APPLIED MICROBIOLOGY, Feb. 1969, p. 286-290

Vol. 17, No. 2
Printed in U.S.A.

Effect of Shaking Speed and Type of Closure on Shake Flask Cultures

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Received for publication 2 December 1968

Growth of microorganisms and biosynthesis of microbial products in shake flasks may be limited by operating conditions which provide inadequate supplies of oxygen. Methods are described for meeting the oxygen requirements of test organisms by using standard baffled flasks with pad-type closures and shaking at relatively high speeds. Growth of *Escherichia coli B* in a rich medium and production of candidin by *Streptomyces viridoflavus* were the test systems used. Flasks shaken at 230 to 385 rev/min gave sulfite oxidation rates of 1 to 8 mmoles of oxygen per liter per min over a useful working volume range (40 to 150 ml in 300-ml flasks). These rates are as high as those obtained in agitated fermentors under usual operating conditions.

Shake flasks are usually considered to be inferior to agitated fermentors for the transfer of oxygen from air to liquid, principally because of the usual employment of unbaffled Erlenmeyer flasks. That the use of baffled flasks results in increases in oxygen transfer rates has been clearly demonstrated (1, 2, 4).

The use of closures (usually cotton plugs) may also affect the oxygen supply to the culture. Chain and Gualandi (1) compared oxygen transfer in open flasks with that of flasks with cotton plugs or aluminum caps and concluded that interference with passage of oxygen into the latter was great enough to limit seriously the usefulness of shake flasks for experimental purposes.

Schultz (11) showed that cotton plugs appreciably restricted oxygen diffusion and demonstrated that growth of *Bacillus megaterium* in cotton-stoppered flasks was less than that in open flasks. He reported a reduction in chlortetracycline production in cotton-stoppered flasks as compared with flasks sparged with air. A complicating factor in these tests was the considerable evaporation that occurred.

Recently, Hirose et al. (6) also measured oxygen diffusion through cotton stoppers with results similar to those of Schultz. They emphasized the need to consider the overall oxygen transfer coefficient, including both diffusibility through the closure and across the gas-liquid interface.

The substitution of thin pads of filtering material for the usual cotton plugs has been reported as a means of increasing gas exchange. Corman et al. (2) used three layers of Rapid-flo discs (Johnson and Johnson Co., New Brunswick, N.J.). Gaden (4) described a closure made of cotton sandwiched between layers of gauze. The latter type of closure was used in our previous studies (9).

In the investigation reported here, we wished to determine the effect of gauze-cotton pads on the growth of an aerobic organism (*Escherichia coli*) and to compare the effectiveness of these pads with cotton and plastic foam plugs. If we found growth limitations with gauze pads similar to those previously reported for cotton plugs, we would then determine if the effect could be overcome by increasing oxygen transfer rates with increased shaker speed. It was shown many years ago that the speed of shaking affects mass transfer of oxygen (1), but most shakers appear to be run at standard speeds, often with no indication that the conditions used are optimal. The effect of shaking speed on a biosynthetic process, candidin production by *Streptomyces viridoflavus*, was also investigated.

MATERIALS AND METHODS

Working stocks of *E. coli* B were kept on nutrient agar slants. *S. viridoflavus* IMRU 3685 inoculum was preserved in a liquid nitrogen refrigerator as described previously (8).

The *E. coli* medium contained (g per liter): K_2HPO_4, 7.88; KH_2PO_4, 3.88; sodium citrate, 0.3; $MgSO_4 \cdot 7H_2O$, 0.075; $(NH_4)_2SO_4$, 2.25; yeast extract (Difco), 5; and Cerelose, 13.5 (sterilized separately). The candidin medium contained (g per liter): soybean meal (Special Nutrient 4-S; A. E. Staley Manufacturing Co., Decatur, Ill.), 30; and Cerelose, 45. Inoculum development was described previously (8, 9). The

amount of inoculum was 10% (v/v). Temperature of incubation was 37 C for *E. coli* and 28 C for candidin.

Triple-baffled, 300-ml Erlenmeyer flasks (no. 597 and no. 598; Bellco Glass, Inc., Vineland, N.J.) were used. The 598 flasks have standard Erlenmeyer necks; the 597 flasks have wide necks (DeLong style) without beaded lips.

