# Continuous Shake-Flask Propagator for Yeast and Bacteria

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**A procedure has been developed for carrying out continuous aerobic bacterial and yeast fermentations on a shake-flask scale. Steady state conditions were readily maintained for long periods. The fermentations behaved essentially in the manner calculable from the amount of oxygen available, as determined by the sulfite oxidation method. The apparatus is simple and convenient to use.**

CONTINUOUS CULTURE METHODS for the propagation of yeasts, molds, and bacteria have been the subject of investigations in many laboratories. Continuous processes for the production of antibiotics, alcohol, and yeast may be classified as single-vessel systems and multiple-vessel systems. Examples of the former type were described by Bilford and others (*4*), Unger and others (*21*), Floro and others (*7*), and Maxon and Johnson (*14*). Two-tank continuous systems for yeast fermentation were described by Keussler (*12*), Ruf and others (*18*), Altsheler and others (*3*), and Adams and Hungate (*1*). The two-vessel system was used by Liebmann and De Becze (*13*) for penicillin fermentations. Owen (*17*) and Victorero (*22*) reported fermentations carried out in a single vessel divided into compartments. Foster and McDaniel (*8*) described a single-vessel semicontinuous process for the production of penicillin.

A method for predicting the time required for the continuous fermentation of specific substrates was reported by Adams and Hungate (*1*). Theoretical aspects of the continuous culture of microorganisms have been studied by Golle (*9*) and Monod (*15*).

A simple small scale continuous aerobic fermentor is a desirable research tool, as a number of fermentations may be carried out simultaneously in a series of fermentors and small amounts of medium may be used. Previously described aerobic fermentors have been of the tank type, designed to handle large amounts of medium. Their complexity makes it difficult to conduct a number of fermentations simultaneously. The method of carrying out continuous shake-flask fermentations described in this paper is simple and convenient, and has been used for a number of fermentations with good success.

### *Apparatus and Procedure*

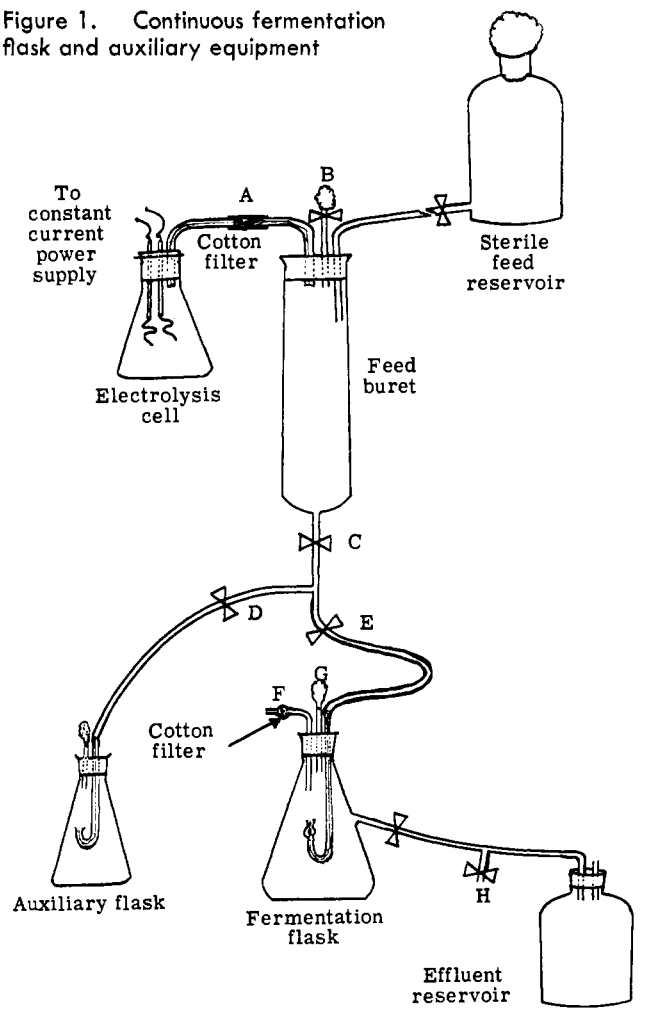
The fermentation apparatus is diagrammed in Figure 1.

Gas, produced at a constant rate in an electrolysis cell, displaces medium which is fed to the fermentor. The volume of medium in the fermentor is held constant by drainage from the side arm in the fermentor flask. The constant current power supply is described in Figure 2. The plate current of the parallel 50L6GT output tubes is held constant by the constant screen voltage supplied by the $OD_8$ regulator tube, and by the inverse feedback supplied by the cathode bias. The device can be assembled, at a cost for parts of approximately $25 including the milliammeter, by anyone familiar with electronic circuits.

