from the cell-free solution.

$$c_L = \sqrt{1/(\beta_L \cdot \rho_L)} \tag{5}$$

By using equation 5, equation 4 can be transformed into:

$$c_S = c_L \left\{ \left[\left(\frac{c_L}{c_P} \right)^2 - \frac{\beta_P}{\beta_L} - \frac{\rho_P}{\rho_L} + 1 \right] x^2 - \left(2 - \frac{\rho_P}{\rho_L} - \frac{\beta_P}{\beta_L} \right) x + 1 \right\}^{-0.5} \tag{6}$$

The differences in sound velocity (Δc) between sound velocity (c_S) in a suspended biomass and the reference measurement taken from the cell-free solution (c_L) can be calculated by:

$$\Delta c = c_S - c_L$$

$$= c_L \left(\left\{ x^2 \left[\left(\frac{c_L}{c_P} \right)^2 - z \right] - x(1 - z) + 1 \right\}^{-0.5} - 1 \right) \tag{7}$$

$$z = \frac{\rho_P}{\rho_L} + \frac{\beta_P}{\beta_L} - 1$$

* Corresponding author.

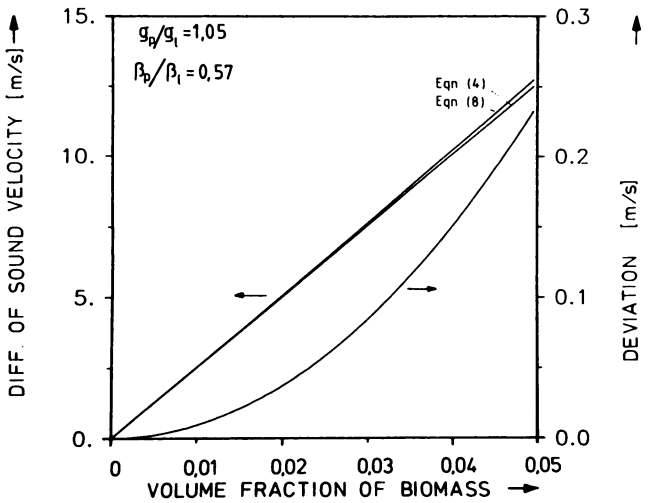

FIG. 1. Increase of sound velocity in a suspension due to the volume fraction of biomass. Simulation of equations 4 and 8 and their differences (T = 25°C).

After a power series expansion of equation 7, the terms of higher orders are neglected on the assumption that $x \ll 1$. The result is a linear relationship between the difference of sound velocities and the volume fraction of biomass. In the area of biotechnology, restricting the volume fraction to small amounts is permissible. If the cell volume (V) is 4 μm^3, a concentration of 10^5 cells per ml corresponds to a volume fraction (x) of 4×10^{-7} and a concentration of 10^{10} cells per ml corresponds to a volume fraction (x) of 4×10^{-2}. The equation 7 can be written as equation 8, which fits well with the experimental results (Fig. 1):

$$\Delta c = 0.5 \cdot c_L \cdot (1 - z) \tag{8}$$

Equation 8 contains not only the quantity being measured, c_L, but also the ratios of densities and compressibilities of biomass and solvent. The ratio of densities is generally greater than 1 and lies $1 < \rho_P/\rho_L < 1.1$. The ratio of compressibilities lies $0.6 < \beta_P/\beta_L < 0.9$. Simulations of equation 8 reveal the influence of density and compressibility on sound velocity (Fig. 2 and 3). Here it is noticeable that a higher biomass density, compared with that of the solution, produces a reduction in sound velocity in the suspension, whereas the comparatively smaller biomass compressibility leads to an increase in sound velocity. Theoretically, sound velocity in a suspension can decrease, as well as increase, if the amount of biomass increases. On the conditions that the density and compressibility of the biomass remain constant and the amount by which the density of the solution changes also remains small, biomass content can be calculated from the differences measured between the sound velocity in the suspension and that of the cell-free solution. The fact that it is necessary to assume that the physical properties of the biomass are constant will be discussed in the last section.

Fluctuations of the solvent density have an almost negligible influence on the sound velocity calculated, according to equation 8, which is demonstrated in Fig. 4.