E. coli B growth tests were made on a model G and on a model V rotary shaker (New Brunswick Scientific Co., New Brunswick, N.J.). They were designated shakers no. 1 and no 2, respectively. The oxygen transfer characteristics of no. 1 were described previously (9). Two model V shakers (no. 3 and no. 4) were used for candidin experiments. All four were variable-speed shakers which rotated in 1-inch (2.54 cm) circles.

The closures tested were gauze-cotton layers [called "Shaken flask closures" by the supplier, Biochemical Processes, Inc., Islip, N.Y.; the closures have been modified somewhat since our previous studies (9)], plastic foam plugs (Idento-Plugs; A. H. Thomas Co., Philadelphia, Pa.), and cotton plugs made by hand from absorbent cotton. These will be referred to, respectively, as "gauze," "foam," and "cotton" closures.

Growth measurements were made with a Klett colorimeter as described previously (9).

Oxygen tension in the cultures and oxygen tension in the gas space were measured with oxygen analyzers (Precision Scientific Co., Chicago, Ill.), modified to fit openings which were made in the bottom and upper sides of Bellco 598 flasks.

Oxygen demands of cultures were determined as described by Phillips and Johnson (10), with a Precision oxygen analyzer. A dropping mercury polarograph (model 106; Delta Scientific Corp., Lindenhurst, N.Y.) was used for determining the oxygen saturation value of the medium.

Oxygen uptake in a 50-liter fermentor (Fermacell; New Brunswick Scientific Co., New Brunswick, N.J.) was measured by continuous monitoring of the oxygen content of the exhaust air with a paramagnetic oxygen analyzer (Beckman Instruments, Inc., Fullerton, Calif.).

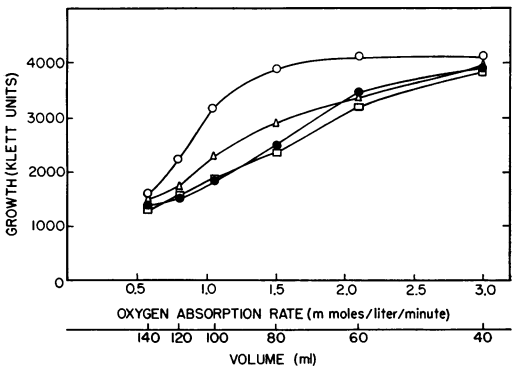

FIG. 1. *E. coli B growth at different oxygen absorption rates (shaker no. 1, no. 598 flasks). Symbols: ○, open flasks: △, gauze closures; □, cotton plugs; ●, plastic foam plugs.*

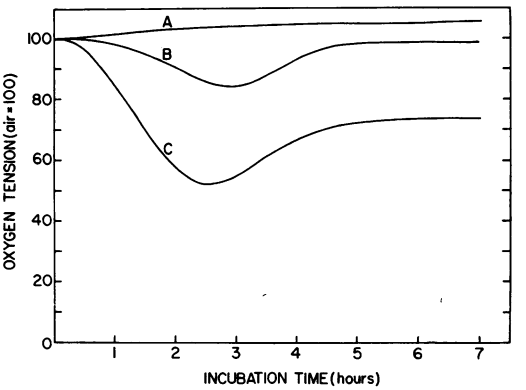

FIG. 2. *Oxygen tension in gas phase of E. coli B shake flasks run at an OAR of 1.05 (shaker no. 1, no. 598 flasks). Symbols: A, open; B, gauze closures; C, cotton plugs.*

RESULTS

Effect of closures on E. coli growth at 230 rev/min. Preliminary tests were run in glucose-salts and yeast extract-glucose-salts (YGS) media at concentrations such that growth levels from 1,000 to 5,000 Klett units were obtained in open 598 flasks with adequate aeration, starting with 50 Klett units and incubating for 7 hr. Growth reached a maximum or nearly maximum level by this time. Comparison of the growth obtained with gauze, cotton, or foam closures with the growth in open flasks at different potential growth levels showed that the use of closures reduced growth at turbidity levels of 1,500 to 2,500 Klett units or above but not at levels below 1,500.

