FERMENTATION

Oxygen Uptake in Shake-Flask Fermentations

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Because of the importance of oxygen as a substrate in fermentation, a shake-flask fermentor was designed, in which the uptake of oxygen during a fermentation is recorded automatically. Information on substrate utilization and product formation under various conditions is necessary if optimal fermentation conditions are to be established. The oxygen requirements of three different fermentations—α-amylase, ustilagic acid, and citric acid—were studied using this equipment.

SHAKE-FLASK TECHNIQUE is widely used in the study of aerobic fermentations. Characteristic features of a fermentation are generally assessed by determining substrate utilization and product formation under various conditions. Such behavior must be understood, if optimal fermentation conditions are to be established. However, in spite of the importance of oxygen as a substrate, technical difficulties introduced by the acquisition of oxygen from the surrounding atmosphere through the cotton plug and the poor solubility of oxygen in aqueous solution usually prevent study of oxygen requirements when chemical changes during fermentation are being considered. The rate of oxygen diffusion through a cotton plug and the gas-liquid interface are the limiting factors. The rate of oxygen diffusion through the gas-liquid interface may be determined by either the polarographic method (*4*, *10*) or the sulfite oxidation method (*3*), but these methods are not suitable for application to the actively fermenting shake-flask cultures at various stages in the fermentation.

This paper describes a specially designed closed shake-flask fermentor in which the uptake of oxygen during a fermentation is recorded automatically. The oxygen requirements of three different fermentations were studied using this equipment.

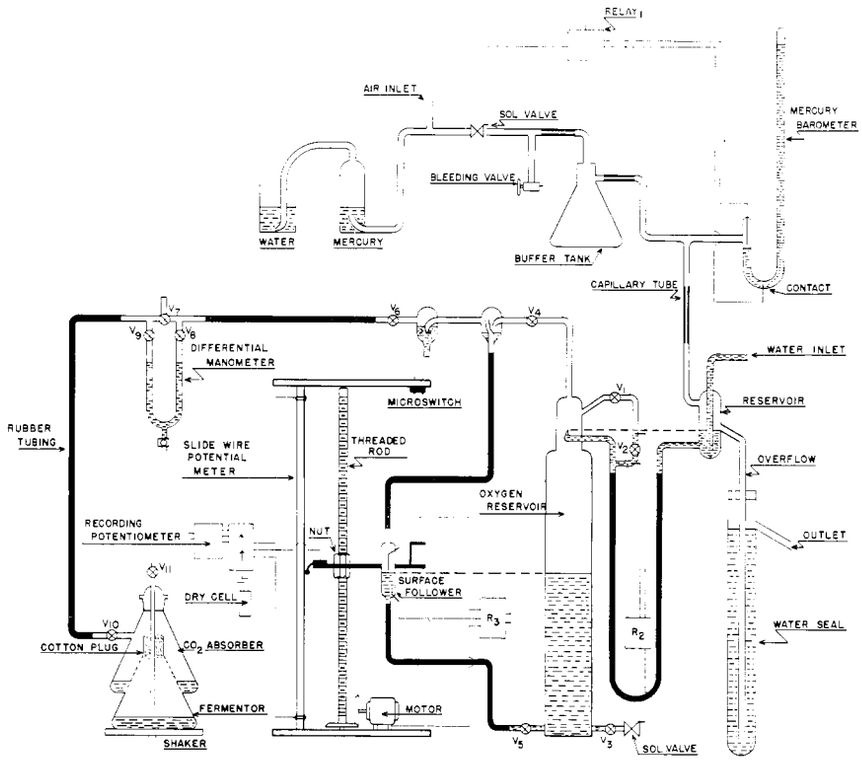
Apparatus

The closed shake-flask fermentor used for this study is similar to that described by Shu and Thorn (*8*), but is of improved form and is provided with facilities for recording oxygen uptake. The apparatus (shown in Figure 1) consists of four major systems: a fermentor attached to a carbon dioxide absorber, an oxygen reservoir and water supply, a surface follower and slide-wire potentiometer, and a manostat. The entire apparatus is assembled in an incubator.

