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The Measurement of the Aeration of Culture Media

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SUMMARY: The aeration of culture media can be measured in terms of ϕ_L (defined below) which is dependent on rate of air flow, agitation, etc. Two methods of measuring ϕ_L are given, based on the polarographic estimation of dissolved oxygen. For penicillin and streptomycin fermentations there is a correlation between the amount of antibiotic produced and ϕ_L .

Recent advances in industrial stirred and aerated fermentations have emphasized the need for a suitable technique to measure aeration in terms of the oxygen available to the organism rather than in terms of the volume of air which is passed through the medium. In the case of submerged cultures, the organism is dependent upon the oxygen dissolved in solution. In order to assess the efficiency of the aeration it is therefore necessary to compare the rate of solution of oxygen into the culture medium with the rate of consumption of oxygen by the organism.

Determination of the oxygen demand

The Warburg apparatus is usually used for measuring oxygen demands, but in the present work it was found convenient to use a polarographic method first used by Petering & Daniels (1938). The culture is saturated with air and then the air supply is cut off. The concentration of dissolved oxygen in the culture medium decreases steadily from the saturation concentration to zero as the organism consumes the oxygen. A typical determination is shown in Fig. 1, from which it will be seen that the graph of the concentration of dissolved oxygen against time is linear, i.e. the oxygen demand is of zero order with respect to time and oxygen concentration over the period of measurement. This behaviour was always found with the organisms studied, and in the following theory it is assumed that the oxygen demand at a particular moment is of zero order. The polarographic measurements required only a few minutes so that no significant growth occurred during the measurement. The oxygen demand of the culture varies comparatively slowly with time as growth proceeds, and by measuring the oxygen demand of samples taken at various times throughout the culture period it is possible to draw the curve connecting oxygen demand with the time after the inoculation of the medium.

It has many times been noted (for a review see Tang, 1933) that the oxygen demand of many organisms is of zero order although only above a critical oxygen tension. Below the critical oxygen tension, the oxygen demand becomes first order. The critical oxygen tensions found are very low. For instance, in the present work the critical oxygen tensions were too low to be apparent from curves such as those shown in Fig. 1, so that the assumption that the oxygen demand is of zero order is justified.

Determination of the rate of solution of oxygen

If a culture vessel is filled with water which has previously been freed from dissolved oxygen by gassing-out with nitrogen, then when the air supply to the vessel is switched on, the concentration of dissolved oxygen rises from zero to the saturation concentration (C_e) as shown in Fig. 2. The rate of solution of oxygen is given by the slope of the tangent to the curve, e.g. when $C = 0.5 C_e$ the rate of solution is the slope of the dotted line. It will be clear that when the concentration of dissolved oxygen is at the saturation concentration, C_e , the slope of the tangent is zero. This is as expected, since once the water has become saturated with oxygen no more oxygen will dissolve from the air. Also, when the concentration of dissolved oxygen is zero, the rate of solution of oxygen is the highest possible rate with the given vessel under the given conditions. This value of the rate of solution will be called the 'maximum solution rate'.

It is convenient to express the curve relating concentration of dissolved oxygen to the rate of solution of oxygen in the form of an equation. From the theory of gas absorption it would be expected that the rate of solution of oxygen is given by the equation

$$\frac{dC}{dt} = \phi_L(C_e - C), \quad (1)$$

where dC/dt is the rate of solution of oxygen at a concentration of dissolved oxygen C , and C_e is the saturation concentration of dissolved oxygen. dC/dt is the rate of solution of oxygen expressed as the rise in the concentration of dissolved oxygen. The total molecules of oxygen dissolving per minute from the air is given by $V(dC/dt)$, where V is the total volume of the liquid medium in litres and C is measured in molecules of dissolved oxygen per litre of medium.

From equation (1) it will be seen that when $C = C_e$, the rate of solution is zero and when $C = 0$ the maximum solution rate is given by $\phi_L C_e$. It is found that equation (1) fits the experimental curves closely.

The theory of gas absorption enables the constant ϕ_L in equation (1) to be considered in terms of other variables, the most important of which are rate of air flow and agitation. The value of ϕ_L is proportional to the rate of air flow over a certain range. Hence ϕ_L is only constant in equation (1) if the rate of air flow is kept constant throughout the experiment. It is to be expected that agitation would increase the value of ϕ_L so that if the culture vessel is fitted with a stirring device this must be kept running at constant speed throughout the experiment. The value of ϕ_L will characterize the efficiency of aeration of a given vessel under fixed conditions of rate of air flow and agitation. Moreover, the effect of these variables on the aeration can be assessed by measuring the value of ϕ_L at various values of the rate of air flow or the rate of agitation, of which a rough measure is given by the speed of the stirrer.

