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Automatic Control of pH Value in Cultures of Micro-organisms

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SUMMARY: Equipment for the automatic control of pH value in cultures of micro-organisms is described. The apparatus was designed for a 2 l. scale continuous culture apparatus. The equipment will continuously control pH value with an accuracy of ± 0.05 unit for periods of many hundred hours. The pH value may be changed at will by turning a knob on the controller. It is sufficient to check the pH meter standardization once every day or two. The main components of the apparatus are standard commercial products.

The control of pH value is a factor of fundamental importance in the cultivation of micro-organisms and must, in general, be controlled within narrow limits to achieve the optimum conditions. To enable one to control the pH accurately over a wide range and to change the pH at will it is essential to use automatic control instruments. Apparatus for the automatic control of pH value is of recent development and requires instruments of relatively complicated design. Morton (1932) seems to have been about the earliest to describe an automatic pH control apparatus. A pH-controlling apparatus applicable to pure cultures of micro-organisms was described by Longworth & MacInnes in 1935. The complicated and delicate nature of their apparatus, which had to be built in the laboratory, must have precluded it from coming into general use. The commercial development of electronic instruments has made automatic pH control instruments much more readily available. Recently, several groups of workers have reported the use of instruments for the automatic control of pH value (Neish & Ledingham, 1949; Kempe, Halvorson & Piret, 1950; Hosler & Johnson, 1953; Wheat, 1953; Lakata, 1954). The present paper describes equipment suitable for controlling accurately the pH value of cultures of micro-organisms for periods of many hundred hours with complete freedom from contamination. Included are details of component design and operating technique not previously described.

APPARATUS

The pH-control equipment was used on a reactor for the continuous culture of micro-organisms. A diagram of the whole assembly is given in Fig. 1. The points of the reactor design relevant to this work are as follows. The vessel was a Pyrex glass pipe with stainless steel base and head plates. The volume of culture was about 2 l. It had automatic temperature control. The agitation was by means of a vaned disk impeller rotated at 500-1000 r.p.m. The impeller, of which the diameter was approximately half that of the vessel, had eight vanes, 15 mm. in height.

The equipment used for pH control in the reactor consists of a pH meter (model 23A, Electronic Instruments Limited, Richmond, Surrey), the output of which besides operating the usual galvanometer is fed to a recorder/con-

Fig. 1. Diagram of apparatus for automatic pH control of reactor.

troller (type 153, Electronik electric contact controller, Honeywell Brown Ltd., Greenford, Middlesex). The latter instrument is a self-balancing potentiometer which gives a continuous record of the pH value as given by the meter, and in addition operates two mercury switches connected to solenoid valves or other

control gear for admitting acid or alkali to the reactor. This enables the pH value of the culture fluid in the reactor to be kept constant, within limits, at any setting of the controller, the setting being immediately adjustable over the range 0–10 pH units by the turn of a knob. The two instruments are housed in a light metal frame separate from the reactor frame to ensure freedom from vibration. As an alternative to the pH measuring and controlling instruments which we have used, we considered an automatic titrimeter, one of the types which have recently been produced commercially for acid-base titration. A preliminary trial of such an instrument indicated that it had neither the accuracy nor the long-term stability which we required for our purpose.

