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Oxidation-Reduction Potential and Growth of *Clostridium perfringens* and *Pseudomonas fluorescens*[1]

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A new apparatus was developed for measuring changes in E_h, pH, and cell numbers. With this apparatus, the relationships of these parameters were studied at initial E_h levels of 200 and 40 mv (pH 7.0), by using *Clostridium perfringens* and *Pseudomonas fluorescens*. One of the strains of *C. perfringens* grew more luxuriantly at the higher E_h, in the presence of small quantities of oxygen, than at the lower one in the absence of oxygen. *P. fluorescens* could grow at a relatively low E_h (40 mv, pH 7.0) in pure culture but not in the presence of *C. perfringens* under the same conditions.

A number of environmental factors affect the growth of bacteria; among these is the oxidation-reduction potential or E_h. This factor has acquired increased significance for the food microbiologist with the advent of the practice of packaging various meat products in evacuated, oxygen-impermeable films. According to Ingram (14), when meat is vacuum-packed in a relatively oxygen-impermeable membrane, the redox potential on the surface of the meat is reduced due to the respiration of the tissues; usually, sufficient oxygen remains to permit the growth of aerobic bacteria. Baran, Kraft, and Walker (2) observed growth of *Clostridium perfringens* in fresh ground beef in both vacuum and nonvacuum conditions; maximal growth, however, was obtained in the shortest period in samples packaged under vacuum.

Although various methods have been described in the literature for studying oxidation-reduction potentials (4, 7, 8, 11-13, 18, 26), none of these methods proved satisfactory for the purpose of measuring changes in E_h, pH, and numbers in pure or mixed bacterial cultures. Therefore, a new apparatus was developed to measure these parameters. In addition, this apparatus was used to study the relationships between these variables in bacterial cultures.

MATERIALS AND METHODS

Apparatus. The apparatus for measuring E_h, pH, and bacterial numbers (Fig. 1) was constructed from a 500-ml, three-neck, roundbottom distilling flask with the bottom flattened. A screw-cap culture tube (15 cm long and 1.5 cm inside diameter) with the bottom removed and a Pyrex tube (ca. 7.5 cm long and 1.5 cm inside diameter) were constructed onto the flask to provide openings for the inoculation tube and the salt bridge. The salt bridge was constructed from a piece of soft glass tubing (1.5 cm inside diameter), heated, and drawn out to capillary size at a place approximately 8.0 cm from one end. The gas inlet tube was constructed from a Pyrex tube (ca. 18 cm long and 0.6 cm outside diameter) and pinched at the bottom to provide two smaller openings. The gas outlet tube was also constructed from a Pyrex tube (0.6 cm outside diameter) but was provided with two glass bulbs to serve as traps. The upper ends of these tubes were plugged with cotton to prevent contamination. The gas inlet tube of the electrode vessel was connected to a gas manifold (not shown) via: (i) latex rubber tubing (ca. 22 cm long); (ii) capillary glass tubing (5 cm long and 0.1 cm bore); and (iii) latex rubber tubing (5 cm long). Use of capillary tubing to connect the gas manifold to the electrode vessel helped maintain a constant bubbling rate. Fine adjustments of bubbling rate were accomplished by placing a screw clamp on the rubber tubing between the gas manifold and the capillary tubing.

Two flow meters connected at the upper ends to a "T" tube were used (sizes 10 and 11, Cole-Parmer Instrument and Equipment Co., Chicago, Ill.) for monitoring prepurified nitrogen alone or to provide mixtures of prepurified nitrogen and compressed air. The size 10 meter was connected to the compressed air tank, and the size 11 meter was connected to the prepurified nitrogen tank. A tube (ca. 7 cm long and 1.5 cm outside diameter) packed with cotton served as a filter and was placed between the gas manifold and the flow meters.

The pH was measured with a general-purpose glass electrode (Corning 476022 or Beckman 42263) and a saturated calomel electrode (Corning 476002 or Beck-

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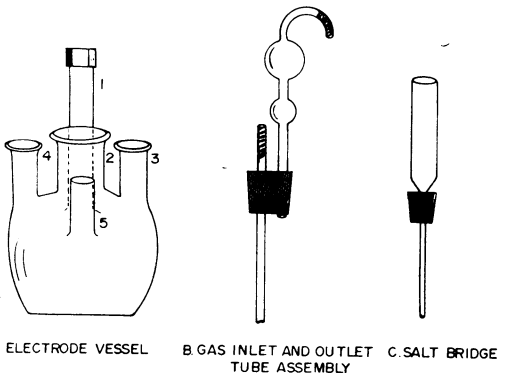