When microorganisms are added to a suspension, not only does the sound velocity change, but also its attenuation. Along with the sound absorption of the solvent and that of biomass, α_L, a scattering coefficient, α_P, enters as a consequence of the difference in acoustic impedance of solvent and cells (1, 2). As the contribution of the absorption due to

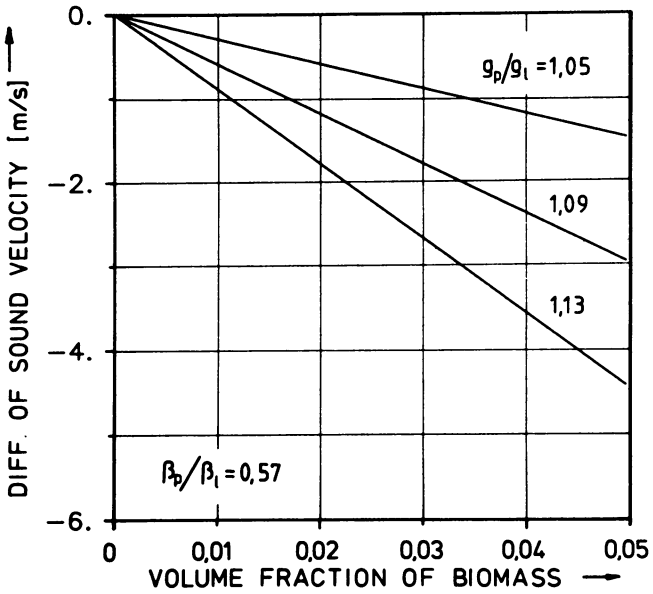

FIG. 2. Influence of the ratio of densities on the sound velocity according to equation 8 (T = 25°C).

the proportion of biomass is of a negligible size, biomass concentration must only be taken into consideration when calculating scattering. According to references 1 and 2, attenuation and scattering coefficients are described as follows:

$$\alpha_L = \eta_L \frac{2\pi^2 \cdot f^2}{\rho_L \cdot c_L^3} \tag{9}$$

$$\alpha_P = x \left[\frac{4}{3} \pi^4 \cdot f^4 \cdot \frac{R^3}{c^4} + \left(\frac{\rho_P}{\rho_L} - 1 \right)^2 \cdot \frac{s}{s^2 + \left(\frac{\rho_P}{\rho_L} + \tau \right)^2} \right] \tag{10}$$

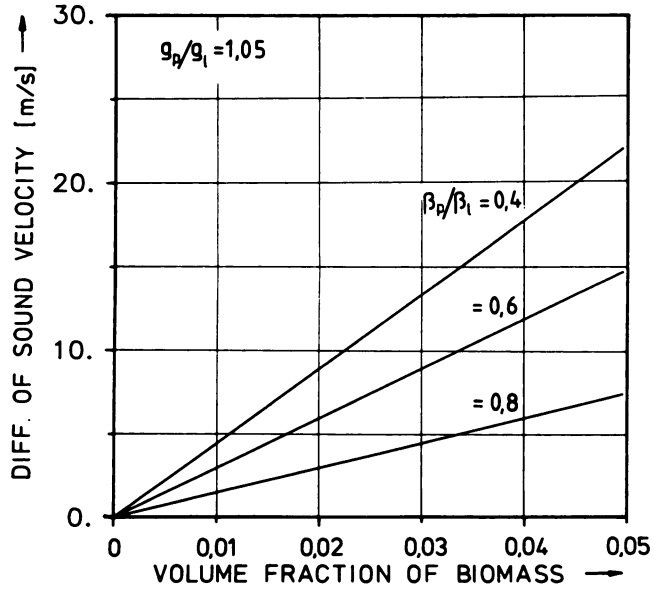

FIG. 3. Influence of the ratio of compressibilities on the sound velocity according to equation 8 (T = 25°C).

