Polarographic Assay of Hydrogen Peroxide Accumulation in Microbial Cultures

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A method is described for determining low concentrations of hydrogen peroxide by using a polarographic oxygen electrode to measure the oxygen released into solution on addition of catalase. A sample can be assayed directly without prior manipulation in 3 min. The method is capable of assaying hydrogen peroxide concentrations as low as 7 μM. The method has proved extremely useful for the assay of hydrogen peroxide secreted into milk by lactic acid bacteria.

Hydrogen peroxide is produced in microbial cultures due to the activity of certain oxidoreductase enzymes. The extracellular concentration is often quite low, especially in cultures which possess strong catalase (EC 1.11.1.6) or peroxidase (EC 1.11.1.7) activities. Special sample preparation is needed for the spectrophotometric assay methods capable of measuring such low concentrations, especially if the growth medium is opaque (1, 4, 5).

Modern oxygen electrodes are capable of measuring small variations in the dissolved oxygen concentration of aqueous liquids. We have taken advantage of this fact to develop a simple assay for hydrogen peroxide in culture media. The addition of excess catalase to a small sample of the untreated culture containing hydrogen peroxide caused the liberation of oxygen, which could be measured with an oxygen electrode, and related to the concentration of hydrogen peroxide using a standard curve.

MATERIALS AND METHODS

Apparatus. Culture samples were assayed for H_2O_2 in a specially constructed cylindrical glass cell (Fig. 1) of approximately 4-ml capacity, sealed at the lower end by insertion of the electrode portion of a Beckman oxygen analyzer (model 77701). The cell was water-jacketed to maintain a constant sample temperature of 25 C. The cell contents were continuously stirred during the assay by means of a Gallenkamp overhead stirrer (model SS540) equipped with a nichrome wire loop as a paddle. The stirrer was set at a speed which provided a flow rate of the sample of at least 55 cm/s, over the surface of the electrode. The adequacy of the flow rate was routinely checked by observing that a steady oxygen meter reading was obtained when no change in dissolved oxygen content was occurring in

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the cell. The oxygen analyzer meter was routinely calibrated to give a reading of 21% saturation in air.

Polarographic assay method. Medium containing H_2O_2 (2.9 ml) was added to the glass cell and stirred to give the desired flow rate. On establishment of a steady reading on the dissolved oxygen meter (or coupled recorder), 0.1 ml of catalase solution containing 50 μg of purified beef liver catalase (Sigma London Chemical Co.) in 0.05 M phosphate buffer, pH 7.0, was pipetted quickly (using a 0.1-ml constriction pipette) into the bottom of the cell. The continuous stirring ensured rapid mixing of the catalase with the other assay constituents. The immediate rise in dissolved O_2 level was noted on the analyzer or recorder.

Test media. The method was tested in a minimal medium, a complex medium, and reconstituted skim milk. The minimal medium contained 2 g of glucose, 1 g of NH_4Cl, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.01 g of $FeSO_4 \cdot 7H_2O$, and 0.01 g of $CaCl_2$ per liter of 0.05 M Na-K phosphate buffer, pH 7.0. The complex medium consisted of 10 g of yeast extract per liter of minimal medium, and the reconstituted skim milk contained 100 g of nonfat milk solids per liter. The media were sterilized in an autoclave at 15 lb/in² for 5 (reconstituted skim milk) or 15 (minimal and complex media) min.

Stability of H_2O_2. The stability of H_2O_2 was measured in sterile reconstituted skim milk and minimal glucose (0.2%) and complex media. H_2O_2 was added to a concentration of 0.134 mM to 100-ml Erlenmeyer flasks containing 50-ml amounts of each medium. The flasks were held at 20 C without agitation for 5 h, and the H_2O_2 concentration was estimated periodically.

The rate of O_2 loss from supersaturated solutions. The rate of O_2 loss from supersaturated solutions to the air above the assay cell was measured. H_2O_2 was added to the assay cell containing reconstituted sterile skim milk or minimal glucose (0.2%) or complex media. The concentrations of H_2O_2 used (0.26 mM) were such that on addition of catalase the dissolved O_2 level in the cell increased to approximately 38% saturation. The dissolved O_2 concentration gradually decreased towards the normal calibra-
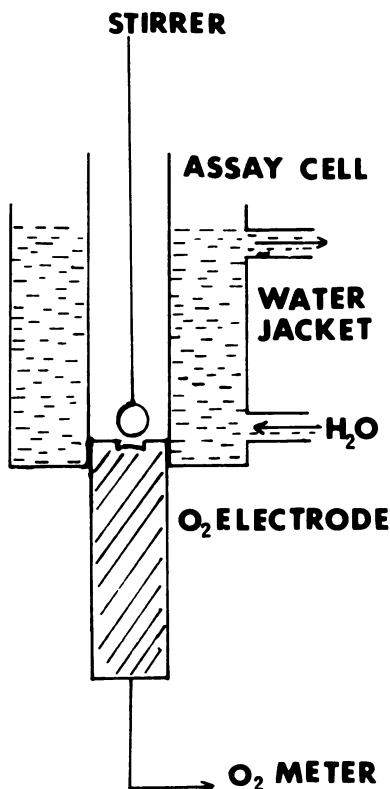
FIG. 1. *Diagrammatic representation of the H_2O_2 assay cell.*