FIG. 1. *Schematic drawing of electrode vessel assembly for measuring E_h and pH of bacterial cultures. (A) electrode vessel; (1) screw-cap tube for introduction and removal of samples; (2) port for holding gas inlet and outlet tube assembly (B); (3 and 4) ports for holding glass and platinum electrodes; (5) port for holding salt bridge (C).*

man 39170). E_h was measured with a platinum inlay electrode (Corning 476060) and a saturated calomel reference electrode. For continuous measurement of E_h or *p*H, a Beckman Expandomatic *p*H meter was connected to a recorder (model VOM-7, Bausch & Lomb, Rochester, N.Y.). A potentiometer of 0 to 500 ohms, in series with a 200-ohm resistor, was attached across the input terminals of the recorder to expand the recorder scale to ± 500 mv with 0 at the center.

Standardization of pH and E_h electrodes. The *p*H changes were measured in millivolts rather than *p*H units to avoid changing the zero setting on the *p*H meter. A calibration curve of *p*H versus millivolt readings was prepared for each set of *p*H electrodes by using a series of five standard buffers (*p*H 4.01, 6.85, 6.99, 7.40, and 9.14, obtained from Beckman Co., Fullerton, Calif.). The buffers were equilibrated at 30 C before millivolt readings were taken. To standardize the E_h electrodes, phthalate buffer of *p*H 4.0 saturated with quinhydrone was used at 30 C; this system has an E_h of 456 mv.

Sterilization of apparatus. A 350-ml amount of Trypticase Soy Broth (Baltimore Biological Laboratory, Baltimore, Md.) was sterilized in the electrode vessel at 121 C for 15 min. The salt bridge, gas inlet, and outlet tube assembly were sterilized at 121 C for 25 min.

Platinum and glass electrodes were sterilized by immersion in a solution containing 0.05% sodium hypochlorite for 15 min, followed by rinsing three times in sterile distilled water and drying with a square of sterile cheesecloth before insertion in the electrode vessel.

KCl-agar was prepared by dissolving 3 g of agar in 100 ml water containing 35 g of KCl. The mixture was autoclaved at 121 C for 15 min. The salt bridge was prepared by pouring the hot KCl-agar into the bridge, held in a near horizontal position to allow the KCl-agar to solidify while slowly flowing out.

To prevent contamination during assembly, all sterile materials were placed in a bacteriological hood equipped with a germicidal lamp. The ultraviolet light was left on 1 to 2 hr before assembly of the electrode vessels.

Equilibration of the medium. After the apparatus was assembled and before inoculation, the flasks were placed in a water bath maintained at 30 ± 0.5 C and connected to the gas manifold. The flow rates of the gases were adjusted so that 120 ml/min registered on the flow meter. The medium was purged with prepurified nitrogen until the E_h equilibrated; this usually required 72 hr. When a more positive E_h was desired, the prepurified nitrogen was mixed with compressed air adjusted to a flow rate of 0.2 to 0.5 ml/min. This gas mixture was bubbled through the medium until a stable E_h was maintained, usually after 48 to 72 hr.

Measurement of oxygen. Percentage of oxygen in the gas mixture was analyzed on a gas chromatograph (model 810, F & M Scientific Corp., Avondale, Pa.), as recommended by Lyle A. Douglas (*personal communication*). A sample of the gas mixture was removed with a 1-ml gas syringe (Precision Sampling Corp., Baton Rouge, La.) and applied to a molecular sieve column (Anasorb, 70/80 mesh, Analabs Inc., Hamden, Conn.). The column [6 ft by 0.25 inch (1.83 m by 0.63 cm)] was activated by heating at 400 C for 4 hr before use. A temperature of 70 C was maintained during gas analyses. The thermal conductivity detector was also maintained at a temperature of 70 C; the bridge current, at 150 ma; and the injection port, at room temperature. Helium served as the carrier gas and was adjusted to a flow rate of 46 ml/min. A standard curve was prepared by analyzing mixtures of gases containing known concentrations of oxygen in nitrogen. A straight-line relationship was obtained by plotting oxygen column height against percentage of oxygen in the gas mixture. From the oxygen column height and by using the standard curve, the percentage of oxygen in the air-nitrogen mixture was determined.