Electrodes. The pH electrodes (supplied by Electronic Instruments Ltd.) consist of a spear type glass electrode with Ag-AgCl half cell and a calomel reference electrode, only the former being within the reactor. The reference electrode is outside and above the reactor, contact with the culture fluid being made with a long KCl bridge. The bridge consists of glass and silicone tubing with an internal diameter of 3 mm. It is filled with saturated KCl solution and terminates within the reactor in a porous ceramic plug which is a bacterial filter and at the other end in a 100 ml. separatory funnel used as KCl reservoir and carrying the reference electrode. The reservoir is about 120 cm. above the reactor in order to provide the necessary head to cause a slight flow (about 5 ml./day) of KCl through the ceramic plug. The glass electrode is fitted in a metal tube in such a way that most of the glass part is outside the tube. The ebonite cap and the cable are inside the metal tube and isolated from the reactor contents by a rubber bush which makes a liquid-tight seal. The spear-type electrode was selected instead of the bulb type because the former can be inserted through a rubber bush without risk of damage. The glass electrode in its holder and the KCl bridge are fitted in the head plate of the reactor by means of rubber bungs. The bungs are held in ports consisting of short lengths of externally threaded stainless steel tubing. The bungs are kept tightly in place by threaded brass caps fitting on the stainless steel tubing. Alternatively, the KCl bridge may be fitted in the base of the reactor. We tried this arrangement but prefer the other as, on the head plate in our installation, the glass part of the KCl bridge is less liable to accidental breakage. The electrodes are arranged so that their tips are at about the 1300 ml. level, that is, well below the normal working level of *c.* 2 l. The standard electrodes as received from the manufacturers had 90 cm. leads, too short for use on the reactor. They are coupled to the pH meter by means of extension leads connected with a normal junction block. A metal-sheathed cable is used for the glass electrode. No trouble has been experienced from this simple method of connexion.

In addition to the electrodes, a resistance thermometer is also connected to the pH meter through the junction block. This resistance thermometer, which is part of the normal temperature compensating device of the pH meter, is housed in a pocket in the base of the reactor. The reference electrode remains at room temperature. The difference in temperature between the two electrodes is compensated for by the isopotential control of the pH meter.

Arrangement for admission of acid and alkali to the reactor

Acid and alkali reservoirs are installed some 90 cm. above the reactor and feed by gravity through glass and silicone tubing. Gravity feed was preferred to pressure feed as it allows a safer installation. The reservoirs consist of Buchner flasks with a useful capacity of 6 l. The side arms carry small air filters packed with fine Fibreglass. The reservoirs are calibrated so that the

Fig. 2. Solenoid-operated pinchcock shown in the open position.

Fig. 3. Apparatus for sterilization of glass electrode by ethylene oxide gas.

amount of acid and alkali used can be measured. The lines from the reservoirs pass through solenoid-operated valves to a port on the reactor head. An arrangement of concentric tubes allows both lines to enter the reactor through the one port. This arrangement consists of a 30 cm. length of Pyrex glass capillary tube inside a second glass tube having minimal clearance. In most of the experiments reported the tubes terminated about 25 mm. above the liquid surface. Thus the rate of flow through the acid and alkali lines could be checked visually. Later, however, this was found to be unnecessary so that the ends of the concentric tubes could be placed below the medium surface. This was found to be advantageous (see below).

For the controlled addition of acid and alkali to the reactor a simple type of solenoid-operated tubing clamp (Fig. 2) has been used. It consists of a spring pressing on the silicone tubing of the acid or alkali line with sufficient force to provide a leak-free seal against the liquid head. When the solenoid is energized the arms of the plunger move forward on each side of the silicone tubing, lift the spring, and allow liquid to flow to the reactor. When the solenoid is de-energized the plunger is returned inside the coil by the spring. The solenoid valves require about 100 mA. to operate them and are energized directly from the 230 V. mains by the mercury switches of the controller. (The solenoids were supplied by Westool Ltd., St Helens, Auckland, Co. Durham, and designated series 500, model 5, rating 2 for 230 V. operation and were modified as shown in Fig. 2).

A commercial type of solenoid-operated pipe line valve has also been tried (magnetic valve, type R.B.G. seat diam. $\frac{3}{8}$ ins. for 230 V. a.c. operation, Teddington Industrial Equipment Ltd., Sunbury, Middlesex). This was used on the alkali line and gave completely satisfactory service for several hundred hours of operation. Eventually, however, the phosphor-bronze valve seat became corroded by the 0.5 N-alkali as expected and leakage occurred which became progressively worse. An all-stainless steel valve of similar type is now being obtained. For this type of valve the current consumption is higher than the permitted maximum (500 mA.) of the mercury switches of the pH controller. A relay (type F 103/3 for 230 V.a.c. operation, Sunvic Controls Ltd., 10 Essex Street, Strand, W.C. 2) was therefore interposed in this case. The time delay of the relay made no detectable reduction in the sensitivity of control.