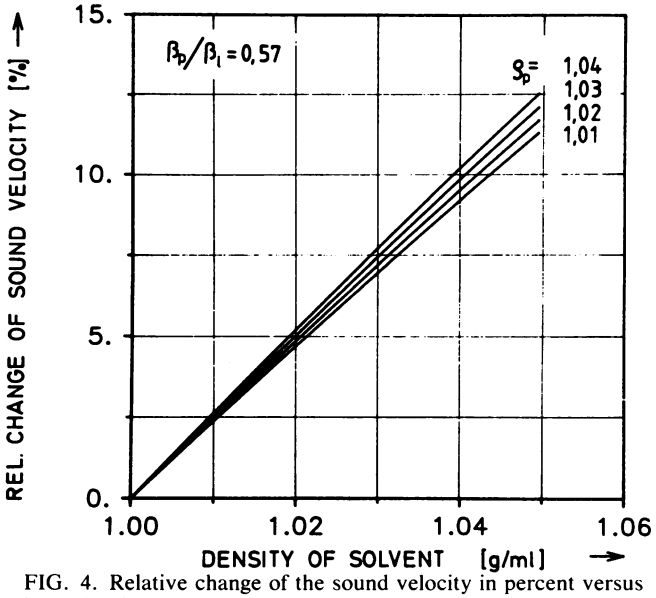

FIG. 4. Relative change of the sound velocity in percent versus solvent density according to equation 8 with ρ_P as a parameter. The ratio of the compressibilities remains constant (T = 25°C).

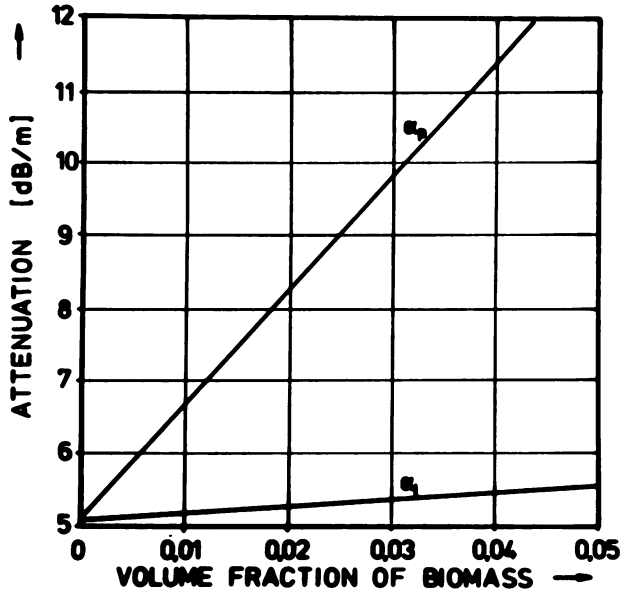

FIG. 5. Calculated sound attenuation in a suspension, versus volume fraction of biomass according to equations 9 and 10 for a frequency of 5 MHz and a diameter of spherical cells of 5 μm.

with $s = \frac{9}{4bR} \cdot \left(1 + \frac{1}{bR}\right)$, $b = \left(\frac{\pi f}{\nu_L}\right)^{1/2}$, and $\tau = \frac{1}{2} + \frac{9}{4bR}$.

Attenuation in a suspension is made up of the following two components:

$$\alpha_S = \alpha_L + \alpha_P \tag{11}$$

This additional attenuation is proportional to the share of particles in the total volume and can be represented as being dependent on the physical properties of density, compressibility, viscosity, and frequency. The changes taking place in the solvent's sound attenuation during a biological process depend primarily on its viscosity. If the viscosity remains roughly constant, then the concentration of biomass, given that the physical properties (density and compressibility) are also known, can be calculated according to attenuation measurements obtained. Figure 5 shows the attenuation calculated according to equations 9 and 10.

MATERIALS AND METHODS

Apparatus. The measuring equipment used (Fig. 6) consists of a computer, an ultrasound pulser/receiver unit, an analog/digital converter, an oscilloscope, and the measuring receptacle itself. The ultrasound pulser/receiver unit used consists of an ultrasonic flaw detector (USIP 11, Krautkrämer). The transducers were broad-band piezoelectric crystals with a nominal frequency of 5 or 10 MHz. The pulse length was around 0.2 μs, and the repetition rate was 200 Hz. The scanning of the signals in the time domain was carried out by using an eight-bit, two-channel, transient digitalizer (R7612D; Tektronix) with a capacity of 2,048 bytes. Its maximum sampling rate is 200 MHz. The digitalized signals were transferred via an IEC-Bus interface to an IBM-AT computer and evaluated.

The suspensions were in a Plexiglas measuring chamber, surrounded by a water bath. The inner length of the Plexiglas chamber measured 10 cm. In the reflection mode the path distance travelled by the pulse corresponds to double the inner length of the chamber. The transducer is outside the measuring chamber and the inner walls of the chamber serve as reflectors. The temperature is controlled with the aid of a thermostat.