The fermentor consists of two 500-ml. Erlenmeyer flasks sealed together as shown in the diagram. The cotton-plugged mouth of the fermentation flask is exposed to the atmosphere of the carbon dioxide absorber. A capillary tube with a stopcock valve, V_{11}, is extended through the cotton plug down nearly to the bottom of the fermentation flask. This tube is used for taking gas samples from the fermentation flask. The oxygen supply line is connected to the side arm of the carbon dioxide absorption chamber with valve V_{10}.

The oxygen reservoir is a cylinder 2.25 feet long and 2.5 inches in inside diameter. The water-inlet tip at the top of the cylinder is connected with rubber tubing to the water reservoir. Between the two valves, V_1 and V_2, of the side arm, there are two electrical con-

Figure 1. Schematic diagram of closed shake-flask fermentor



tacts, normally closed by the presence of water. Whenever an excess pressure builds up in the oxygen reservoir after the introduction of excess water, owing to accidental causes such as power failure, the water level in the side arm will be pushed below the contacts and the circuit will thus be opened. The opening of the circuit activates an electronic relay, R_2, to open the solenoid valve at the bottom of the oxygen reservoir. This allows the release of some water, until the pressure in the oxygen reservoir is restored to the original value and a closed circuit is again formed.

A tube (with valve V_5) at the bottom of the reservoir is connected with rubber tubing to the surface-following bulb. A capillary tube leads from the top of the oxygen reservoir to the fermentor unit and the surface-following bulb. A water-sealed check valve is situated between V_4 and V_6 to prevent the gas in the fermentor from diffusing into the oxygen reservoir. This valve has a rubber serum cap at the bottom, from which water can be withdrawn or injected with a hypodermic needle. The differential manometer with a three-way stopcock, V_7, is used to balance the pressure between the fermentor and the oxygen reservoir at the start of the fermentation. Running water is supplied to the water reservoir and overflow to the water seal. The overflow tubing must be sufficiently wide so that water will not fill up the entire tubing. The atmospheric pressure in the water reservoir is kept constant by connecting it to a manostat.

The manostat consists of a mercury barometer with two electrical contacts, a low pressure (40 mm. of mercury above atmospheric pressure) air supply controlled by a solenoid valve, and a pressure-damping device. The damping device consists of a bleeding valve, a buffer tank, and a series of capillary tubings each of 0.75-mm. inside diameter and 4 inches long. It reduces the pressure oscillations caused by the operation of the solenoid valve to an amplitude of not more than $^1/_8$ inch of water. The solenoid valve is operated by an electronic relay, which in turn is activated by the electrical contacts of the barometer. Because of the high frequency of operation, a specially designed solenoid valve (see Figure 2) is employed. The plunger of the solenoid presses and releases the rubber tubing connecting the air supply and the pressure-damping device.

Figure 2. Solenoid valve for air pressure control

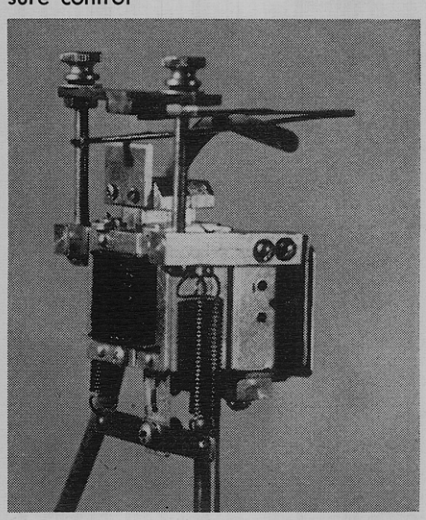
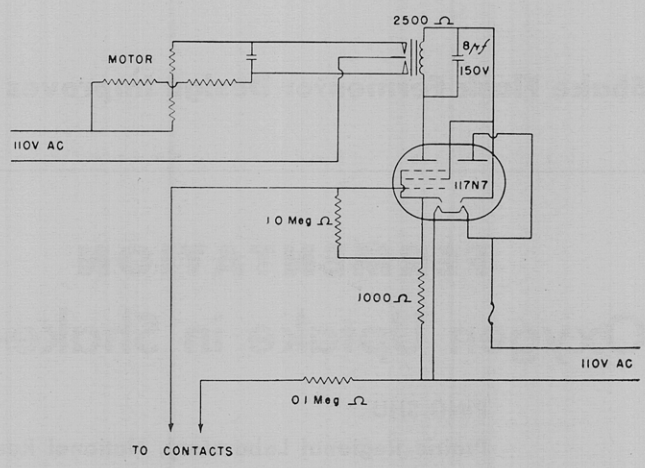