tion level of 21% O_2 saturation. The decrease in dissolved O_2 content of each medium was recorded over a 2- to 3-min period, starting with a concentration of 37% O_2 saturation.

Growth of test culture. *Streptococcus lactis* C10 was used to demonstrate H_2O_2 accumulation during growth. Cultures were grown in 2-liter quantities of sterile reconstituted skim milk in a New Brunswick bench top fermenter at 25 C and agitated at 250 rpm. The pH was maintained constant at pH 6.6 by the automatic addition, on demand, of 1 M NaOH from a burette. The lactic acid developed by the culture was calculated from the amount of NaOH needed to maintain the pH at 6.6. Samples were withdrawn periodically to determine H_2O_2 concentrations.

RESULTS

Assay time. Figure 2 is a typical recorder tracing showing the increase in dissolved O_2 content on addition of purified catalase to a sample of reconstituted skim milk containing H_2O_2. The level of O_2 in freshly sterilized media (or growing cultures) was often less than that of air (21% saturation). However, a steady reading in the oxygen analyzer meter was invariably established in 1 to 2 min, after which 50 μg of

catalase was added. The maximum increase in dissolved O_2 content was reached in approximately 1 min, making a total assay time of 2- to 3-min duration.

Standard curves. Standard curves were constructed from data relating the increase in dissolved O_2 content on addition of catalase to known concentrations of H_2O_2, as determined by sodium thiosulfate titration. The H_2O_2 solutions were in minimal or complex media or in reconstituted skim milk. Similar graphs were obtained for the minimal and complex media used (Fig. 3). The relationship between increases in dissolved O_2 content and H_2O_2 concentration was linear as far as 2.0 mM. H_2O_2 concentrations as low as 7 μM could be accurately estimated (standard deviation of 5.4% at 6.8 μM). The skim milk standard curve obtained was similar to the other two, except that the increase in dissolved O_2 content was slightly greater at any given H_2O_2 concentration. The relationship between increases in dissolved O_2 content and H_2O_2 concentration in skim milk was found to be linear as far as 2.0 mM. The fact that the dissolved O_2 increase, on addition of catalase to identical concentrations of H_2O_2, was greater in sterile reconstituted skim milk than in sterile minimal glucose or complex media was investigated further. The higher readings were not due to a greater stability of H_2O_2 in skim milk than in the other media. H_2O_2 was considerably less stable in skim milk than in either minimal glucose or complex media (Fig. 4). A possible explanation for the relatively high readings in skim milk is its greater capacity to retain O_2 than either minimal glucose or complex media. Supersaturated
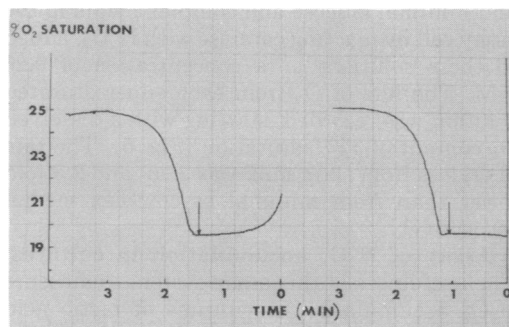