The electrolysis cell consists of a flask of approximately 1 *N* sulfuric acid. The two electrodes (No. 32 platinum wire, sealed in glass, is satisfactory) are connected to the current supply. Ten to 20 such cells may be operated in series from one power supply. The cell is connected to the feed buret, which is constructed from a 65 × 500 mm. borosilicate glass test tube. The stopper of the feed buret bears three tubes, one for connection to the electrolytic cell, one connecting with a large reservoir of sterile medium, and the third for escape of gas when the buret is filled from the reservoir. The lower opening of the buret is connected, by a rubber tube, to the fermentation flask and to an auxiliary flask. The auxiliary flask connection is used only during a short equilibration period following the filling of the buret.

The fermentation flask is fitted with a rubber stopper bearing three holes, one for the feed tube, one for the air inlet tube, *F*, and one for a cotton-stoppered air outlet tube and sampling

Figure 1. Continuous fermentation flask and auxiliary equipment



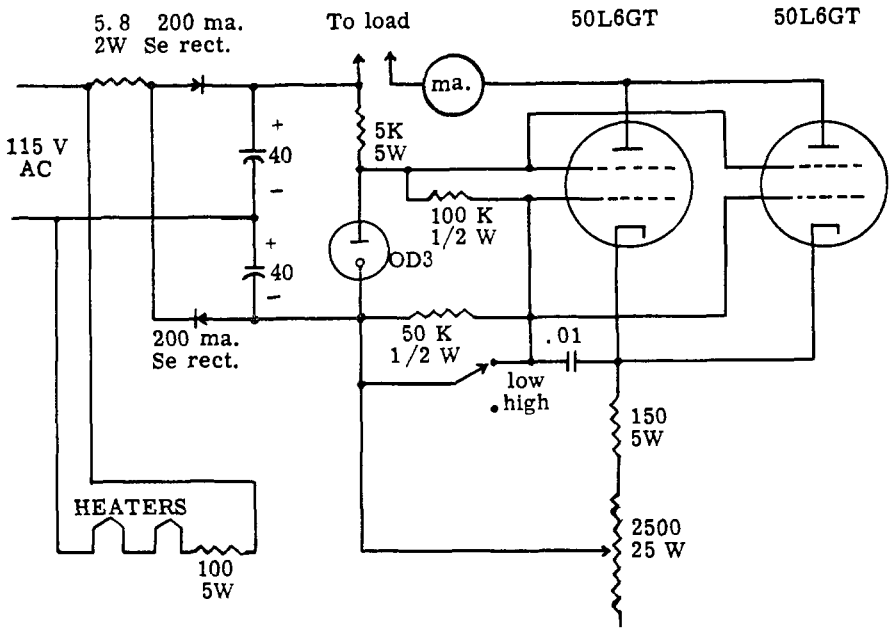



Figure 2. Constant current source

With switch in high position, current output is adjustable from 20 to 150 ma., and on low range, from 7 to 30 ma. Output current is substantially independent of line voltage fluctuations between 100 and 130 volts, and of voltage drop through the load as long as this does not exceed 80 volts

port, *G*. The construction of the feed tube has been described by Soltero and Johnson (*20*). It is a capillary glass tube, turned upward at its lower end, and with a small bulb blown in the upturned portion. This construction prevents air bubbles from rising through the feed tube during incubation on the shaker. The air inlet tube, a section of which is packed with cotton for air filtration, is connected, through any suitable throttling device, to the compressed air line. The air flow rate used is low, 10 ml. per minute being ample. At low air rates, the simple air filter used is adequate.

The 500-ml. fermentation flask is fitted with a side arm which allows the broth to be discharged from the flask by the rotary action of the shaker. When a flask that contains liquid is placed on a shaker, the liquid, during rotation, rises along the sides of the flask to a level that is determined by the amount of liquid present and the speed of rotation of the shaker. The height at which the side arm is placed on the fermentation flask is determined by the above factors, and may be estimated by means of a calcium carbonate slurry. A volume of 1% calcium carbonate slurry equivalent to the desired fermentation volume is placed on the shaker and agitated for 5 minutes. The height to which the slurry rises on the sides of the flask is indicated by a line of calcium carbonate deposited at this level. The side arm is attached at this point. It is important to place each flask firmly in the same position on the shaker each time it is used. If these precautions are carefully observed, the residual volume in the flask has been found to vary about 5% when 75 to 110 ml. of medium are used.