Figure 3. Relay motor circuit for surface - follower operation



The surface-following bulb with two electrical contacts is attached to a platform fixed on nut 1 inch long. With the aid of a square guide bar the nut may move freely up and down as the threaded rod turns in one direction or the other. The threaded rod is made of shaft rod 2 feet long and $^3/_4$ inch in diameter with $^1/_{16}$-inch thread, and is mounted on ball bearings at both ends. The rod is turned at a speed of 2 r.p.m. by a 1-r.p.m. Bodine KY C22 motor (Bodine Electric Co., Chicago, Ill.) coupled through a friction drive. The direction of the motor rotation is controlled by an electronic relay, R_3, which in turn is activated by the contacts in the surface-following bulb. The circuit diagram is shown in Figure 3. As the water level in the oxygen reservoir increases, the rise of the water level in the surface-following bulb closes the circuit and turns the threaded rod in such a direction that the bulb moves upward until the circuit is opened again. The rotation of the motor will then be reversed, and the bulb will be lowered until the circuit is closed. Thus, the bulb oscillates above and below the height of the water level inside the oxygen reservoir. The amplitude of this oscillation is less than $^1/_{32}$ inch. Glass surfaces in the vicinity of all the electrical contacts are coated with water-repellent Desicote (Beckman Instruments, Inc., South Pasadena, Calif.).

The slide-wire potentiometer is made of No. 36 gage Nichrome wire (28 ohms per foot) stretched along the insulated surface of the square guide bar. The moving arm of the potentiometer is made of a brass roller contact attached with insulation to the platform which carries the surface-following bulb. The current is supplied by a 1.5-volt dry cell and is limited by two variable resistors (10,000 and 1000 ohms) connected in series with the wire. The potential drop across the entire wire is adjusted to 9.5 mv. Three terminals of the wire potentiometer are connected to the Brown recording potentiometer with a chart speed of $^1/_2$ inch per hour.

In the course of a fermentation, the oxygen uptake by the organism upsets the pressure equilibrium in the closed system. An equivalent amount of water is then drawn into the oxygen reservoir, resulting in the displacement of oxygen to the fermentor. The amount of displacement is proportional to the distance traveled by the surface-following bulb, which in turn is recorded by the recording potentiometer.

In carrying out a fermentation in the apparatus, the oxygen reservoir is first filled with water through V_3 and the solenoid valve, with V_4, V_6, and V_7 opened and V_5 closed. The water in the reservoir is then displaced by oxygen admitted through the open end of the differential manometer, with V_7 and the solenoid valve kept open. After V_7 is closed and the relay R_2 is set for operation the water level in the oxygen reservoir adjusts itself until the oxygen pressure reaches the set value of the manostat. Then, V_5 is opened, the surface-following bulb is lowered manually to the level of the water in the oxygen reservoir, and the threaded rod is coupled to the motor.