Calculation of E_{h7}. The formula adopted by Leistner and Mirna (19) was used to calculate E_{h7}: $E_{h7} = E + E_{ref} + 60.1\ (pH \times -7.0)$. This formula describes the E_h of a system at *p*H 7.0 and 30 C. E is the measured potential; E_{ref} is the potential of the reference electrode versus the normal hydrogen electrode; 60.1 is equal to the term 2.303 RT/F at 30 C; and $(pH \times -7.0)$ is the *p*H correction term. This formula permits comparison of data obtained from different experiments.

Organisms and preparation of cultures. Two strains of *C. perfringens* were used: Hobbs strain HR2 obtained from H. E. Hall and strain number 9 (type B) originally obtained from Paul Ellner. The clostridia were kept in Cooked Meat Medium (Difco Laboratories, Detroit, Mich.), supplemented with 3.8% reinforced clostridial medium (RCM; Consolidated Laboratories, Inc., Chicago Heights, Ill.). Stock cultures were kept at room temperature. To prepare a *C. perfringens* culture, 1 ml of a stock culture was transferred into a tube of Cooked Meat Medium previously steamed and cooled. The culture was incubated at 30 C for 16 hr.

Pseudomonas fluorescens strain F21 isolated in our laboratory from chicken (23) was kept in Trypticase

Soy Broth, transferred bimonthly, and kept at refrigeration temperatures. To prepare a culture, a loopful of the stock culture was transferred to a tube containing 10 ml of Trypticase Soy Broth and incubated at 30 C for 48 hr.

Inoculation and enumeration. One or more vessels were inoculated with a mixture of *C. perfringens* and *P. fluorescens* to yield 10 cells/ml and 10^6 cells/ml, respectively. Two controls were included, one containing *C. perfringens* and the other containing *P. fluorescens* to yield numbers of cells per milliliter similar to those in the mixed culture.

C. perfringens was enumerated on the medium suggested by Angelotti et al. (1), except that sodium sulfadiazine and polymyxin B sulfate were omitted. Plates were incubated under a nitrogen atmosphere at 37 C for 24 hr in an anaerobic incubator (National Appliance Co., Portland, Ore.). *P. fluorescens* was enumerated on the agar suggested by King, Ward, and Raney (16). Spread plates were incubated at 30 C for 48 hr.

RESULTS AND DISCUSSION

Establishment of a uniform E_h value between flasks of broth within one experiment and between experiments performed at different times has always been a problem. Some workers have discarded flasks registering E_h values falling outside the acceptable range; others have developed tedious, but accurate, methods for obtaining uniform E_h values (7, 8, 26). In our experiments, uniform E_h values were obtained only after soaking the flasks in 3 N HCl and rinsing with deionized water. Values differed by as much as 150 mv before soaking; after soaking, however, variations seldom exceeded 10 mv. Excessive variations in E_h possibly were caused by adsorption of detergents on the glass surfaces.

The E_h of Trypticase Soy Broth after equilibration with purified nitrogen was 40 mv (*p*H 7.0); in the presence of 0.4 to 0.6% oxygen (by volume) in the gas mixture, the system equilibrated at 200 mv (*p*H 7.0). Addition of an inoculum to the broth caused a slight fluctuation, but the E_h normally returned to its original value within a few hours.

Typical reproducible E_h, *p*H, and growth curves are plotted for *C. perfringens* HR2 in Figs. 2 and 3 and for *P. fluorescens* F21 in Figs. 4 and 5. Curves for *C. perfringens* number 9 (type B) were essentially the same as those for HR2 and are not shown. Maximum total counts for *C. perfringens* HR2 were little influenced by initial E_h of the medium (Table 1; Fig. 2); on the other hand, total counts for *C. perfringens* number 9 (type B) were enhanced at the higher initial E_h of 200 mv. Sufficient observations have not been made with various strains and types of *C. perfringens* to determine the extent to which such

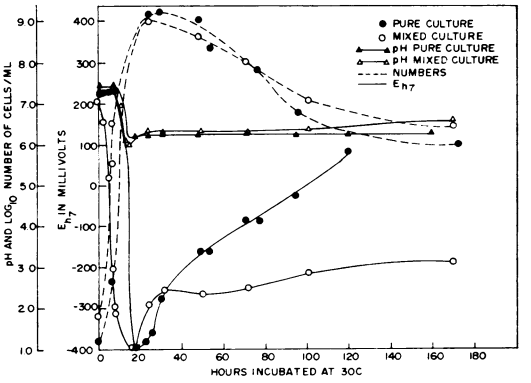

FIG. 2. *Effect of P. fluorescens F21 on changes in E_h, pH, and growth of C. perfringens at an initial E_{h7} of 200 mv and in the presence of 0.4 to 0.6% oxygen. Each point represents the average of three observations.*

