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Fermentor for Small-Scale Submerged Fermentations

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The need for equipment to facilitate the transition of submerged fermentations from shaker flasks to plant scale fermentors has long been recognized. Recently several papers (Bartholomew *et al.*, 1950; Brown and Peterson, 1950; Fortune *et al.*, 1950; Lumb and Fawcett, 1951; Humfeld, 1947; Rivett *et al.*, 1950; and Saeman, 1947) have described equipment for this purpose. The fermentors consist of either large Pyrex jars, or stainless steel containers, equipped with metal covers and devices for securing them. The accessories, such as agitators, aerators, air outlets, sampling cocks, and baffles are fitted to the cover. The assembly is placed in a constant-temperature water bath. In general, the fermentors have been used successfully, but not without such limitations as breakage and difficulties in handling, excessive sterilization times due to the poor heat conductivity of glass, sterilization of medium without agitation, and lack of temperature control of the individual fermentors.

In the Bureau of Agricultural and Industrial Chemistry, pan and rotary drum fermentors were used in early investigations on the production of organic acids by fermentation. These units were valuable in experimental work, but it was found difficult to translate a process directly from them to the present day deep-tank fermentors. Consequently, a 20-liter stainless steel fermentor for semi-pilot plant experimentation, which is similar to industrial installations and permits adequate variation and control of fermentation conditions such as temperature, agitation, aeration, and sterilization was designed at the Northern Utilization Research Branch.

A number of these 20-liter fermentors have been in continuous use since 1947. Data obtained from them, particularly with regard to aeration, agitation, foam control, culture medium, temperature and time of incubation, have been utilized in designing large-scale experiments. The 20-liter fermentors have also often been used to produce sufficient quantities of fermented liquors for isolation, characterization, and toxicological tests of new compounds.

The present report describes in detail the 20-liter stainless steel fermentor and accessories, its operation, and some experimental results obtained. A comparison of data obtained from a battery of these fermentors and others having a greater capacity is made for three typical fermentations.

DESCRIPTION OF FERMENTOR

A general view of 12 stainless steel fermentors at the Northern Utilization Research Branch is shown in figure 1. A line drawing of a fermentor, the accessories, and the various connections is given in figure 2.

The fermentor is a cylindrical, one-half jacket stainless steel vessel with a dish-shaped bottom and rounded corners 8¾ inches in diameter and 20 inches high, having a total capacity of 20 liters. The vessel is fitted with a stainless steel cover, agitator, aeration device,

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antifoam control, inoculation and view ports, thermometer well, and a combination sample and drain cock. All metal parts are made of 316 stainless steel having a smooth No. 4 finish. Wing nuts and lugs, which are fastened to the side of the vessel, each on a pivot, are used to secure the cover. When the lid is removed, the bolts tip out of the way to facilitate cleaning of the fermentor. Airtight seals between cover and fermentor are made with cork, lead, asbestos, rubber, or asbestos-reinforced rubber gaskets. Gaskets containing rubber are preferred at this Laboratory and have been used continuously for 12 to 18 months without appreciable deterioration. The asbestos gaskets are least satisfactory.

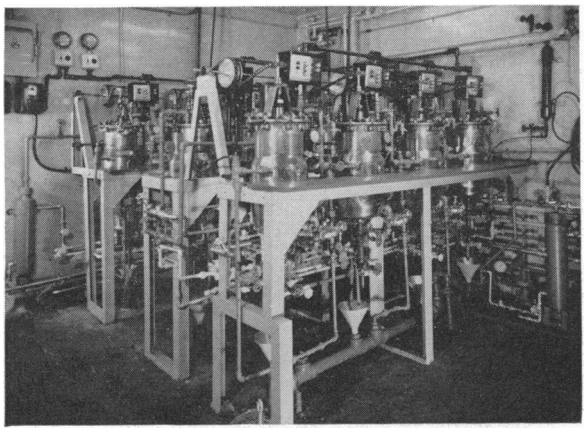

FIG. 1. A battery of twelve 20-liter stainless steel fermentors.