The empty fermentor is sterilized. The glass stopper with the capillary tubing wrapped with the cotton plug is sterilized separately from the unit. After the inoculated medium has been pipetted into the fermentation compartment, 50 ml. of $2N$ sodium hydroxide is pipetted into the absorption compartment. The cotton plug wrapped around the capillary tubing is put into position at the mouth of the fermentor flask with the help of long forceps. With V_{10} and V_{11} in control, the unit is repeatedly evacuated and filled with oxygen-containing gas of the desired composition. At the end of this

process, the pressure inside the unit is made slightly higher than that under which the fermentation is to be conducted, and stopcocks are closed. The unit is then connected to the differential manometer and allowed to reach temperature equilibrium. With V_7 closed and V_8, V_9, and V_{10} open, the excess pressure in the fermentor is released through V_{11} until the differential manometer indicates the equilibrium. Water is then injected into the check valve to make the seal. The fermentor and the oxygen reservoir are connected by turning V_7.

With a continuous supply of oxygen, the yield of products and rate of fermentation obtained with this fermentor are similar to those of ordinary 500-ml. Erlenmeyer shake-flask fermentations. The precision of this apparatus was tested with the sulfite oxidation method and was within 5% error.

Fermentation and Analytical Methods

To illustrate the use of the apparatus in determining the characteristic oxygen uptake of different fermentations and estimating their optimal oxygen requirements, three fermentations were studied —α-amylase production by *Aspergillus niger* PRL 558, ustilagic acid production by *Ustilago zeae* PRL 119, and citric acid production by *A. niger* (Wis.) 72–4. These three fermentations were chosen because they represent three different types of physical consistency of the fermentation broth and have different respiratory quotients.

For α-amylase production, the fermentation was done in a medium containing 2% yeast extract, 3% soluble starch, 0.5% calcium carbonate, and under the conditions described by Shu and Blackwood (*6*). The yield of α-amylase was determined by a method used by Blackwood (*2*) after rupturing the mycelium.

For ustilagic acid fermentation, the medium of Thorn and Haskins (*9*) was used, except that the corn steep liquor was omitted or replaced by 0.06% beet molasses. Ustilagic acid was determined by the method described (*9*).

For the citric acid fermentation, cerelose was purified and fermented by the method of Shu and Johnson (*7*). The citric acid yield was determined by titration with 0.1*N* sodium hydroxide solution.

Fifty milliliters of medium was used for each fermentation, with the exception of citric acid, for which 25 ml. of medium was used. All fermentations were carried out on a rotary shaker of $1^3/_{16}$-inch eccentricity running at 210 r.p.m. The extent of aeration was varied by changing the oxygen partial pressure in the fermentor unit.

When necessary, the adequacy of the oxygen supply was tested by increasing the shaker speed or the oxygen tension in the fermentor flask. Any increase in the oxygen uptake rate indicated that the oxygen supply was not sufficient. The maximum oxygen supply may be estimated by determining the oxygen uptake rate of sulfite (5%) oxidation in culture broth or in the presence of the washed cell mass at the desired phase of the fermentation. The cell weights were determined gravimetrically. The cells, separated from the fermentation broth by filtration or centrifugation, were washed thoroughly with distilled water and dried at 60° C. under vacuum. When carbonate was present (as in α-amylase production) the cells were washed with dilute hydrochloric acid. For the cell mass of *U. zeae*, ustilagic acid was removed by washing with hot methanol.

The relation between the rates of oxygen uptake and product formation was established by determining the average increase in product concentrations and the average oxygen uptake rate during the period of a fermentation, when both the oxygen uptake and the product accumulation rates were constant or nearly constant. The oxygen uptake rate is expressed as millimoles per hour per liter of medium.

Results

In α-amylase fermentation, the mycelial growth was filamentous and mushy. The characteristic oxygen uptake curve (Figure 4) indicated that, after the initial lag phase, the rate of oxygen uptake decreased with time. This phenomenon was more pronounced at reduced oxygen tension. The maximum oxygen uptake in air was 19.2 millimoles per hour per liter of medium and that under oxygen was 56 millimoles per hour per liter.