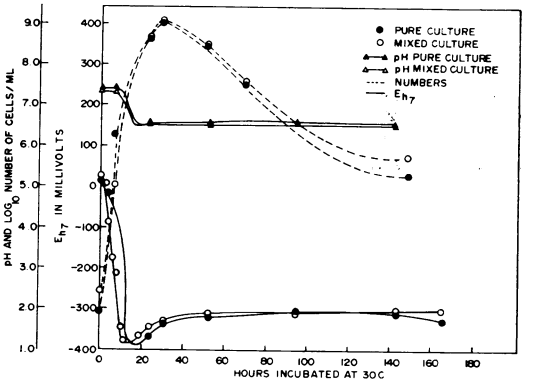

FIG. 3. *Effect of P. fluorescens F21 on changes in E_h, pH, and growth of C. perfringens HR2 at an initial E_{h7} of 40 mv and in the absence of oxygen. Each point represents the average of three observations.*

variations in reaction to the presence of oxygen may occur. *C. perfringens* is known to be aerotolerant (4, 9, 17) under certain conditions in blood and muscle tissues; it can survive hyperbaric oxygen treatment (15). No reference has been found in the literature, however, indicating that oxygen enhances growth. Observations on E_h and growth (4, 17) have usually been limited to initiation of growth at certain E_h levels. Limiting E_h values for growth of *C. perfringens* have been reported to range from −125 to 287 mv (*p*H 7.0; Table 2). *P. fluorescens* mixed with *C. perfringens* at an initial ratio of 10^6 to 10 did not have a marked effect on the growth of *C. perfringens* at either initial E_h. At an initial E_h of 200 mv, growth of *C. perfringens* with the subsequent rapid drop in E_h during the first 24 hr had

little or no effect on *P. fluorescens*. *P. fluorescens* is classified as an aerobe (24; *Bergey's Manual*, 7th ed.) and would theoretically require media of positive E_h values (12). Apparently, the low but constant oxygen tension supported the growth of *P. fluorescens* under adverse E_h conditions. This assumption is supported by the observation that growth of the pseudomonad was slow in pure culture and nonexistent in mixed culture with *C. perfringens* in the absence of oxygen (initial E_h of 40 mv; Fig. 5).

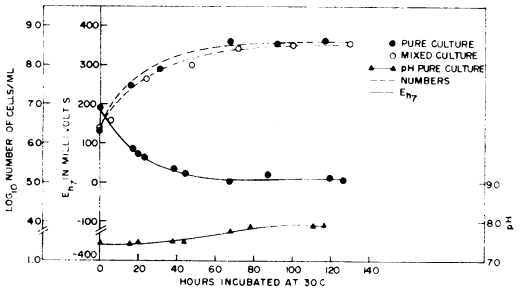

FIG. 4. *Effect of C. perfringens HR2 on growth of P. fluorescens F21 at an initial E_{h_7} of 200 mv and in the presence of 0.4 to 0.6% oxygen. Each point represents the average of three observations.*

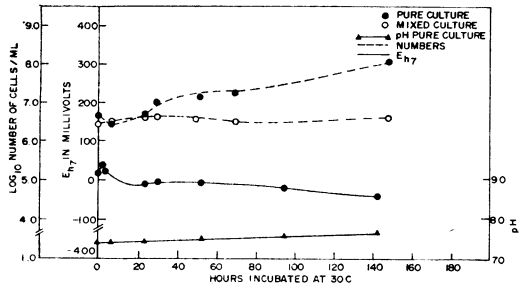

FIG. 5. *Effect of C. perfringens HR2 on growth of P. fluorescens F21 at an initial E_{h_7} of 40 mv and in the absence of oxygen. Each point represents the average of three observations.*