The maximum solution rate, $\phi_L C_e$, can thus be controlled by adjusting the value of ϕ_L by varying the rate of air flow or the rate of agitation. It can also be controlled by altering the value of C_e . By Henry's Law, the value of the saturation concentration (C_e) is proportional to the partial pressure of oxygen

in the gas phase. For instance, if the gas passed through the medium is changed from air containing 20 % oxygen to pure oxygen, the value of C_e would be increased five times. Thus if the oxygen were passed at the same rate as the air, and if the agitation were kept constant, so that ϕ_L was the same in both instances, the value of the maximum solution rate would also be increased five times.

In the case of tall industrial vessels where the hydrostatic head is large, it might be expected that the pressure inside a bubble of air at the bottom of the vessel would be large enough to affect the value of C_e . This correction is necessary when values of C_e are being measured, but the value of the maximum solution rate, $\phi_L C_e$, is relatively insensitive to changes in the total pressure since an increase in the pressure on a bubble will decrease its surface area and this will decrease the value of ϕ_L . The two effects nearly cancel each other, and $\phi_L C_e$ is proportional to $P^{\frac{1}{2}}$ where P is the total pressure. Thus for moderate-sized vessels, the value of the maximum solution rate measured at any point in the vessel can be taken as correct over the whole vessel as far as the pressure effect is concerned.

Another effect which might be important for tall vessels is that as oxygen is consumed from a bubble, the value of the partial pressure of oxygen in the bubble will decrease, affecting C_e , and also the surface area of the bubble will decrease, affecting ϕ_L . Most aeration devices are so inefficient that this effect need not be considered. A treatment of the problem, however, has been given by Pattle (1950), who has produced very efficient aeration devices on a laboratory scale.

Aeration of a respiring culture

As mentioned above, the oxygen demands of cultures can frequently be expressed by the equation

$$\frac{dC}{dt} = k_0, \quad (2)$$

where k_0 is a constant, the zero order oxygen demand.

If the contents of a culture vessel are aerated we have that (rate of increase in concentration of dissolved O_2) = (rate of solution) - (rate of consumption). Expressing this in terms of equations (1) and (2)

$$\frac{dC}{dt} = \phi_L(C_e - C) - k_0. \quad (3)$$

As the concentration of dissolved oxygen rises, the rate of solution of oxygen will fall until eventually it reaches a value equal to the rate of consumption by the organism. When this is so, $dC/dt = 0$ and, representing the steady concentration maintained in the culture vessel by C_s , we have from equation (3) that

$$C_s = C_e \left\{ 1 - \frac{k_0}{\phi_L C_e} \right\}. \quad (4)$$

Equation (4) shows that C_s is always less than C_e , but that $C_s \approx C_e$ when $\phi_L C_e$ is very much greater than k_0 .

The conditions of inadequate aeration

It is often convenient to relate the yield of a metabolic product or the consumption of a medium constituent to the aeration rate. In this case the degree of aeration can be considered adequate when the maximum yield or consumption is obtained. However, this is not a fundamental measure, since the level of adequate aeration so defined will depend on the particular aspect of metabolism studied. Since the purpose of aeration is to supply the organism with the oxygen it needs, the adequacy of aeration can be unambiguously defined in terms of the relationship between the oxygen demand of the organism and the rate of supply of oxygen. A culture will be adequately aerated at a particular instant if the maximum solution rate $\phi_L C_e$ is greater than the oxygen demand of the organism. It is a consequence of the fact that the oxygen demand is of zero order that when the culture is adequately aerated, the oxygen demand will be independent of the rate of aeration.

When the culture is inadequately aerated, $\phi_L C_e < k_0$. This means that an organism which could consume oxygen at a rate k_0 is consuming oxygen only at a rate $\phi_L C_e$. The actual mechanism by which the organism consumes the oxygen at this rate will vary. It may be a rate-determining diffusion across the surface of, or into, the organism or else unsaturation of the enzymes reacting with the oxygen. In these cases, restoring the aeration to an adequate level within a short time should restore the oxygen demand to the original value k_0 .

It is clear that maintaining the aeration at an inadequate level may alter the course of the metabolism, for instance by limiting growth or inducing a partial change to an anaerobic metabolism. In this case, restoring the aeration to an adequate level would result in an oxygen demand different from that of a similar culture which had been adequately aerated throughout. Thus in measuring adequacy of aeration, the reference state should be that of a culture which has been adequately aerated throughout.