Changes in the initial oxygen partial pressure (68 to 700 of mercury) in the fermentor flask caused the oxygen uptake rate to vary from 4 to 54, and the mycelium growth to vary from 6.5 to 15.0 grams per liter. When the oxygen uptake rate was higher than 10, there was only a little effect on the rate of amylase production. The maximum efficiency of enzyme synthesis by the mycelium occurred at an oxygen uptake rate of about 20 (Figure 5). The maximum rate of oxygen demand of the fermentation varied between 50 and 54 millimoles per hour per liter of medium (Figure 6). In the ustilagic acid fermentation, the mycelial growth was yeastlike under the fermentation conditions. The broth consistency increased slightly toward the end of the fermentation. After an initial lag phase, the rate of oxygen uptake was

Figure 4. Oxygen uptake curve of α-amylase fermentations under oxygen and air

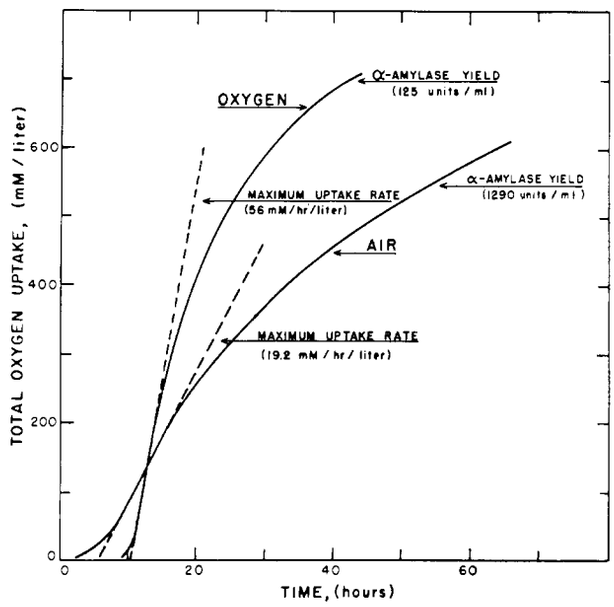
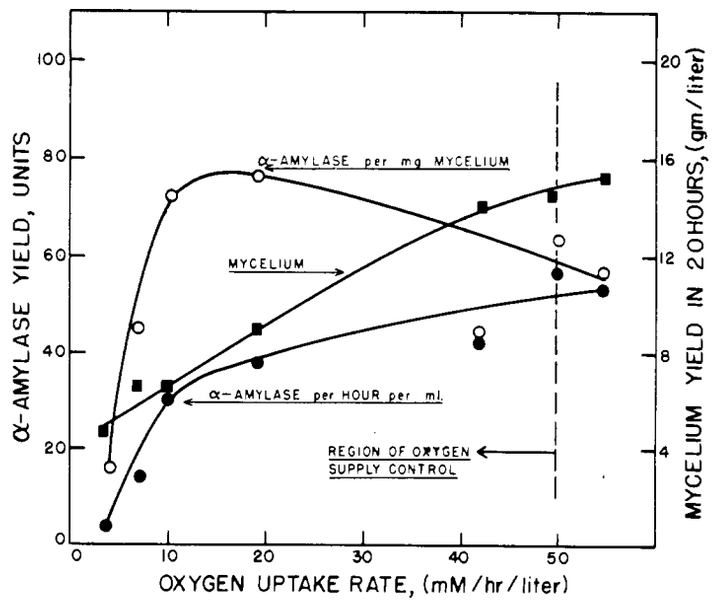