Methods. All measurements were carried out by the ultrasonic pulse-echo technique. A high-frequency ultrasonic pulse is generated in a piezoelectric transducer and transmitted. This is reflected onto a reflector, received again by the transducer, and transformed into an electric signal. The time period covered from the transmission to the reception of the signal is scanned by the transient digitalizer, and the digitalized signal is stored in the computer. During the signal analysis the total travel time, t, of the signal is calculated. The sound velocity can be worked out when the signal's path length is known. The pressure amplitude of the impulse is also recorded to enable the calculation of sound attenuation.

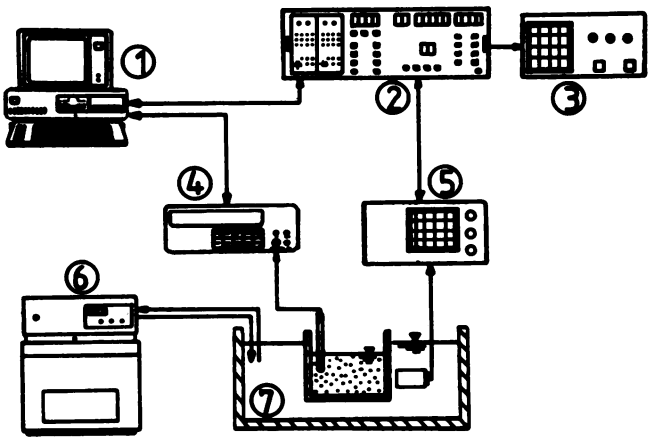

FIG. 6. Block diagram of the apparatus used: 1, IBM-AT computer; 2, transient recorder; 3, oscilloscope; 4, temperature measuring instrument; 5, ultrasonic pulse receiver; 6, thermostat; 7, water bath with chamber and transducer.

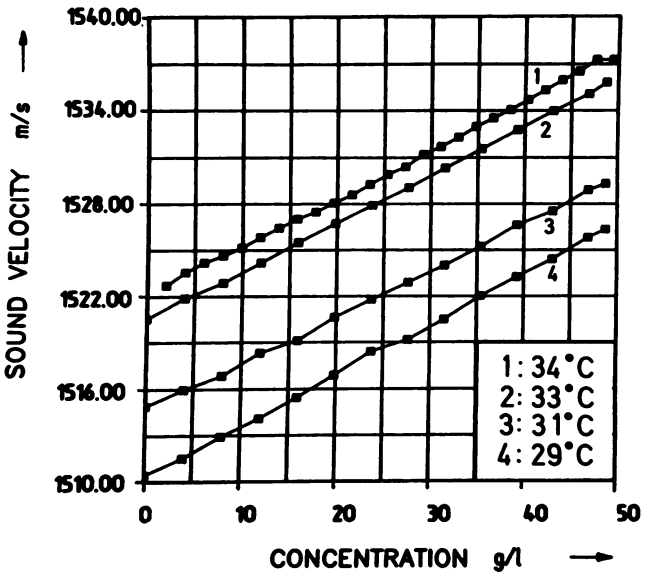

FIG. 7. Temperature dependence of the sound velocity in a glucose-water solution.

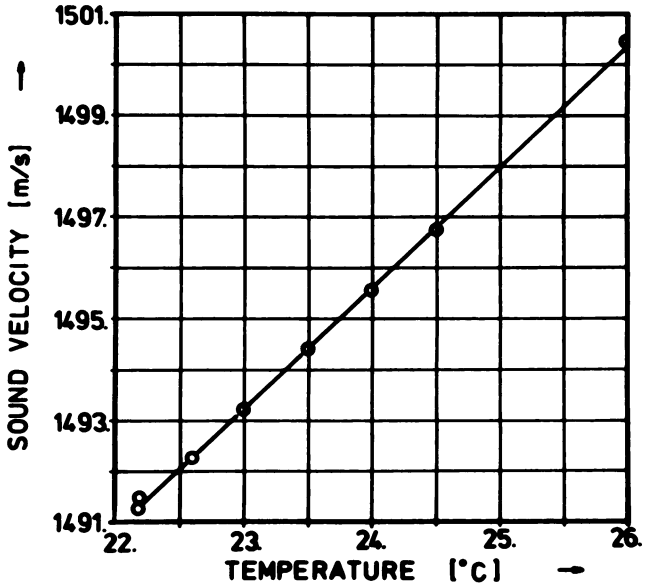

FIG. 8. Temperature dependence of the sound velocity in a suspension of *S. tendae*.