The acid and alkali reservoirs and lines and the reactor are enclosed by shields of transparent Perspex sheet as a protection against possible breakage of the acid or alkali containers. For similar reasons all electrical control gear is grouped together on one panel fed from one 15 amp, 230 V. outlet outside the field of possible liquid contamination so that the entire electrical supply can be immediately cut off in the event of accident.

Acid and alkali for pH correction. 0.5 N- H_2SO_4 and 0.5 N-KOH were used at first. In continuous culture work, however, 1.5 N-alkali was used in order to decrease the volume of neutralizing material required.

Culture media. Two types of medium were used: a medium made from casein hydrolysate and yeast, and a defined medium consisting of glucose, ammonium sulphate, 0.2 % (w/v) KH_2PO_4 and traces of mineral salts.

Antifoam. The antifoam used was Alkaterge C diluted with liquid paraffin 1/10 and 3/10. Alkaterge C is a fatty acid derivative supplied by Commercial Solvents of Terre Haute, Indiana, U.S.A.

RESULTS

Sterilization of apparatus

The reactor without the glass electrode was sterilized in the autoclave. The end of the KCl bridge with the ceramic plug was sterilized *in situ* in the reactor. Some difficulty was experienced in completely filling the KCl bridge without

trapping air in the part within the reactor. It was later found that the best method of filling this part of the bridge was to sterilize it with a small temporary reservoir of KCl attached. During autoclaving the air was completely eliminated from the tube and replaced by KCl solution from the reservoir. After this the temporary reservoir was removed and connexion made to the proper reservoir, containing the reference electrode. Since the ceramic plug at the reactor end of the KCl bridge is a bacterial filter there is no necessity to sterilize the bridge system completely.

The acid and alkali supply units were sterilized separately. The KOH was sterilized in its reservoir. The sulphuric acid, in order to avoid any corrosion of the autoclave which might be caused by steam sterilization, was prepared by adding concentrated acid from a sterile pipette to the reservoir containing sterile distilled water. The acid and alkali lines were connected to the reactor with sterile precautions.

The glass electrodes available could not be sterilized by autoclaving because this caused failure of the insulation; some workers in America, it should be noted (e.g. Hosler & Johnson, 1953; Wheat, 1953), reported that they used steam-sterilized electrodes. Sterilization with liquid disinfectants is complicated and lengthy because means of protecting the cable and connexion from the disinfectant must be devised and washing with sterile water is necessary to remove the disinfectant. To overcome these difficulties, Neish & Ledingham (1949) used ultraviolet irradiation for sterilization of electrodes. This method too has its complications, for example, the need for crevice-free electrodes (see Watson, Clement & Muirhead, 1950). We decided to use ethylene oxide vapour as sterilizing agent for the glass electrode. Its main advantages are: (a) the whole electrode assembly including the cable can be immersed in the gas without causing corrosion or affecting the insulation; (b) on withdrawing from the gas the electrode may be easily freed from the sterilizing agent. The method adopted was to insert the electrode in its tube into a cylinder as shown in Fig. 3. The cylinder was evacuated by a water pump and filled with ethylene oxide gas and air in about equal proportions. The cylinder was then closed and left for 4 hr. at room temperature; 4 hr. were sufficient to ensure sterility but no experiments were made to find the minimum time required. After sterilization, the ethylene oxide vapour was largely displaced from the tube by evacuation and filling with sterile air three or four times. Finally the electrode was removed from the cylinder and inserted into the port on the reactor head. This operation was done against a stream of sterile air from the reactor to minimize the chance of contamination.