Figure 5. Relation between oxygen uptake rate and rate of α-amylase formation



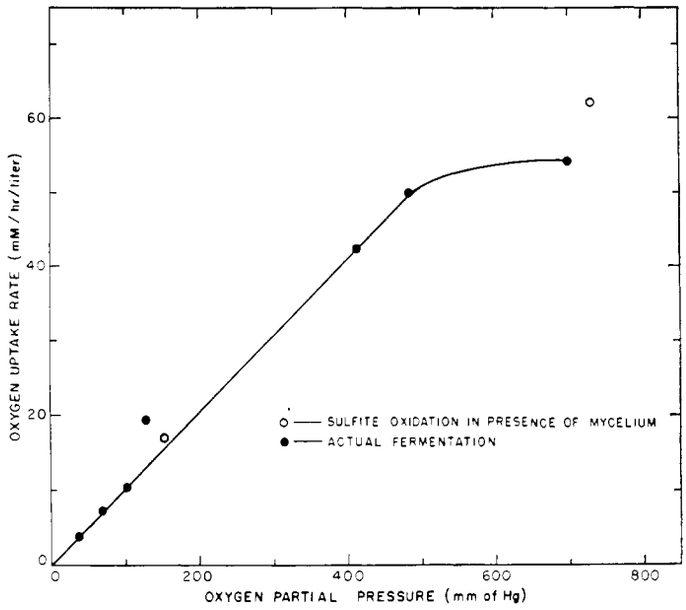


Figure 6. Relation between oxygen partial pressure and oxygen uptake rate in α-amylase fermentation

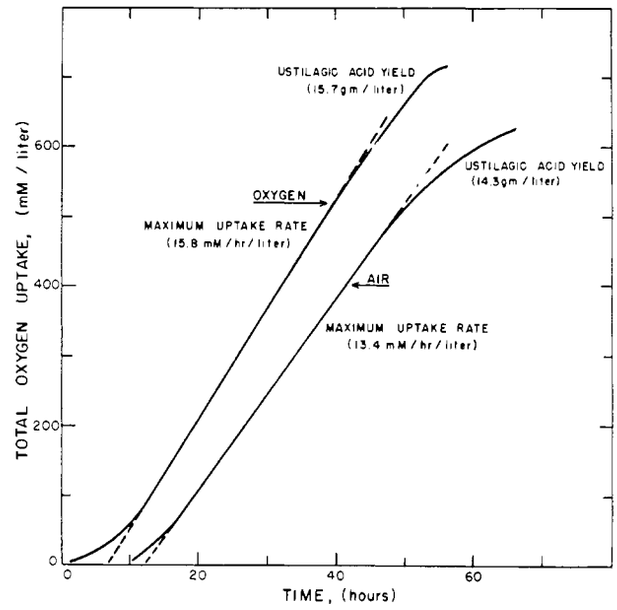


Figure 7. Oxygen uptake curve of ustilagic acid fermentation under oxygen and air

practically constant until near the end of the fermentation (Figure 7); under oxygen it was slightly higher than under atmospheric air (15.8 and 13.4, respectively). The maximum rate of oxygen demand of this fermentation was about 19.2 (Figures 8 and 9). The rate of ustilagic acid production was parallel to the rate of oxygen uptake.

In citric acid fermentation, the mycelial growth was sandy and tended to settle to the bottom of the flask when not stirred. The general shape of the oxygen uptake curve was similar to that of ustilagic acid fermentation (Figure 10). The maximum oxygen uptake rate under oxygen atmosphere was 27.6 and that under air was 8.4. The acid-producing rates were 1.02 and 0.325 gram per hour per liter, respectively. The ratios of the rates of oxygen uptake and acid production between these two runs were equal.

Discussion

The oxygen uptake rate in a fermentation is governed either by the maximum oxygen demand of the fermentation or by the rate of oxygen supply. The magnitude of the former depends upon cell concentration as well as the specific oxygen demand of the organism—that is, the maximum rate of oxygen utilized by a unit cell weight of the organism. On the other hand, the rate of the oxygen supply is affected by changes in suspended matter. The change of this variable in the course of a fermentation is an inherent characteristic of a fermentation process. The pattern of this change varies with the organism employed and the fermentation conditions.

In the period when the oxygen demand is the limiting factor the oxygen uptake curve may be concave if the specific oxygen demand and/or the cell concentration increases with time; convex if the specific oxygen demand and/or the cell concentration decreases with time; or a straight line if the effects of these two factors counterbalance each other or if there are no changes in both variables during this period.