For further studies, we selected the YGS medium as specified earlier in this paper. It gives 4,000 to 4,500 Klett units (5.5 to 6.0 g, dry weight, of cells per liter) at volumes which give OAR values (oxygen absorption rates by sulfite oxidation measurement) of 1.5 mmoles per liter per min or above. When the YGS medium was used at volumes to give OAR values of 0.55 to 3.0 (shaker no. 1), there were decided differences between open and capped flasks over most of the range (Fig. 1). Evaporation amounted to 4 to 6 ml in open flasks and 0.5 to 1 ml in capped flasks. The volume losses were made up before turbidity readings were made. The readings showed no difference between losses made up at the end of 7 hr and losses made up in small increments during incubation.

At the higher volumes there were small but consistent differences between gauze caps and

both cotton and plastic foam. To determine whether the differences were significant, 15 flasks of each were run at an OAR of 1.05 in five experiments with three replicates each. Differences between open and closed flasks and between gauze and both cotton and foam closures were found to be significant at probability levels of less than 0.01. No significant difference was found between cotton and foam closures.

Oxygen content of the gas space and dissolved oxygen concentration in culture fluids. Measurement of the oxygen content of the gas space of flasks run at an OAR of 1.05 showed decreasing oxygen tensions during the first 2.5 to 3 hr (Fig. 2); this drop was greater with cotton than with gauze closures. A slight upward drift in the readings usually occurred in open flasks, probably because the probes could be calibrated for only a short time before use.

There were variations in dissolved oxygen curves from different runs, but the typical picture was a drop to near zero in 2.5 to 3.5 hr (Fig. 3), with the earliest drop occurring with cotton plugs; gauze closures were next, and open flasks last. Since the cultures were run under oxygen transfer-limiting conditions, as shown by growth response (Fig. 1), an oxygen deficiency developed in all instances; only the times at which it occurred were different.

During the period of dropping dissolved-oxygen levels, there were fluctuations in the oxygen curves, usually occurring at fairly regular intervals. These fluctuations were similar to those described by Harrison and Pirt (5) for *Klebsiella aerogenes* in continuous culture, except that they occurred at higher levels of dissolved oxygen and were of only a few minutes duration.

Effect of increased shaking speed on oxygen transfer, growth, and closure inhibition. The most likely methods for eliminating inhibition by closures were: (i) use of thinner closures, (ii) use of a different kind of culture container or flasks with different baffling to increase the oxygen transfer rate, or (iii) an increase in speed of shaking to increase the transfer rate. We ruled out (i) because of the increased danger of contamination and (ii) because we knew of no commercially available culture containers which seemed to be superior to those we were using. The possibility of passing air through the flasks or of enriching the gas phase with oxygen was thought to be impractical.

Increasing the shaker speed from 230 to 320 rev/min increased the OAR twofold in baffled flasks over most of the volume range tested

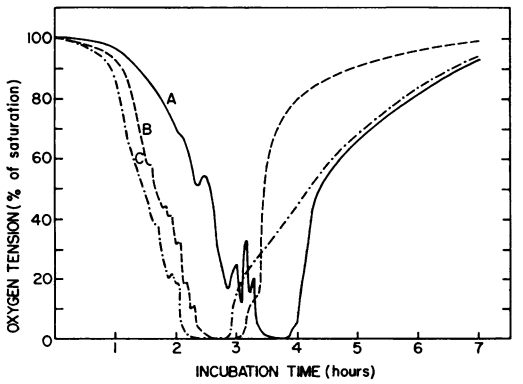

FIG. 3. *Dissolved oxygen tension in E. coli B shake flasks run at an OAR of 1.05 (shaker no. 1, no. 598 flasks). Symbols: A, open; B, gauze closures; C, cotton plugs.*