The cover is drilled with three $\frac{3}{8}$-inch holes for the air outlet, inoculum inlet, and antifoam inlet, and a $1\frac{1}{16}$-inch hole for the electrode of the antifoam system. There are two $2\frac{1}{4}$-inch ports equipped with Pyrex sight glasses. A metal cylinder containing a light source is placed over one of the ports to furnish illumination, and the second port is used to view the contents of the fermentor. A $\frac{1}{2}$-inch packing gland is located in the center of cover for the agitator shaft. A plaited, square, graphite impregnated asbestos cord is used as the packing material and makes a satisfactory seal. Recently, a molded, wedge-shaped, split ring packing, also made of graphite impregnated asbestos, has been found to make excellent seals with less maintenance.

The agitator shown in figure 3 consists of a hub $1\frac{1}{2}$ inches in diameter and six blades $1\frac{1}{8}$ by $1\frac{1}{2}$ inches secured to the hub with set screws making the diameter of the agitator $5\frac{1}{4}$ inches. It is possible to pitch the blades at any angle to give the desired agitation. The propellor is mounted $\frac{1}{2}$ inch above the sparger to a $\frac{1}{2}$-inch shaft which is driven by a Reeves[2] variable speed

[2] The use of names of commercial products or firms in this paper is not to be construed as an endorsement by the authors over similar products. Products used in this work are from the following firms: Reeves Pulley Company, Chicago, Ill.; Manheim Manufacturing and Belting Company, Manheim, Pa.; and The Carborundum Company, Niagara Falls, N. Y.