E_h curves for mixed cultures of the organisms were similar to those observed for *C. perfringens* in pure culture (Fig. 2, 3, 4, and 5). Differences were observed, however, after the minimal E_h had been attained; E_h values for *C. perfringens* in pure culture became more positive at a more rapid rate than those for the mixed culture (Fig. 2) in the presence of oxygen. In the absence of oxygen, the E_h curves for pure and mixed cultures were practically identical. In Fig. 2 and 3 a rapid decrease in E_h is evident, followed by a more or less sharply defined minimum. This minimum in E_h for cultures of *C. perfringens* has also been observed by others (20; M. C. B. Borromeo, M.S. Thesis, Iowa State University, 1969). Lepper and Martin (20) have suggested that the rapid drop in E_h coincides with the formation of hydrogen gas, one of the metabolic products of *C. perfringens*. They proposed that the low E_h measurement was the result of the activation of the platinum electrode in the presence of molecular hydrogen and that actually a hydrogen electrode was formed. On the other hand, Cannan, Cohen, and Clark (5) have found that hydrogen is produced only after low E_h values are achieved. Ferredoxin, which functions as an electron-mediating catalyst for hydrogen formation and utilization, has an E_{m_7} of −417 mv (25); E_{m_7} is equal to the E_h at the midpoint of a symmetrical titration curve at *p*H 7 (6). Therefore, the low values of E_h in cultures of *C. perfringens* probably were not due to activation of the platinum electrode but due to low potential substances such as ferredoxin. In contrast to *C. perfringens*, the E_h curves for *P. fluorescens* leveled off at approximately 10 mv (*p*H 7.0) in the presence of oxygen and at −40 mv (*p*H 7.0) in the absence of oxygen.

For both strains of *C. perfringens*, larger *p*H changes were observed when the initial E_h was 200 rather than 40 mv. Numbers of cells did not necessarily correlate with the extent of change in *p*H in the two systems. In the presence of oxygen (200 mv), the lowest *p*H was 6.2 with approxi-

TABLE 1. *Effect of initial E_h and oxygen on the growth of Clostridium perfringens and Pseudomonas fluorescens*

Organism	Maximum level of numbers of organisms/ml[a]				Inoculum
	E_{h_7} = 200 mv, O_2 present		E_{h_7} = 40 mv, O_2 absent		
	Pure culture	Mixed culture	Pure culture	Mixed culture	
C. perfringens HR2	1.5×10^9	9.7×10^8	1.0×10^9	1.0×10^9	20
C. perfringens B no. 9	3.1×10^8	2.0×10^8	1.5×10^6	3.0×10^6	20
P. fluorescens F21	3.8×10^8	2.0×10^8	2.0×10^7	3.1×10^6	10^6

[a] Average of three observations.

TABLE 2. *Limiting redox potentials for Clostridium perfringens*

E_h reported[a] (mv)	E_{h7} calculated[d] (mv)	Cultural conditions	Reference
230 to 250	169 to 189	Hartley's tryptic digest broth; *p*H 6.0; 37 C	4
200 to 250[b]	237 to 287	Peptone-agar broth; *p*H 7.6; 37 C	22
194 to 238[b]	194 to 250	Tryptone-beef extract-yeast extract-glucose-NaCl, *p*H 7.0–7.2; 37 C	21
160	123	Gelatin-yeast or peptone-meat extract; *p*H 6.4; 38 C	10
−125[c]	−125 to −113	Tyrode's peptone medium with vitamin C; *p*H 7.0–7.2; 37 C	17

[a] Saturated calomel electrode used as reference electrode.
[b] Growth observed only from a large inoculum.
[c] Measured colorimetrically with indigo carmen.
[d] Calculated with the formula of Leistner and Mirna (19).

mately 8×10^7 cells/ml. In the absence of oxygen, the lowest *p*H was 6.5 and the number of cells per milliliter was 2.5×10^8. Minimum values for E_h and *p*H occurred at the same time in cultures of *C. perfringens*, after which only a slight rise was observed. Similar *p*H patterns were observed by Mead (21) for cultures of *C. perfringens*.

Observations on E_h, *p*H, and cell numbers have been highly reproducible with the apparatus and techniques described above. With this apparatus, the relationships of the two organisms differing widely in growth requirements were studied. These organisms were chosen because both occur to some extent in fresh meat products, the pseudomonads usually to a much greater extent than the *Clostridium*. Conditions for growth, except for temperature, would be similar to those found in air- and vacuum-packaged meats. The results showed that initial E_h and dissolved oxygen content of the culture medium played an important role in the growth of the two organisms. One of the two strains of *C. perfringens* grew more luxuriantly in the presence of small quantities of oxygen. *P. fluorescens* could grow at a relatively low initial E_h (40 mv) in pure culture but not in the presence of *C. perfringens* under the same conditions. At temperatures lower than 30 C which are used for storing meat products, the growth responses probably would be quite different, particularly since *C. perfringens* grows poorly or not at all below 15 C (3).

ACKNOWLEDGMENTS

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