In the course of a fermentation these variables generally first increase and then decrease or remain constant. In a batch process at the beginning and end of a fermentation the oxygen supply is often sufficient. In the period when the rate of oxygen supply is limiting, an increase of suspensoid with time results in a convex oxygen uptake curve, while the gradual decrease of this variable produces a concave oxygen uptake curve. A straight line may be formed when there are no significant changes in such variables. The oxygen supply can be the limiting factor only at the middle portion of the oxygen uptake curve.

The characteristics of the oxygen uptake curve when oxygen supply is the limiting factor and its relation to the accumulation of the product under investigation are of greatest interest in the aeration studies.

The differences in the characteristics of the oxygen uptake curve of α-amylase fermentation from those of the ustilagic acid and citric acid fermentations reflect the differences between the effect of their growing cells on the rate of oxygen supply. The mushy filamentous mycelium formed in α-amylase production reduced the oxygen supply much more effectively than the yeastlike and sandy cell masses formed in ustilagic acid and citric acid fermentations. The reduction of the rate of oxygen supply was found to be an exponential function of the concentration of the filamentous cell mass (5). The curvature of the oxygen uptake curve of the α-amylase fermentation was more pronounced in low oxygen tension than in high oxygen tension, because at higher oxygen tension the oxygen supply becomes a limiting factor at a higher mycelium concentration. The higher the mycelium concentration, the less will be the effect exerted upon the oxygen supply by the change of the cell concentration.

The oxygen uptake rates were constant for both ustilagic and citric acid fermentations under either air or oxygen. However, the oxygen demand of the ustilagic acid fermentation was considerably lower than that of citric acid fermentation, as shown by the different oxygen uptake rates under an atmosphere of air and of oxygen. This difference was small in ustilagic acid fermentation as compared with citric acid fermentation. The higher aeration demand of citric acid fermentation was partly due to the high sugar content of the medium and partly due to the requirement of a high oxygen tension for oxygen consumption by the organism under the fermentation conditions. The rate of sulfite oxidation which indicates the maximum rate of oxygen supply was found to be much higher than the actual uptake by the fermentation where oxygen supply is limiting.

In all three fermentations the rate of product formation is proportional to the rate of oxygen uptake at the region where rate of oxygen supply is low. In ustilagic and probably in citric acid fermentations the rate of the product formation is limited by the rate of the specific oxygen demand of the organism. In α-amylase fermentation, however, the rate of product formation reaches a saturation value when the oxygen uptake rate is far below the value of the maxi-

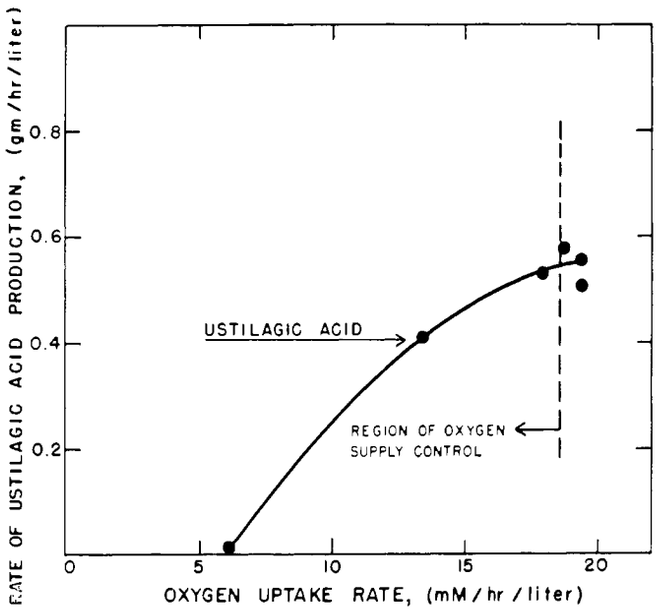


Figure 8. Relation between oxygen uptake rate and rate of ustilagic acid formation