EXPERIMENTAL

The concentration of dissolved oxygen was measured with the polarograph, using a dropping mercury electrode. The general technique used was similar to that of Petering & Daniels (1938). The method used is specific for dissolved oxygen. Air was supplied to the polarographic cell through a sintered glass disk in order to achieve a high value of ϕ_L . All samples taken contained enough inorganic salts to be polarographed directly, and no maxima on the wave were found. In exceptional circumstances it might be necessary to add supporting electrolyte to the sample before it was polarographed. When possible, the polarographic cell was kept in the same incubator as the fermenter; otherwise the cell was placed in a thermostat at the same temperature as the fermenter.

The potentiometer of the polarograph was fixed at -0.7 V. which corresponded to the plateau at the top of the first oxygen wave. The current between the electrodes was taken as proportional to the concentration of dissolved oxygen. A dummy sample was taken to adjust the controls of the polarograph. The zero was established by bubbling nitrogen through the sample to remove

all the dissolved oxygen and then adjusting the galvanometer deflexion to a suitably low value, using the zero-setting control. The sample was then saturated with air and a suitable deflexion of the galvanometer obtained by adjusting the galvanometer shunt. The instrument was then ready for use.

Measurement of oxygen demands

Samples of fermenting cultures were taken, placed in the polarographic cell and saturated with air. The air supply to the cell was then cut off and the concentration of dissolved oxygen measured at various times. When the sample had such a high oxygen demand that the value of C_s in the cell was much less than C_e , then the value of the current corresponding to C_e was obtained by inhibiting the oxygen demand with a trace of cyanide or other suitable inhibitor, and saturating again with air. The value of the zero was then determined to correct for any increase in current due to the inhibitor.

The polarograph measures the oxygen concentration in arbitrary units (see Lewis & Mackenzie, 1947). It is thus most convenient to express the results of oxygen demand experiments in terms of t_0 , the time in minutes taken to reduce the concentration of dissolved oxygen from C_e to zero, a straight line being drawn through the experimental points, neglecting any tailing-off effect at low concentrations of dissolved oxygen.

Measurement of ϕ_L values

Two methods were employed, the gassing-out method based on equation (1) and the sampling method based on equation (4).

Gassing-out method. The vessel under test was filled with either the fermenting medium with the oxygen demand inhibited, or else with a solution of potassium chloride containing a trace of methyl red to eliminate the maximum on the oxygen wave. The vessel was arranged as for an actual fermentation. After a preliminary gassing-out with nitrogen, the aeration train was switched on and simultaneously a stop-clock was started. Throughout the determination the rate of air flow, usually measured with a calibrated capillary flowmeter, was kept as uniform as possible. At convenient intervals samples were removed and the concentration of dissolved oxygen determined polarographically. A graph of $\log(C_e - C)$ against t was drawn. By integrating equation (2) it can be shown that the slope of this plot is $-\phi_L/2.303$, whence ϕ_L can easily be calculated.

The sampling method. The value of ϕ_L can be determined from equation (4) when C_s , C_e and k_0 are known. The values of C_e and k_0 can be obtained when the oxygen demand of a sample is measured. The value of C_s was obtained by taking a sample from an actual fermentation at a noted time, putting it quickly into the polarographic cell and then measuring the concentration of dissolved oxygen at various times. The graph of these results, extrapolated to the time at which the sample was taken, gave the value of C_s . The value of ϕ_L could then be calculated using equation (4). A correction was necessary when the partial pressure of oxygen in the gas passed through the culture vessel was different

from that in the gas used in aerating the polarographic cell. To apply the correction, Henry's Law was assumed to be valid. It will be assumed as a convention that $C_e = 1$ for water in equilibrium with air at 1 atm. pressure.

RESULTS

A typical result of an oxygen-demand experiment is shown in Fig. 1. Usually good straight lines were obtained, but irregularities were sometimes observed, especially with penicillin fermentation samples. This was due to interference by the mycelium, and possibly the anti-foam agent, with the dropping of the mercury from the capillary. With some samples measurements were hardly possible. In general, it was found that the oxygen demand of penicillin and streptomycin fermentations was associated with the mycelium and that the medium itself usually had a negligible oxygen demand.

Fig. 1. Graph of concentration of dissolved oxygen against time for a culture initially saturated with air. C_e = saturation concentration of oxygen.

The results of a typical determination of ϕ_L using the gassing-out method is shown in Fig. 2 in which C and $\log_{10}(C_e - C)$ are plotted against t . Fig. 3 shows the result of a determination of ϕ_L using the sampling method.