Standardization of pH meter with electrodes in the reactor

Two methods of standardizing the pH meter were used. The pH value of a sample of medium from the reactor was determined by another pH meter. The reactor pH meter was then set to read the found pH value of the medium. A second method dispensed with the need for two pH meters; a second set of electrodes only is required. The reactor pH meter was connected to a pair of electrodes outside the reactor and standardized, using buffer solutions. Using

the external electrodes the pH value of a sample of the medium in the reactor was determined. The reactor electrodes were then connected to the meter and the meter set to read the previously found pH value of the medium.

Evaluation of the pH control system

The installation has been subjected to comprehensive tests by using it to control the pH value of cultures of *Aerobacter aerogenes*. Two different culture media were used: (1) the casein hydrolysate and yeast medium in which without control the pH value rose during a culture; (2) the defined medium in which growth of the organism without control caused a decrease in pH value. The quantity of organism obtained in these cultures was 4–8 mg. dry wt./ml. The apparatus was operated continuously under growth conditions for periods of several hundred hours. This was done (a) by successive batch runs in which most of the old culture was removed daily and replaced by fresh medium, and (b) by using the system in continuous culture.

Figs. 4–6 show the results of various tests. Fig. 4a shows the stability of the pH meter reading in sterile casein hydrolysate + yeast medium over a period of 7 hr. Fig. 4b shows the drift of the pH value of a batch culture of the organism in the casein hydrolysate + yeast medium without control. The chart with the same medium and the pH controlled at pH 6.55 is shown in Fig. 4c; the exponential increase in the frequency of acid addition which paralleled the growth curve, is apparent.

The first pH-controlled run showed that it was necessary to limit the rates at which acid or alkali were delivered when the solenoid-operated valves were open. This was done partly by the use of some capillary tubing in the feed line and partly by an adjustable Hoffman screw clamp. A suitable flow-rate was found to be roughly 0.05 ml./sec. If this rate was much exceeded overshoot of the control point occurred and hunting of the controller resulted. Once the flow-rate of the acid and alkali had been limited in this way it was found that the control equipment was capable of maintaining a given pH value within ± 0.05 unit for very long periods provided that the culture pH tended to change only in one direction. If the change of pH value brought about by the organism changed from, say, a downward drift to an upward one the mean pH value was displaced by 0.09 pH unit, although the variation about the mean was still ± 0.05 unit; this point is illustrated in Fig. 5a. The reversal of the pH drift was effected by using a mixture of the two types of media. This change in the mean is due to the fact that in order to minimize operation of the solenoid valves and help to prevent hunting of the controller there is a small range of pH close to the control point within which neither solenoid valve is operated. The width of this neutral zone in the present instrument, preset by the manufacturers, is 0.08 pH unit.

The presence of a foam layer above the culture fluid acts as a barrier to the entry of acid or alkali if the inlet is above the liquid surface and thus decreases the sensitivity of the control system. This is clearly shown in Fig. 5b, where the width of the chart-trace increases as foam builds up and resumes normal limits after the addition of antifoam agent. The antifoam oil did not affect the

Fig. 4. In the three charts reproduced, the heavy vertical lines represent 30 min. intervals. The numbers are actual pH units so that one small horizontal division represents 0.1 unit. (a) Part of chart of 4-day stability test of Electrode/Meter system with sterile casein hydrolysate yeast medium in the reactor. (b) pH record of batch growth of *Aerobacter aerogenes* in casein + yeast medium without pH control. The arrow indicates point of inoculation. (c) pH record of batch growth of *A. aerogenes* in casein + yeast medium with automatic pH control by addition of 0.5 N-sulphuric acid. The pH value was set at 6.55. The arrow indicates point of inoculation.