Fermentation medium contained 0.06% beet molasses

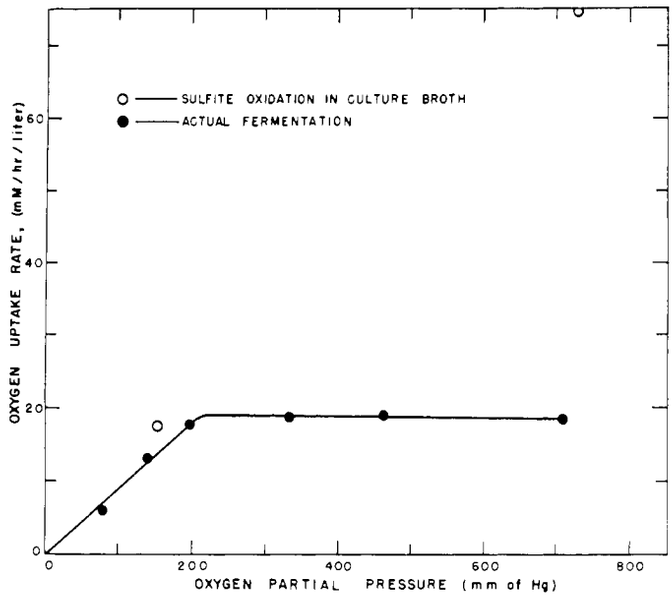


Figure 9. Relation between oxygen tension and oxygen uptake rate in ustilagic acid fermentations

Fermentation medium contained 0.06% beet molasses

mum oxygen demand of the fermentation.

The above illustration represents three different types of relation between oxygen uptake and product formation: α-amylase production, a type where the optimal oxygen requirement for the fermentation is far below the maximum oxygen demand; ustilagic acid production, a type where the optimal oxygen requirement for the fermentation is the same as the maximum oxygen demand; and citric acid fermentation, a type similar to the ustilagic acid fermentation, except that a comparatively high oxygen tension is necessary for maximum rate of its utilization by the organism.

The optimal oxygen requirement of the second and third types may be estimated by the polarographic method suggested by Wise (*10*) and Hixson and Gaden (*4*), or by the sulfite oxidation method suggested by Cooper *et al.* (*3*). The variations in the oxygen uptake rate during the course of a fermentation may be estimated by the combined use of these two methods (*1*), assuming that the effect of the change in cell concentration on the oxygen supply rate is insignificant.

The optimal oxygen requirement of the first type, however, cannot be correctly determined by these methods, the value obtained with the polarographic method is not correct in the region where oxygen supply rate is the limiting factor and the use of the titrimetric sulfite oxidation method is not practical in the fermentation broth or in the presence of living cells.

Summary

A closed shake-flask fermentor, constructed to simulate the fermentation conditions of a cotton-plugged 500-ml. Erlenmeyer shake-flask culture and provided with a device for automatically recording the oxygen utilization during the entire period of a fermentation, provided a means of direct measurement of the oxygen uptake without disturbing the fermentation system, and made possible study of the oxygen utilization characteristics of various fermentations. The relation between oxygen uptake rate and the rate of product formation could be established and the optimum aeration requirement correctly determined. This method is particularly useful in the determination of the optimal aeration level for a fermentation in which the optimal level is far below that of the over-all oxygen demand of the culture.

Tests were made with α-amylase, ustilagic acid, and citric acid fermentations, representing three different types of culture broth consistency and respiratory quotients. In α-amylase fermentation the oxygen supply was gradually reduced, owing to the growth of the organism. The optimal oxygen uptake rate for maximum α-amylase production was far below the maximum rate of oxygen demand. For ustilagic acid fermentation, the maximum rate of oxygen demand was very low and the rate of acid production was parallel to the oxygen uptake rate. In both fermentations, the organism efficiently utilized oxygen at low tensions. In citric acid fermentation the rate of acid production was parallel to the oxygen uptake rate, and high oxygen tension was necessary for a high rate of oxygen utilization.

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Received for review September 8, 1953. Accepted November 13, 1953. National Research Council Paper No. 3132, Prairie Regional Laboratory.

Figure 10. Oxygen uptake curves of citric acid fermentations under oxygen and air