Results obtained with the sampling method were much less accurate than those given by the gassing-out method. The difficulty inherent in the former method is that it is necessary for C_s to be large for a good extrapolation to be possible, but as C_s becomes larger, the value of $(C_e - C_s)$ becomes smaller and finally comparable with the experimental error in determining the concentrations of dissolved oxygen. The method can thus only be used when $C_s \approx \frac{1}{2}C_e$. This has to be arranged either by adjusting the air flow or by waiting until the oxygen demand has reached a suitable value. The advantage of the sampling method is that it can be used for examining fermentations in large production vessels which cannot be released for test purposes. The ordinary sampling points can be used during the routine fermentation providing that they yield

a representative sample. By replication, the value of ϕ_L can be determined to within about 20 %. Greater accuracy is possible where polarographic measurements are subject to less irregularity, e.g. with yeast fermentations.

Fig. 2

Fig. 3

Fig. 2. Graph of concentration of dissolved oxygen (C) against time for a vessel filled with potassium chloride solution initially oxygen-free. The upper graph is a plot of $\log (C_s - C)$ against time for the same results. The ϕ_L value obtained from the upper curve is 0.045 min.^{-1} and the full lower curve has been calculated using this value.

Fig. 3. Curve A: graph of concentration of dissolved oxygen (in arbitrary polarographic units) against time. Sample initially saturated with air. Curve B: sample from fermentation vessel removed at zero time and then placed in polarographic cell. The value of ϕ_L for this vessel is 0.85 min.^{-1} .

A cylindrical vessel 1 m. tall and 5 cm. in diameter, aerated through a 1 in. coarse sintered disk, was used for some laboratory tests, the gassing-out method being used. The results are shown in Fig. 4. The plot of ϕ_L against G , the rate of air flow, is linear over the range measured. Also it is shown in Fig. 4 that ϕ_L/G is independent of the volume of liquid in the vessel. This shows that the arbitrary convention of measuring aeration as volumes of gas/volume of medium/min. is meaningless.

Fig. 5 shows the effect of stirring on the ϕ_L value of a 50-gallon stirred fermenter, using the sampling method of measuring ϕ_L . It is clear that stirring has considerably increased the value of ϕ_L .

Fig. 6 shows a graph of the maximum titre, determined by bio-assay, produced in a streptomycin fermentation (*Streptomyces griseus* strain 5001 in salt glucose peptone medium) against $\phi_L C_s$ for various vessels in which the fermentation was conducted. Although the points show considerable scatter the correlation is convincing. When $\phi_L C_s > 1.0$ no increase in titre is given by

increasing the aeration. The value of the peak oxygen demand in these fermentations which occurred at the peak of growth corresponds to a value $\phi_L C_e = 1.0$.

Fig. 7 shows a similar curve for penicillin fermentations (*Penicillium chrysogenum*, strain Q176, in a cornsteep liquor medium). This graph shows broadly the same characteristics as those obtained with the streptomycin fermentations.

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 4. Curve A: graph of ϕ_L (in min.^{-1}) against G , the rate of air-flow in l./min. Curve B: graph of ϕ_L/G against V , the volume of liquid in litres.

Fig. 5. Graph of ϕ_L (in min.^{-1}) against rate of stirring for a 100-gallon vessel fitted with a stirrer. Rate of air flow constant at 15 cu.ft./min.

Fig. 6. Graph of maximum titre of streptomycin (at 75 hr.) against $\phi_L C_e$. $\circ-\circ$, 5 l. stirred round-bottomed flasks; $\bullet-\bullet$, 100 gallon vessel with stirrer. $\times-\times$, 50-gallon vessel with stirrer. Each point is the average of five to ten experiments.

Fig. 7. Graph of titre, at 72 hr. after inoculation, of penicillin fermentations against $\phi_L C_e$. $\circ-\circ$, 10 l. aspirators. $\times-\times$, 50-gallon vessel with stirrer. Each point is the average of five to ten experiments.

The results shown in Figs. 6 and 7 were given by vessels in which the higher ϕ_L values were obtained either by a low rate of air flow and considerable mechanical stirring or else by fine aeration and no stirring. In spite of this, no correlation has so far been observed between titre and rate of stirring except in as far as the rate of stirring affected the value of ϕ_L . In this way the deter-

mination of ϕ_L values should allow a separation to be made of the two distinct effects of aeration and agitation. No effect of the size of the vessel is obvious in Figs. 6 and 7. This suggests that as far as aeration is concerned results obtained with small vessels may be used to predict the behaviour of much larger vessels. A similar result has been recorded for the case of yeast (Olson & Johnson, 1949).