Fig. 5. In the charts reproduced the heavy vertical lines represent intervals of 1 hr. The numbers represent actual pH units. (a) Part of pH chart showing change-over from alkali addition to acid addition. (b) Part of pH chart showing the effect of foaming in the reactor on sensitivity of control. Arrows indicate where antifoam agent was added. (c) Part of pH chart showing extremely rapid working of control system to maintain a constant pH value against a continuous alkali leak.

electrodes in any way. This was shown by adding the antifoam in concentrations up to 10 ml./l. Complete elimination of interference by foaming was achieved by arranging for the point of entry of acid and alkali to be below the liquid surface.

As mentioned earlier, at one stage of the work a solenoid valve which was being tested became corroded and a continuous leak of alkali to the reactor occurred. Even under these conditions of heavy demand the pH controller maintained the pH value at the set point and indeed the control was better than ± 0.05 unit as shown in the enlarged tracing in Fig. 5c. These conditions were maintained for 18 hr. until the alkali valve was replaced.

Fig. 6. Part of pH chart showing an alteration of the control point from pH 6.75 units to pH 7.24 units during a continuous culture. The incoming medium had a pH of 6.20. The heavy vertical lines represent intervals of 1 hr. The numbers represent actual pH units.

The smooth and rapid changeover from one pH value to another when desired is illustrated in Fig. 6. By means of the apparatus we have grown *Aerobacter aerogenes* in continuous culture at controlled pH values ranging from 4.75 to 8.25.

Over 2000 hr. of trouble-free operation of the pH control equipment have been obtained, mostly in continuous runs of 300–600 hr. In this period regular checks of the standardization of the reactor pH meter were made, and it was found that the maximum error was ± 0.02 pH unit in 24 hr. Whenever samples were taken from the reactor for analytical purposes their pH value was measured by another meter. In this way it was checked that the pH value indicated by the reactor electrodes was in fact the true pH value of the culture. In no case did the difference between the two meters exceed 0.05 pH unit.

Limits of precision of pH control

No attempt has been made to assess the limits of precision of pH control attainable with the particular combination of instruments and control gear available. As stated above, control within ± 0.05 pH unit was obtained in the reactor with the acid and alkali inlets just above the liquid surface. Still greater accuracy (± 0.03 pH unit) was attained by having the acid and alkali inlets below the liquid surface. Several independent variables impose limits

on the accuracy attainable, such as the sensitivity and stability of the pH meter/electrode system, the speed of response of the control instrument/control gear system, the positioning of electrodes and point of entry of the correcting fluid and the efficiency of mixing in the reactor. Ideally the operation of the acid or alkali valve, entry of acid or alkali into the reactor, and registering of the new pH value should be instantaneous. Some workers, for various reasons, have sited the electrodes outside the reactor. This may be done when a continuous flow system is used or by circulating the culture through a loop. This may lead to appreciable time lags in the operation of various parts of the system and there may also be insufficient mixing in the region of the electrodes. Wheat (1953), for instance, reported that the pH control system he used was much more trouble-free when the electrodes were inserted inside the culture vessel rather than in a loop with circulation of the medium. A discussion and mathematical treatment of time lags and other factors involved in precision of control have been given by Greer & Chaplin (1943).

Life of glass electrodes

The conditions of continuous culture are exacting for all of the control equipment used since not only must a precise control be achieved but it must be maintained for relatively long periods of time. Before the start of this work, no information was available as to what useful life could be expected of a glass pH electrode continuously immersed in culture fluid. During the work described in this report, three glass electrodes have been used. The first of these failed after about 300 hr. use, but examination by the manufacturers disclosed that the fault was in a piece of poor-quality cable used for the electrode lead and not in the electrode itself. The second electrode failed after a similar time period. Here the fault may have been due to deterioration of the glass membrane. Unfortunately, this electrode was broken when removing it from the reactor for testing, so a proper examination could not be made. The third glass electrode is still in use after about 1600 hr. made up of four separate runs. It appears, therefore, that under the conditions within the reactor a life of several hundred hours at least can be expected from an average commercial glass electrode. In any case the design of the present electrode assembly and reactor head permits the substitution of either of the electrodes or the KCl bridge without difficulty should it become necessary during a culture.

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