To increase the reliability of the measurements, the results represent averages taken from each of a series of 30 separate measurements. When using this measuring method, the maximal measuring error in sound velocity can be calculated by

$$\Delta c = -\frac{D}{t^2} \cdot \Delta t + \frac{\Delta D}{t} \qquad (12)$$

As the path length D is constant, the only remaining source of error is the scanning accuracy of the A/D converter. The error of measurement is at the most 10 ns at a sampling rate of 200 MHz. The level of inaccuracy when calculating sound velocity is therefore ±0.05 m/s. To minimize the error due to temperature fluctuations of the thermostat, the temperature was always exactly recorded at 0.01 K during a series of measurements, and the calculated sound velocity was converted using a reference temperature. It can be assumed that the temperature remained constant during a single measuring procedure lasting only a few milliseconds.

Cell concentration was determined by ascertaining the dry weight. The microorganisms were cultivated in culture bottles or in fermentors with standard culture media.

RESULTS

Experiments carried out to ascertain how far sound velocity depends on temperature in water-glucose solutions and suspensions of microorganisms revealed an almost linear relationship (Fig. 7 and 8). However, such a linear approximation is only valid for a very small temperature range. Generally, the quadratic influence of temperature on sound velocity must be taken into consideration. In Table 1 the temperature gradients of a number of solutions are shown. These temperature gradients emphasize the necessity and the importance of keeping the temperature constant, measuring the temperature precisely, and converting the results by using a reference temperature. By comparing the figures in Table 1 it can be seen that the conversion of sound velocities in suspensions at the given reference temperature, using the temperature gradient of water of 2.29 m/(s · K) (between 20 and 35°C), causes a measuring error which is under the measuring accuracy of our equipment.

For the series of measurements undertaken, we produced a series of dilution shakes composed of different microorganisms and determined, in both the suspensions and solvents, the sound velocity and attenuation. Figure 9 shows a characteristic curve of *Saccharomyces cerevisiae* in an aqueous medium. By using the multiphase model, it can be seen that the measurements taken and the predictions made correspond to each other to a large degree. In Fig. 10 the characteristic curves of a number of different microorganisms investigated are shown. Figure 11 shows the dependency of attenuation in a suspension of *Streptomyces tendae* on concentration.

DISCUSSION

Measurements made to date of morphologically different microorganisms reveal a great degree of similarity to the theoretical model. According to the theoretical model, there is an almost linear dependence between the volume fraction of biomass and the difference between the two sound velocities. Attempts made to determine biomass concentration

TABLE 1. Temperature coefficient of the sound velocity of different liquids in small temperature ranges

Medium	Temp coefficient (m/s · K)	Concn range (g/liter)	Temp range (°C)
Water	2.29		20–35
Water + glucose	2.27	0–50.0	22–34
Water + peptone	2.49	0–10.0	28–32
Nutrient broth[a]	2.36		22–26
Suspension of yeast	2.57	0–30.0	21–33
Suspension of *Lactobacillus*[a]	2.15	0–0.4	24–34
Suspension of *S. tendae*	2.43	0–0.4	22–26

[a] Czapek Dox medium.

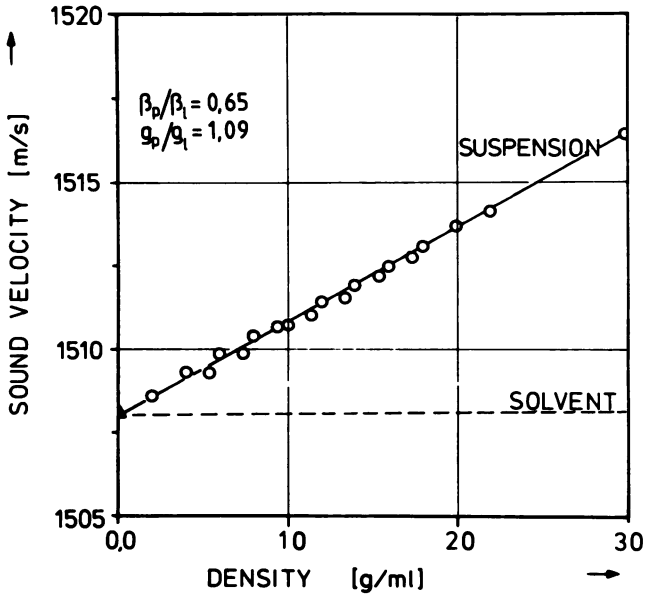

FIG. 9. Sound velocity in a suspension of *S. cerevisae* versus concentration (T = 28°C).