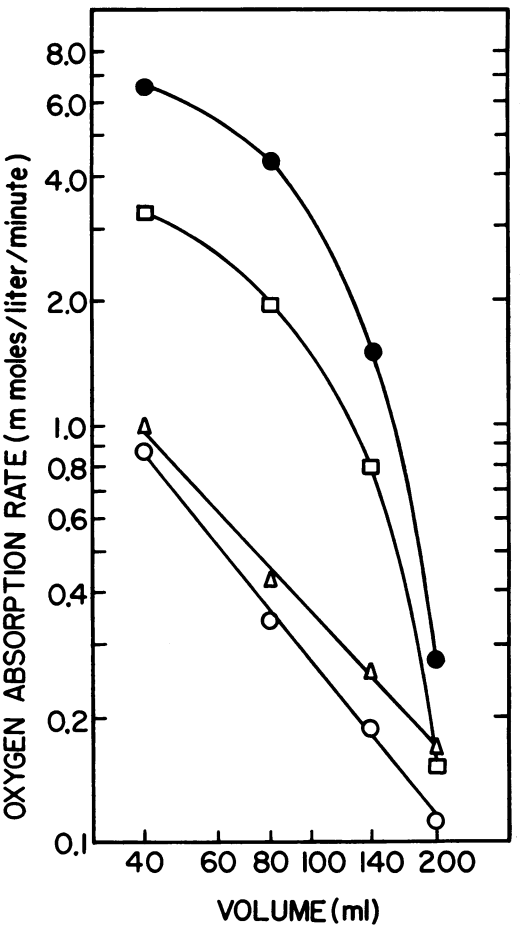

FIG. 4. *Sulfite oxidation rates with different volumes shaken at 230 and 320 rev/min (shaker no. 2). Symbols: ○, 230 rev/min, unbaffled flasks; △, 320 rev/min, unbaffled flasks; □, 230 rev/min, no. 598 flasks; ●, 320 rev/min, no. 598 flasks.*

(Fig. 4). The OAR measurements were made in open flasks. A further increase in speed to 385 rev/min gave an OAR of 8.0 with 40 ml of sulfite solution.

At 320 rev/min, the only closures which could be tested with cultures were gauze pads, since at this speed cotton and foam plugs both became wet in baffled flasks. Growth tests were made with Bellco 598 and Bellco 597 flasks. The latter have openings about 65% larger in cross section than the former. Increasing the speed of shaking from 230 to 320 rev/min increased the volumes at which maximum growth was obtained and eliminated closure inhibition up to 80 to 100 ml (Fig. 5). With gauze-capped flasks, growth was heavier in no. 597 than in no. 598 flasks at volumes above 100 ml. With 120- and 140-ml volumes, the differences between the two types of flask were statistically significant at the 0.01 probability level. Increasing the shaking speed to 385 rev/min with 40-ml volumes gave no increase in *E. coli* growth.

Production of candidin in shake flasks. Candidin production was also strongly affected by aeration conditions (Fig. 6). The half maximum yield points on the curves were at volumes equivalent to an OAR of 1.3 at 230 rev/min and 1.5 at 300 rev/min. Very low yields were obtained with unbaffled flasks. The medium which was used had been developed in experiments with 50-ml volumes in no. 598 flasks shaken at 245 rev/min (OAR, 2.9).

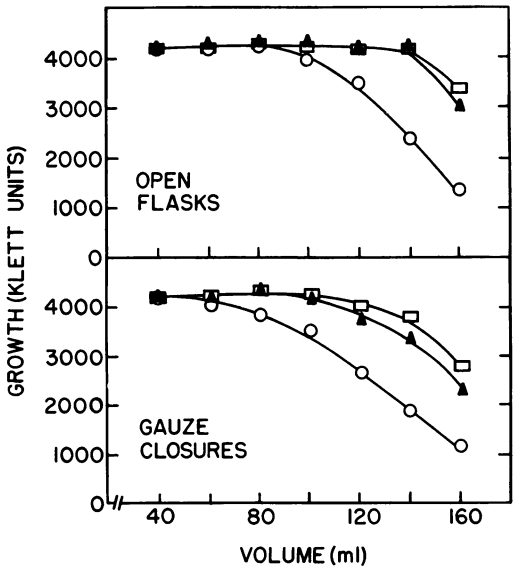

FIG. 5. *E. coli B growth with different volumes shaken at 230 and 320 rev/min (shaker no. 2). Symbols: ○, 230 rev/min, no. 598 flasks; ▲, 320 rev/min, no. 598 flasks; □, 320 rev/min, no. 597 flasks.*