DISCUSSION

The polarographic method of measuring oxygen demands shows clearly that dissolved oxygen is consumed by micro-organisms. The correlation between the *rate of solution* of oxygen and the antibiotic production of the organisms grown in different vessels shows that any effect due to some cells obtaining oxygen directly from air bubbles is very small. Moreover, the number of cells which could retain a contact with an air bubble sufficient to satisfy their oxygen demand must be small compared with the bulk of growth throughout the vessel.

The only previous reference found to the determination of ϕ_L values was published by Olson & Johnson whilst the present work was in progress. They determined the value of $\phi_L C_e$ by aerating a vessel filled with sodium sulphite solution containing copper ions. The concentration of dissolved oxygen in the liquid was always very close to zero, and the oxygen dissolving reacted quickly with an equivalent amount of sulphite ion to form sulphate. The value of $\phi_L C_e$ was obtained by measuring the amount of sulphite oxidized in a given time. Experiments in this laboratory have shown that the sulphite technique does not give the same value for $\phi_L C_e$ as the polarographic method. This is to be expected, since the rate of reaction of sulphite ions with oxygen is so fast as to affect the diffusion gradient across the air/liquid interface.

The fact that C_s is less than C_e has been tacitly assumed by many investigators. A clear demonstration, however, has already been given by van Goor & Jongbloed (1942) using a Warburg apparatus and estimating dissolved oxygen by a chemical method. They showed that the break-down from zero-order behaviour of the oxygen demand of brain-tissue of rats occurred at lower concentrations of dissolved oxygen than would be expected from the assumption that the oxygen tension in the medium was equal to the partial pressure of oxygen in the gas. Using the present methods on their graphs it seems that the ϕ_L value of the Warburg apparatus which they used was less than 0.2 min.^{-1} . This is rather low compared with many of the oxygen demands encountered in the present work, and confirms what is already known, namely, that the rate of transfer of oxygen from the gas phase of a Warburg apparatus to the liquid phase may be a limiting factor in measuring rates of oxygen uptake.

Certain general conclusions may be drawn from Figs. 6 and 7. The fact that the titre becomes independent of the rate of solution of oxygen above the value corresponding to the peak oxygen demand is due to the fact that the oxygen demand is of zero order. When the culture is adequately aerated, the oxygen demand, and thus the aerobic metabolism, is independent of the rate of aeration. When the culture is inadequately aerated, the rate at which oxygen is

available is limited by the rate at which oxygen dissolves into the medium. Hence, if the antibiotic is produced in association with an aerobic process, the rate of antibiotic formation will become dependent on the aeration.

It is interesting that the titre falls off so rapidly with inadequate aeration. If the maximum solution rate is fairly close to the peak oxygen demand, the organism will be adequately aerated over most of the growth period and then slightly under-aerated over a relatively short period. This period, however, seems to be a critical one for antibiotic production, and considerable loss occurs if the required supply of oxygen is not then available.

It is quite likely that, with some of the simple apparatus which is frequently used for screening large numbers of strains, failure to obtain the best titre of which a strain is capable might be due to poor aeration. For instance, a 1 l. conical shake flask, containing 300 ml. of medium and oscillating 90 times per min. with a 7 cm. throw, had a maximum solution rate of 0.4. Again, a large test-tube, fitted with a wash-bottle head and aerated at 1 volume per volume per min., had a maximum solution rate of 0.3. A similar tube aerated through a sintered disk gave a maximum solution rate of 0.5, which is some improvement on the simple test-tube. Adequate aeration was obtained in this apparatus by increasing the rate of air flow. When this was done only 1 % of the oxygen in the air bubbled through the medium was being consumed by the organism. This apparatus does not compare unfavourably for efficiency of aeration with most of the culture vessels commonly used.

This waste of oxygen is not usually important on a laboratory scale, but on an industrial scale the cost of aeration may be very considerable. It is therefore important to design efficient large-scale vessels, and by suitable small-scale experiments to find out the minimum aeration which is required for economical running of the plant. Although, so far, little attention has been paid to the point, it would probably be useful to try to select strains which give a good titre with little aeration.

The technique described should be applicable to any type of submerged cultures. It is likely that most of the oxygen demands encountered would be of zero order, and in this case any given metabolic process should eventually become independent of the rate of aeration. However, the method is applicable to any culture in which the oxygen demand can be expressed as a function of the oxygen tension, and in this case k_0 in equation (2) would be replaced by the appropriate function.

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