from the sound velocity of the suspension alone have failed. This is due to the fact that during the fermentation process, the different influences brought about by changes in densities and compressibilities compensate each other, so that sound velocity remains roughly constant. Due to the nutrient consumption, sound velocity decreases, and as a result of the increase in biomass, sound velocity increases. Therefore a reference measurement in a cell-free solution must be carried out. A filtration of the reference sample, which is therefore necessary, presents, however, only a few technical problems. The accuracy of the reading of biomass content can be seen, on the one hand, by the gradient of the characteristic curve (Fig. 10) and, on the other hand, by the accurate measurements taken of sound velocity and sound attenuation. When yeast cells were used, this resulted in a resolution of biomass of 10^{-4} of the total volume, corresponding to a concentration of around 10^6 cells per ml.

The advantage of this method lies in the fact that it can also be employed when cell concentration is high because the depth of ultrasonic penetration is also sufficiently large.

The method described here to measure sound velocity is based on two assumptions. First, it is assumed that the composition of a solvent changes during a biological process while its density, however, remains roughly constant. The second assumption is that the density and compressibility of biomass are constant. Whereas the first assumption applies to many processes and can be proved to do so, the second assumption can only be verified with difficulty. In relevant literature there is no information on the compressibility of cells. The only exception is to be found in experiments on erythrocytes (9). In these experiments compressibility was measured using readings of sound velocity. We also used this method to determine the unknown compressibility of biomass. By this way the compressibility of microorganisms can be calculated with the aid of characteristic curves, which show the relationship between sound velocity and concentration after equation 8 is solved for the compressibility.

In this way the technique for taking practical measurements is determined. When the physical properties and