FIG. 2. *Typical recorder tracings showing the time course of the H_2O_2 assay. The oxygen analyzer was calibrated to give a reading of 21% saturation in air. The assay samples, containing 0.156 mM H_2O_2 in sterile reconstituted skim milk, were added to the cell at zero time. After establishment of an equilibrium, catalase was added at the time indicated by the arrows.*
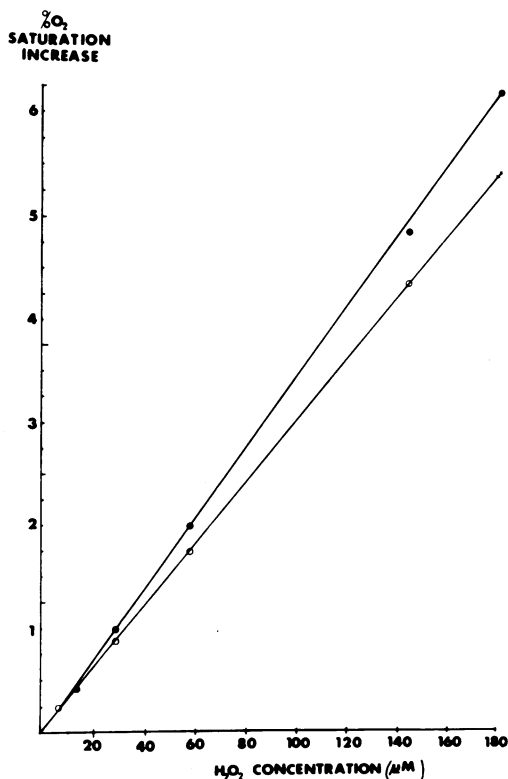
FIG. 3. *Standard curves relating the increase in dissolved oxygen content (as % O_2 saturation increase) on addition of catalase to solutions containing known concentrations of H_2O_2. The oxygen analyzer was calibrated to give a reading of 21% saturation in air. Symbols: O, minimal and complex media (data points coincident);* ●, *reconstituted skim milk.*

solutions of O_2 were made in sterile skim milk and minimal glucose and complex media in the assay cell by reacting catalase with H_2O_2, added to these solutions at a concentration of 0.26 mM. The loss of O_2 from each supersaturated solution was recorded, starting with a dissolved O_2 content of 37% saturation (Fig. 5). The rate of O_2 loss from skim milk was 2.25- and 2.5-fold lower than from minimal or complex media, respectively.

Assay of H_2O_2 accumulation in cultures. The usefulness of this assay system to measure H_2O_2 accumulation in cultures of lactic acid bacteria growing in opaque media, such as milk, was established in several experiments, of which that represented in Fig. 6 is a typical example. H_2O_2 accumulation was measured in a culture of *S. lactis* C10 growing in sterilized reconstituted skim milk in a fermenter. The culture was continuously stirred at 250 rpm. H_2O_2 accumulated gradually, following a pro-

duction course roughly parallel to that of acid development, and reached a concentration in excess of 0.1 mM after 6 to 7 h.

DISCUSSION

The outstanding features of this assay method are speed, simplicity, and sensitivity. Once the oxygen analyzer has been calibrated, samples can be routinely assayed for H_2O_2 in 2
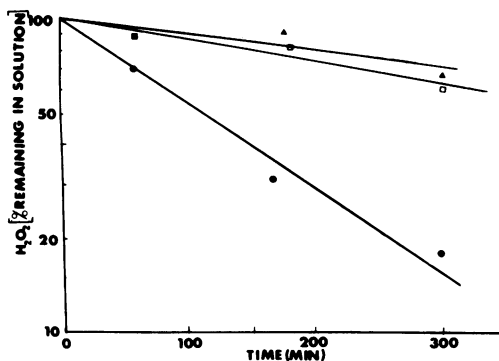

FIG. 4. *Decomposition of H_2O_2 in sterile media as a function of time at 20 C. Symbols:* ●, *reconstituted skim milk;* ▲, *minimal glucose (0.2%) medium;* □, *complex medium.*
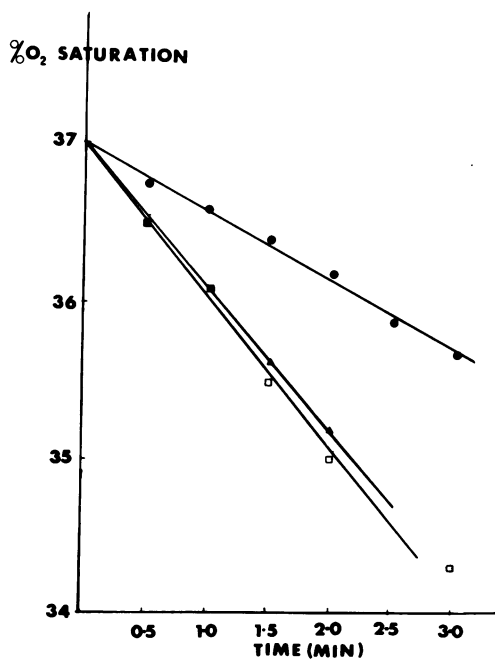

