Polarographic Assay of Hydrogen Peroxide Accumulation in Microbial Cultures

PATRICK M. DEMPSEY, JOSEPH O'LEARY, and SEAMUS CONDON
Dairy and Food Microbiology Department, University College, Cork, Ireland

Received for publication 1 October 1974

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 H₂O₂ 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 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 SS40) 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 56 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 the cell. The oxygen analyzer meter was routinely calibrated to give a reading of 21% saturation in air.

Polarographic assay method. Medium containing H₂O₂ (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 O₂ 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 NH₄Cl, 0.2 g of MgSO₄·7H₂O, 0.01 g of FeSO₄·7H₂O, and 0.01 g of CaCl₂ 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 H₂O₂. The stability of H₂O₂ was measured in sterile reconstituted skim milk and minimal glucose (0.2%) and complex media. H₂O₂ was added to a concentration of 0.154 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 H₂O₂ concentration was estimated periodically.

The rate of O₂ loss from supersaturated solutions. The rate of O₂ loss from supersaturated solutions to the air above the assay cell was measured. H₂O₂ was added to the assay cell containing reconstituted sterile skim milk or minimal glucose (0.2%) or complex media. The concentrations of H₂O₂ used (0.26 mM) were such that on addition of catalase the dissolved O₂ level in the cell increased to approximately 38% saturation. The dissolved O₂ concentration gradually decreased towards the normal calibra-
catalase was added. The maximum increase in dissolved O₂ 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 O₂ content on addition of catalase to known concentrations of H₂O₂, as determined by sodium thiosulfate titration. The H₂O₂ 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 O₂ content and H₂O₂ concentration was linear as far as 2.0 mM. H₂O₂ 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 O₂ content was slightly greater at any given H₂O₂ concentration. The relationship between increases in dissolved O₂ content and H₂O₂ concentration in skim milk was found to be linear as far as 2.0 mM. The fact that the dissolved O₂ increase, on addition of catalase to identical concentrations of H₂O₂, 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 H₂O₂ in skim milk than in the other media. H₂O₂ 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 O₂ than either minimal glucose or complex media. Supersaturated

![Diagram of the H₂O₂ assay cell](image)

**RESULTS**

**Assay time.** Figure 2 is a typical recorder tracing showing the increase in dissolved O₂ content on addition of purified catalase to a sample of reconstituted skim milk containing H₂O₂. The level of O₂ 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

![Typical recorder tracings showing the time course of the H₂O₂ assay](image)
production 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$_2$O$_2$ in 2

![Graph](image1)

**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$_2$O$_2$. 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$_2$O$_2$, 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$_2$O$_2$ accumulation in cultures.**

The usefulness of this assay system to measure H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ accumulated gradually, following a pro-

![Graph](image2)

**FIG. 4.** Decomposition of H$_2$O$_2$ in sterile media as a function of time at 20°C. Symbols: ●, reconstituted skim milk; ▲, minimal glucose (0.2%) medium; ○, complex medium.

![Graph](image3)

**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.
from the air within does samples indicate that dissolved be land (5) and Ferrier measured, ten of O₂ above the method of Gilliland (5) and Ferrier et al. (4).

The establishment of a steady reading for dissolved O₂ content of assay samples within 1 to 2 min (Fig. 2) may seem surprising. Some samples had O₂ levels lower than that of the air above the assay cell (21% saturation). O₂ would be expected to diffuse into the stirred sample from the air above the cell. The steady dissolved O₂ reading attained before catalase addition, within 1 to 2 min after starting an assay, indicates that O₂ diffusion into the assay cell does not contribute significantly to the dissolved O₂ 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 H₂O₂ 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), H₂O₂ 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 O₂ concentrations in skim milk, compared with the other two media, is the former's greater capacity to retain O₂ in supersaturated solution (Fig. 5). From Fig. 3 it is evident that the greatest discrepancies between skim milk O₂ levels and minimal and complex media O₂ levels occurred at the higher concentrations of dissolved O₂, i.e., where the degree of supersaturation was greatest. It is likely that more O₂ is lost from solution during assay from minimal and complex media than from skim milk, and that the discrepancy increases as the concentration of H₂O₂ in the assay cell increases.

The method described has been successfully applied to the assay of H₂O₂ 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 H₂O₂ accumulation resembled those previously obtained for aerated cultures of S. lactis (6) and a Lactobacillus plantarum strain (8). The concentration of H₂O₂ 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 H₂O₂ accumulation. The concentration of H₂O₂ 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 H₂O₂ 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.

**Fig. 6.** Accumulation of H₂O₂ in a stirred culture of S. lactis C10 in sterilized reconstituted skim milk. Symbols: O, H₂O₂, •, growth as developed lactic acid.
The polarographic method allows, therefore, the easy detection of \( \text{H}_2\text{O}_2 \) concentrations reported to inhibit lactic acid bacteria. In addition, this assay method could find application in food industries which routinely use \( \text{H}_2\text{O}_2 \) as a sterilizing agent. Low, but still unwelcome, levels of residual \( \text{H}_2\text{O}_2 \) could be easily detected in any liquid or solid food after emulsification.

ACKNOWLEDGMENTS

We would like to express our appreciation to Liam Burgess and Pat Higgins for technical assistance.

LITERATURE CITED

1. Amin, V. M., and N. F. Olson. 1967. Spectrophotometric determinations of hydrogen peroxide in milk. J. Dairy Sci. 50:461–464.
2. Amin, V. M., and N. F. Olson. 1967. Effect of temperature on stability of hydrogen peroxide in milk. J. Dairy Sci. 50:1336–1338.
3. Anders, R. F., D. M. Hogg, and G. R. Jago. 1970. Formation of hydrogen peroxide by group N streptococci and its effect on their growth and metabolism. Appl. Microbiol. 19:608–612.
4. Ferrier, L. K., N. F. Olson, and T. Richardson. 1970. Analysis of hydrogen peroxide in milk using titanium tetrachloride. J. Dairy Sci. 53:598–599.
5. Gilliland, S. E. 1969. Enzymatic determination of residual hydrogen peroxide in milk. J. Dairy Sci. 52:321–324.
6. Gilliland, S. E., and M. L. Speck. 1969. Biological response of lactic streptococci and lactobacilli to catalase. Appl. Microbiol. 17:797–800.
7. Hogg, D. McC., and G. R. Jago. 1970. The inactivation of lactoperoxidase by group N streptococci. J. Dairy Res. 37:457–459.
8. Price, R. J., and J. S. Lee. 1970. Inhibition of Pseudomonas species by hydrogen peroxide producing lactobacilli. J. Milk Food Technol. 33:13–18.
9. Subramanian, C. S., and N. F. Olson. 1968. Effect of hydrogen peroxide on activity of lactic cultures in milk. J. Dairy Sci. 51:517–519.