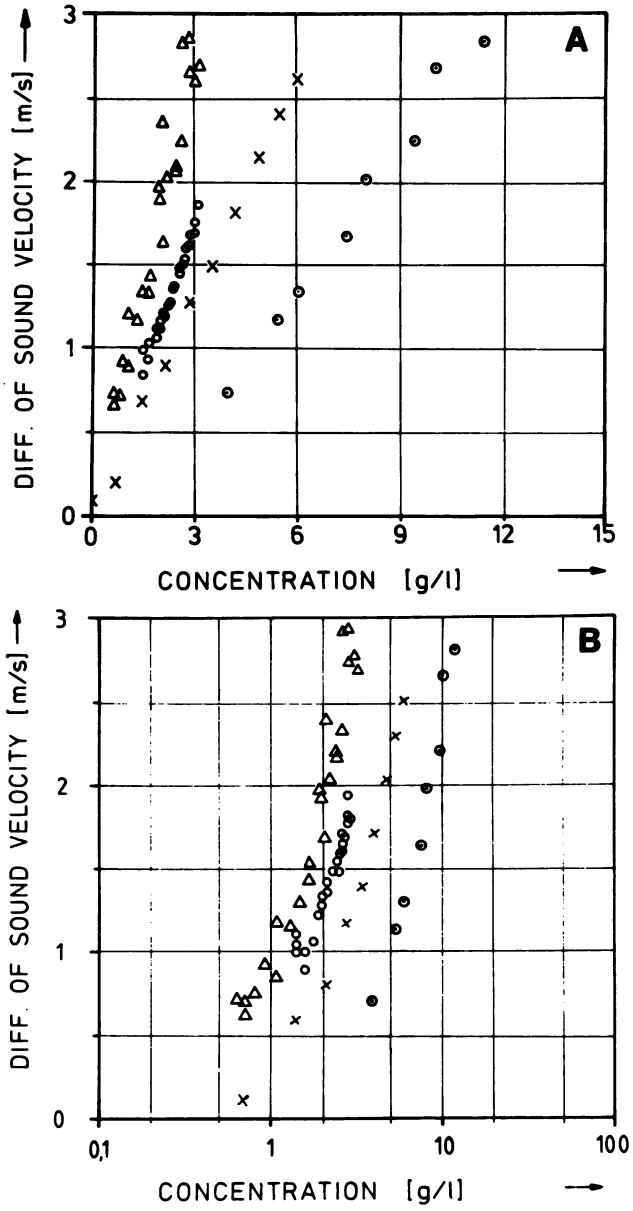

FIG. 10. Sound velocities in suspensions of different microorganisms versus concentration (T = 28°C). (A) Logarithmic scale; (B) linear scale. Symbols: ⊙, *S. cerevisiae*; ○, *Aspergillus niger*; Δ, *Penicillium chrysogenum*; ×, *Lactobacillus bulgaricus*.

especially the cell compressibility are known, equation 7 can be used to calculate the concentration from the sound velocity measured. When the densities and compressibilities of each phase are unknown, the first thing that must be done is to draw up a calibration curve of the relationship between velocity and concentration for each organism investigated. By doing this, two things are achieved: the physical characteristics can be calculated, and the curve can also be directly used to determine concentration of biomass.

At this time we were not able to take on-line measurements during fermentation with the realized system, because the smallest sample quantity needed for a measurement is 400 ml which results in an unacceptable loss of volume in the reactor.

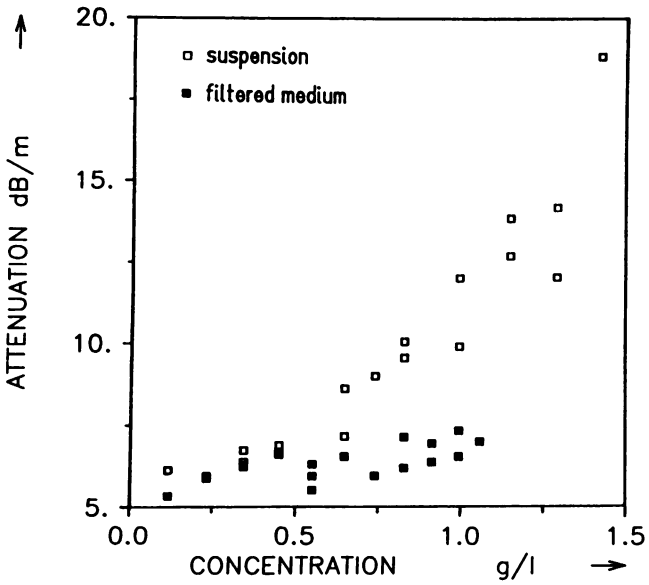

FIG. 11. Sound attenuation in a suspension of *S. tendae* and solvent, versus volume fraction of biomass.

Owing to the necessity of taking a reference measurement in the solvent, the sound measurements were taken outside the reactor. When biomass is determined by sound attenuation measurements, however, rather than by sound velocity measurements, one has the added advantage of being able to carry out these measurements inside the reactor. Figure 12 shows the construction of a sterilizable sonic probe for installation in the reactor itself. In this case it is necessary to minimize the influence of bubbles in the reactor, using constructional solutions as well as statistical methods of signal analysis. The disadvantage of this method is that in comparison with the sound velocity measuring method the resolution is rather small. In our experiments we only obtained a resolution of 0.2 dB/m, corresponding to a biomass concentration of around 10^8 cells per ml. A prerequisite for using this technique is that the absorption of the solvent, compared with the attenuation caused by biomass, is negligible. The correlation between attenuation and biomass can be obtained by using equations 9 and 10 or by drawing up a calibration curve.

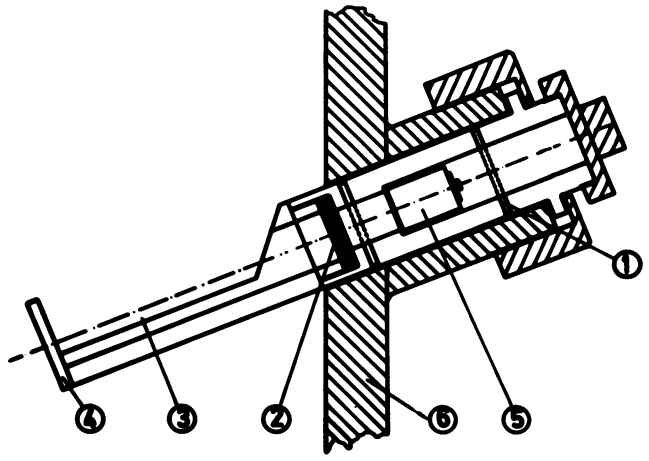

FIG. 12. Ultrasonic probe for the measuring of the sound attenuation in a bioreactor: 1, rubber seal; 2, interlayer; 3, sound path; 4, reflector; 5, transducer; 6, reactor wall.