The side arm of the fermentation flask is connected, by means of rubber tubing, to a reservoir for collection of effluent. A T-tube, *H*, is provided for convenient sampling of the effluent. The fermentation flask is mounted on a Gump rotary shaker, but the remainder of the equipment is mounted in a stationary position.

For a short fermentation, the feed reservoir may be omitted. When large volumes of feed are to be added, however, a reservoir is preferable to a very large feed buret, because, if a large volume of gas space is present above the medium in the feed buret, a change in barometric pressure will cause a very appreciable change in feed rate. Changes in temperature will also have an effect, but, because the fermentations are carried out in a constant temperature room, this occasions no difficulty.

A continuous fermentation is set up as follows: All parts except the electrolysis cell are sterilized in the autoclave. The feed reservoir contains sterile medium. The electrolysis cell is connected at *A*, and the buret is filled from the feed reservoir, allowing air to escape through *B*, which is temporarily opened. *C* and *D* are now opened, and a small amount of medium flows into the auxiliary flask during pressure equilibration. *D* is then closed, *E* is opened, and the output of the current supply is set at the desired value. The current necessary for any desired feed rate is readily calculated, and may be checked during the fermentation by the rate at which the feed buret empties. A tube through which flows a slow stream of air (about 10 ml. per minute) is connected at *F*. At the beginning of a continuous fermentation, the fermentation flask ordinarily contains a suspension of organisms in spent medium, produced by a preliminary batch fermentation in the same flask.

The fermentation apparatus described may be used to study the effect of aeration efficiency on the growth of the microorganisms. The aeration efficiency of the flasks was determined by the sulfite method of Cooper, Fernstrom, and Miller (*6*). The aeration efficiency of a 500-ml. Erlenmeyer flask containing 100 ml. of medium was found to be 0.3 millimole of oxygen per liter per minute. This value may be altered by using different amounts of fermentation medium or by indenting the sides of the flask. With the continuous apparatus described, it was found unsatisfactory to use volumes of liquid less than 60 to 75 ml. Indentations in the flask must be small and must be placed near the base of the flask, or control of foam and of volume in the flask becomes difficult.

### Fermentation and Analytical Methods

*Saccharomyces cerevisiae* A batch fermentation, which preceded the continuous fermentation and was carried out in the same flask, provided the desired cell population. The synthetic medium used for production of yeast in the batch fermentation was similar to medium A described by Olson and Johnson (*16*), except that no L-asparagine was used and the glucose concentration was reduced to 1.25%. Medium B of Olson and Johnson (*16*) was used for the continuous process with the following modifications: glucose 0.87%, sodium citrate 1.1 grams per liter, and 50% potassium hydroxide 2.2 ml. per liter.

Inoculum for the batch fermentation was provided from pure cultures of *Saccharomyces cerevisiae* y-30 that had been transferred to 25 ml. of medium A in a 500-ml. Erlenmeyer flask and incubated at 30° C. on a Gump rotary shaker at 250 r.p.m. for 18 hours. The inoculum was 0.5% by volume. This corresponds to 0.5 mg. of yeast (dry matter) per 100 ml. of medium.

When the desired population was obtained in the batch fermentation and all alcohol had been utilized by the yeast (about 20 to 24 hours), the continuous fermentation was begun. In the early stages of the continuous process frequent samples were taken to determine when steady-state conditions were established within the fermentor. At frequent intervals samples were removed aseptically and tested for the presence of contamination by the method of Green and Gray (*10*) as modified by Maxon and Johnson (*14*). Glucose was determined by the method of Shaffer and

Somogyi (*19*). The yeast dry weight and ethyl alcohol were determined by the procedures described by Maxon and Johnson (*14*). The pH of each sample was determined immediately after removal by means of a glass electrode.

***Azotobacter vinelandii*** For *Azotobacter*, a nitrogen-free mineral salts medium, modified from that of Burk and Lineweaver (*5*) was used. Its composition was: magnesium sulfate, 0.020%; potassium monohydrogen phosphate, 0.09%; potassium dihydrogen phosphate, 0.023%; calcium sulfate, 0.001%; ferric citrate and sodium molybdate, each 3 p.p.m. The pH of the salts medium was adjusted to 7.0 prior to autoclaving and the carbon source, sucrose, was added aseptically after the autoclaved salts had cooled.