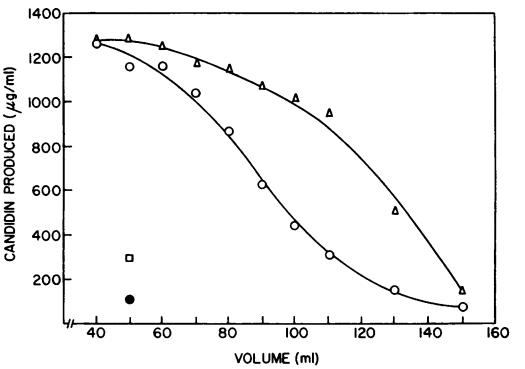

FIG. 6. *Candidin produced with different volumes shaken at 230 and 300 rev/min. Symbols: ○, 230 rev/min, no. 598 flasks; △, 300 rev/min, no. 598 flasks; ●, 230 rev/min, unbaffled flasks; □, 300 rev/min, unbaffled flasks.*

Oxygen requirements of E. coli B and S. viridoflavus. One question we wished to answer was whether provision for high oxygen transfer rates might be required for many shake flask cultures or whether those used in these studies were unusually high in oxygen requirement. To provide a basis for comparison with other cultures, the oxygen uptake of *E. coli* B was determined in a 50-liter fermentor run at a high oxygen transfer rate (OAR, 3.3). The fermentor growth curve was identical with that in shake flask controls run at the same OAR. The uptake peak was 1.16 mmoles per liter per min after 5 hr of incubation. Measured oxygen demand at this time (also the peak) was 0.93 mmoles per liter per min. The observed higher uptake than demand was similar to that reported by Siegell with yeast (Ph.D. Thesis, Columbia Univ., New York, N.Y., 1963). When run in a 50-liter fermentor at an OAR of 2.9, the candidin fermentation gave yields equal to shake flasks run at the same OAR. The maximum oxygen uptake rate was 0.68 mmoles per liter per min. In comparison with values reported previously for bacteria and streptomycetes (3, 10, 13), the observed uptake rates for *E. coli* B and *S. viridoflavus* were not exceptionally high.

DISCUSSION

Whether growth limitations occur in shake flasks depends, on the demand side, on the total oxygen requirement of the culture, and, on the supply side, on (i) the physical conditions employed (shaking speed, type of flask, and type of closure) and (ii) the rheological properties of the culture.

Since the total demand of a culture depends on its specific demand and on the amount of growth, and since the latter depends in turn on the oxygen supply rate, the maximum requirement can be determined only by increasing supply rates until no further increases in growth (or product formation) occur. This can be done by combining shaker speeds and culture volumes to cover OAR ranges as high as 8 mmoles per liter per min. In previous reports, OAR levels up to 4.05 were obtained with 15-ml volumes in 500-ml unbaffled flasks (7), and OAR levels up to 9.5 were obtained with 20-ml volumes in indented flasks (12). Such small volumes in flasks as large as 500 ml are not suitable for most shake flask work.

The OAR values reported here, in comparison with previously reported fermentor values, and the associated culture tests suggest that the oxygen requirements of most nonviscous cultures can be satisfied in shake flasks in a workable volume range. Viscous cultures pose special problems. Whether the same procedures will be successful with these cultures can only be determined by test.

The upper level of effective shaking speed was not established in these experiments, since 320 rev/min was adequate for the test systems used. An increase in speed to 385 rev/min gave no increase in *E. coli* growth. This is about the upper limit of the New Brunswick shaker, although other shakers with higher speeds are available.

The use of wide-mouth flasks seems indicated for maximum rate of gas exchange through the flask closures. However, only in borderline cases was an effect on *E. coli* growth detected.

Some filamentous organisms present special problems in shake flasks due to growth on the flask walls. The problem is accentuated by the use of baffled flasks. With the candidin-producing culture, periodical shaking by hand to wash off the wall growth minimized the problem.

Evaporation may also be a problem if flasks are run for several days. Weight losses can be made up periodically with sterile water, although the procedure is time consuming. Maintaining a moderately high relative humidity in the shaker room may also help.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant AI-07310 from the National Institute of Allergy and Infectious Diseases.

Perry Brett, Bellco Glass, Inc., provided the flasks modified to hold oxygen probes. Excellent technical assistance was provided by Gail Harris.

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