Future prospects. To be able to take full advantage of the velocity measurement technique, we are currently developing an acoustic-resonant interferometer (7) in which the total sample volume for both comparative measurements will only be 5 ml. The acoustic interferometric method does not measure the travel time of a pulse, but measures the resonance frequency of the measuring chamber filled with suspension. The changes which take place in resonance frequency are a linear function of sound velocity in the measuring solution. Such an interferometric cell can be coupled with a bioreactor via a sampling system. While taking measurements, care must be taken to ensure that the measuring solutions are free of bubbles, as the gaseous phase influences sound velocity. In principle it is conceivable that such a chamber could be integrated with a bypass line with return, as the transducer can be separated from the actual substance to be measured by a coupling medium. We found an initial attempt to construct an interferometric chamber directly inside a batch reactor described reference 6. The apparatus constructed consists of two membranes with piezoelectric properties lying parallel to one another in a reactor. One of these membranes is driven with an alternating current voltage, and from the other membrane readings are taken on the amplitude of the output voltage received. By converting the signals taken in reference 6 to sound velocities, a plausible interpretation of the relationships between the density and compressibility of the solution measured is possible.

It is very unlikely that the microorganisms can be harmed by the installation of the sonic probe into the reactor, as the intensity of the ultrasonic pulse (<10 mW/cm^2) lies in the diagnostic area.

A decisive advantage of the interferometric method is that the accuracy of the measurements taken, and thereby the resolution of biomass content, is increased by a factor of 5, a large improvement on previously used methods. In addition, as the measuring system is smaller, a practical and reasonably priced system for determining biomass has become available.

ACKNOWLEDGMENT

We are grateful to the Deutsche Forschungsgemeinschaft for their support of this project (DFG Fa 111/7-1).

LITERATURE CITED

1. **Allegra, J., and S. A. Hawley.** 1972. Attenuation of sound in suspensions and emulsions: theory and experiments. J. Acoust. Soc. Am. **51**:1545–1557.
2. **Bergmann, L.** 1954. Der Ultraschall. Stuttgart Hirzel Verlag, Stuttgart.
3. **Davis, M. C.** 1978. Coal slurry diagnostics by ultrasound transmission. J. Acoust. Soc. Am. **64**:406–410.
4. **Gladwell, N., C. Javanaud, R. C. Chivers, and L. Anson.** 1987. Measurements and interpretation of ultrasonic velocity and attenuation in low concentration polystyrene dispersions. Ultrasonics **25**:340.
5. **Harris, H. C., and D. B. Kell.** 1985. The estimation of microbial biomass. Biosensors **1**:17–84.
6. **Ishimori, Y., I. Karube, and S. Suzuki.** 1981. Determination of microbial population with piezoelectric membranes. Appl. Environ. Microbiol. **42**:632–637.
7. **Sarvazyan, A. P.** 1982. Development of methods of precise

ultrasonic measurements in small volumes of liquids. Ultrasonics **21**:151–154.
8. **Sarvazyn, A. P., A. G. Lyrchikov, and S. E. Gorelov.** 1987. Dependence of ultrasonic velocity in rabbit liver on water content and structure of the tissue. Ultrasonics **25**:244–247.
9. **Shung, K., and B. A. Krisko.** 1982. Acoustic measurement of erythrocyte compressibility. J. Acoust. Soc. Am. **72**:1364–1367.
10. **Urick, R. J., and W. S. Ament.** 1949. The propagation of sound in composite media. J. Acoust. Soc. Am. **21**:115–119.
11. **Watson, J. D., and R. Meister.** 1963. Ultrasonic absorption in water containing plankton in suspension. J. Acoust. Soc. Am. **35**:1584–1589.