The inoculum for the batch phase, which preceded the continuous fermentation, was provided from a pure culture of *Azotobacter vinelandii* O that had been transferred to 100 ml. of the modified Burk's medium containing 1.7% sucrose in a 500-ml. flask. The flask was incubated at 30° C. on a Gump rotary shaker at 250 r.p.m. for 20 hours. The inoculum was 1% by volume and contained about 3 mg. of dry cells per 100 ml. of medium. Nitrogen was determined by a micro-Kjeldahl procedure described by Johnson (*11*).

## Results and Discussion

Cells of *Saccharomyces cerevisiae* y-30 and of *Azotobacter vinelandii* O have been grown in continuous culture by the method described, in order to establish the reliability of the procedure. With both organisms, the behavior in continuous culture was approximately that expected from the aeration efficiency of the fermentor used and the characteristics of the organism.

Data on a continuous yeast fermentation are given in Table I. As may be seen from the table, a steady state could be maintained for long periods, with good cell yields. The lack of alcohol production indicated that the sugar feed rate was low enough to be within the limits imposed by the aeration efficiency. The productivity observed was greater than 80% of the productivity theoretically possible at the aeration level employed. Theoretical productivity was calculated from formula 16 of Maxon and Johnson (*14*).

Preliminary experiments with *Azotobacter* showed that yields with this organism were low, and its growth rate was slow. In order to ensure that the growth rate was limited by available oxygen, excess sucrose was fed; in the fermentation described about one third of the sucrose fed appeared in the effluent. This procedure resulted, as might be expected, in very efficient use of the oxygen entering the solution but not in more rapid growth or in increased yields. Data on an *Azotobacter* continuous fermentation are given in Table II. No difficulty was experienced in maintaining a steady state. The productivity actually observed was about 25% greater than that calculated [by the formula of Maxon and Johnson (*14*)] from the aeration efficiency. This discrepancy is too large to be attributable to the difference in composition between *Azotobacter* cells and yeast cells. The reason for this disparity is not known.

The fixation of between 16 and 17 γ of nitrogen per ml. per hour during the continuous phase compares favorably with the fixation values reported by other workers (*2*) who used the batch process with similar effective aeration values. This rapid fixation of nitrogen is possible in the continuous process because a high population of growing cells is maintained in the fermentor.

**Table I. Continuous Propagation of Yeast in Shake-Flask Fermentor**

| | |
|---|---|
| Volume, ml. | 80 |
| Aeration efficiency, millimole $O_2$ per liter per minute | 0.37 |
| Glucose concentration in feed, mg. per ml. | 8.7 |
| pH of feed | 6.7 |
| Retention time, hours | 8 |
| Yeast yield on added sugar, % | 40.8 |
| Productivity, gram per liter per hour | 0.444 |
| Ethyl alcohol in effluent, gram per liter | <0.04 |

| Time[a], Hr. | pH | Cell Dry Weight, G/L. | Residual Glucose[b], G/L. |
|---|---|---|---|
| 0 | 2.8 | 3.75 | |
| 18 | 5.3 | 3.55 | 0.2 |
| 66 | 5.6 | 3.55 | 0.15 |
| 96 | 5.5 | 3.55 | 0.2 |
| 119 | 5.6 | 3.60 | 0.15 |
| 163 | 5.5 | 3.55 | |

[a] From beginning of continuous phase.
[b] Reducing material in effluent, calculated as glucose.

**Table II. Continuous Propagation of *Azotobacter* in Shake-Flask Fermentor**

| | | | |
|---|---|---|---|
| Volume, ml. | 110 | Cell yield on added sugar, % | 20.2 |
| Aeration efficiency, millimole $O_2$ per liter per minute | 0.27 | Nitrogen fixed, mg. per liter per hour | 16.6 |
| Sucrose in feed, mg. per ml. | 17.0 | Productivity, gram per liter per hour | 0.168 |
| Retention time, hours | 12.7 | | |

| Time[a], Hr. | pH | Cell Yield, G./L. | Cell Protein (N × 6.25), G./L. | Sucrose Utilized, Mg./Ml. |
|---|---|---|---|---|
| 0 | 6.4 | 2.20 | 0.224 | . . |
| 19 | 6.45 | 2.12 | 0.216 | 10.6 |
| 47 | 6.4 | 2.15 | . . . | . . |
| 66 | 6.4 | 2.20 | 0.213 | 10.8 |
| 90 | 6.35 | 2.10 | 0.199 | 10.3 |
| 116 | 6.45 | 2.07 | 0.203 | 10.6 |

[a] From beginning of continuous phase.

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