FIG. 5. *Rate of O_2 lost from sterile solutions with greater than 21% (air saturation concentration) of dissolved O_2. Symbols:* ●, *reconstituted skim milk;* ▲, *minimal glucose (0.2%) medium;* □, *complex medium.*
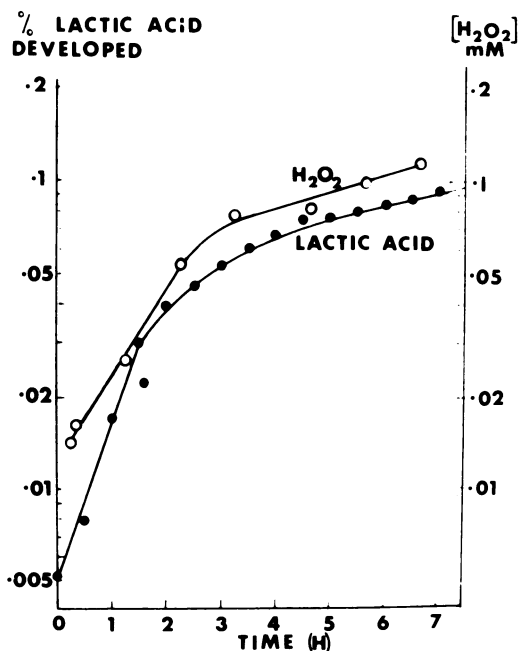
FIG. 6. *Accumulation of H_2O_2 in a stirred culture of S. lactis C10 in sterilized reconstituted skim milk. Symbols:* O, H_2O_2; ●, *growth as developed lactic acid.*

to 3 min. Samples of cultures can be assayed directly without prior treatments such as removal of cells or production of clear filtrates from opaque media, such as milk, steps which are essential in spectrophotometric assays (1, 4, 5). Spectrophotometric assays have the additional disadvantage that complex media ingredients may absorb significantly at the wavelengths employed in such assays, thus causing interference.

The sensitivity of the polarographic assay method described compares favorably with that of reported spectrophotometric assays. Concentrations as low as 7 μM could be reliably measured, which means that the assay is five to ten times as sensitive as the methods of Gilliland (5) and Ferrier et al. (4).

The establishment of a steady reading for dissolved O_2 content of assay samples within 1 to 2 min (Fig. 2) may seem surprising. Some samples had O_2 levels lower than that of the air above the assay cell (21% saturation). O_2 would be expected to diffuse into the stirred sample from the air above the cell. The steady dissolved O_2 reading attained before catalase addition, within 1 to 2 min after starting an assay, indicates that O_2 diffusion into the assay cell does not contribute significantly to the dis-

solved O_2 level of assay samples over the short assay time.

The present work indicates that calibration curves constructed from data obtained using a particular medium are only applicable to media of similar composition. Dissolved oxygen increases, obtained from the breakdown of given concentrations of H_2O_2 in reconstituted skim milk, were greater than those in chemically defined or broth media. This is not due to a greater stability of hydrogen peroxide in skim milk. As other workers have previously shown (2, 5), H_2O_2 was quite unstable in sterile skim milk (Fig. 4) and much more unstable than in minimal glucose or complex media. The most likely explanation for the higher dissolved O_2 concentrations in skim milk, compared with the other two media, is the former's greater capacity to retain O_2 in supersaturated solution (Fig. 5). From Fig. 3 it is evident that the greatest discrepancies between skim milk O_2 levels and minimal and complex media O_2 levels occurred at the higher concentrations of dissolved O_2, i.e., where the degree of supersaturation was greatest. It is likely that more O_2 is lost from solution during assay from minimal and complex media than from skim milk, and that the discrepancy increases as the concentration of H_2O_2 in the assay cell increases.

The method described has been successfully applied to the assay of H_2O_2 in cultures of lactic acid bacteria such as *S. lactis* C10 (Fig. 6). The concentrations produced were well within the range of the assay. The pattern of H_2O_2 accumulation resembled those previously obtained for aerated cultures of *S. lactis* (6) and a *Lactobacillus plantarum* strain (8). The concentration of H_2O_2 accumulated in the aerated skim milk culture after 7 h was 0.1 mM, which is similar to that reported by Hogg and Jago (7) for the same strain. The data of Fig. 6 also show that the rate of acid production by *S. lactis* C10 in aerated skim milk culture was being inhibited at a very low developed acidity. Other workers (3) have had a similar experience with this strain, growing under aerobic conditions, and attributed the inhibition to H_2O_2 accumulation. The concentration of H_2O_2 at the time that acid development was being inhibited in the C10 culture of the present study (Fig. 6) ranged from 0.03 to 0.1 mM. Work at this (M. Keane, M.Sc. dissertation, University College, Cork, 1973) and other laboratories (3, 9) has shown that addition of H_2O_2 at concentrations ranging from 0.029 to 2.3 mM inhibited acid production by several strains of lactic streptococci, including *S. lactis* C10 growing in milk.

The polarographic method allows, therefore, the easy detection of H_2O_2 concentrations reported to inhibit lactic acid bacteria. In addition, this assay method could find application in food industries which routinely use H_2O_2 as a sterilizing agent. Low, but still unwelcome, levels of residual H_2O_2 could be easily detected in any liquid or solid food after emulsification.

ACKNOWLEDGMENTS

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