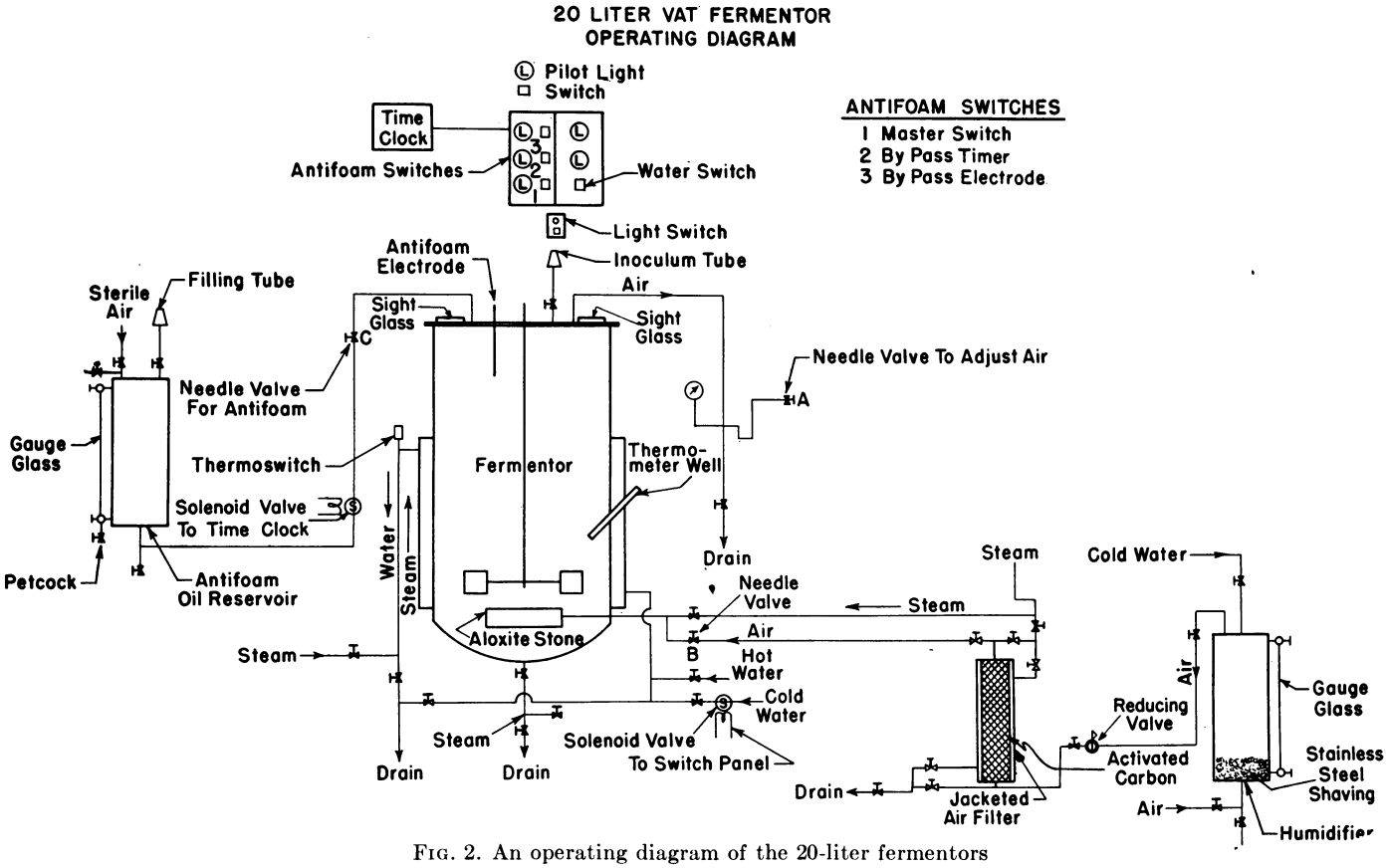

FIG. 2. An operating diagram of the 20-liter fermentors

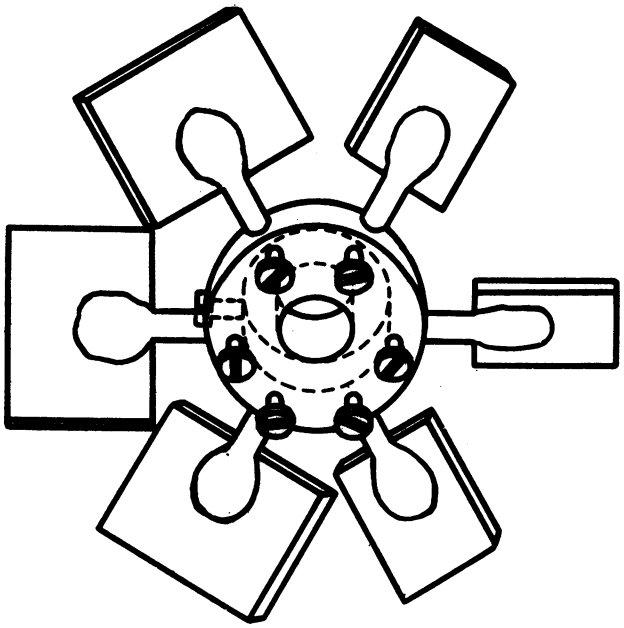

FIG. 3. Variable-pitch propeller

drive through a range of 50 to 350 rpm. Changes in the speed of individual agitators are possible by varying the size of pulleys on either the drive or agitator shaft. The use of Veelos Belting,[2] "B" section, facilitates the necessary changes in the length of belts when odd sized pulleys are used. Other types of drives, such as chain or direct drives, are less satisfactory.

The inoculating port consists of a short nipple, a valve, and a tapered fitting with a screw cap to cover the tapered portion. A small hole in the side of the cap near the threads permits passage of steam and condensate during sterilization.

The fermentor is equipped with a foam control system consisting of an electrode; electrical timer, the function of which is described later; a needle valve, C; a solenoid valve; and antifoam reservoir as shown in figure 2. The electrode, which is shown in figure 4 along with the antifoam system wiring diagram, is an automotive spark plug having a 3-inch prong inserted through the cover to make contact with the foam. In the development of the electrode two types of insulation material, namely porcelain and hard rubber, and various designs were tried. The porcelain insulation proved to be more durable and to have less tendency to foul than hard rubber. Spark plugs with elongated prongs have been most successful.

Air is dispersed in the culture medium through an Aloxite stone[2] (Number 1 porosity) shown in figure 5. A threaded ½-inch stainless steel pipe welded to a flat disc of stainless steel 2⅞ inches in diameter forms the core of the sparger. Ten 5/32-inch holes in the pipe permit the passage of air from the core through the Aloxite stone. The assembly is screwed into a port 1⅜ inches from the bottom of the fermentor. After each fermentation the sparger is taken apart for cleaning and is soaked in HNO_3 for 24 hours and thoroughly washed with water.

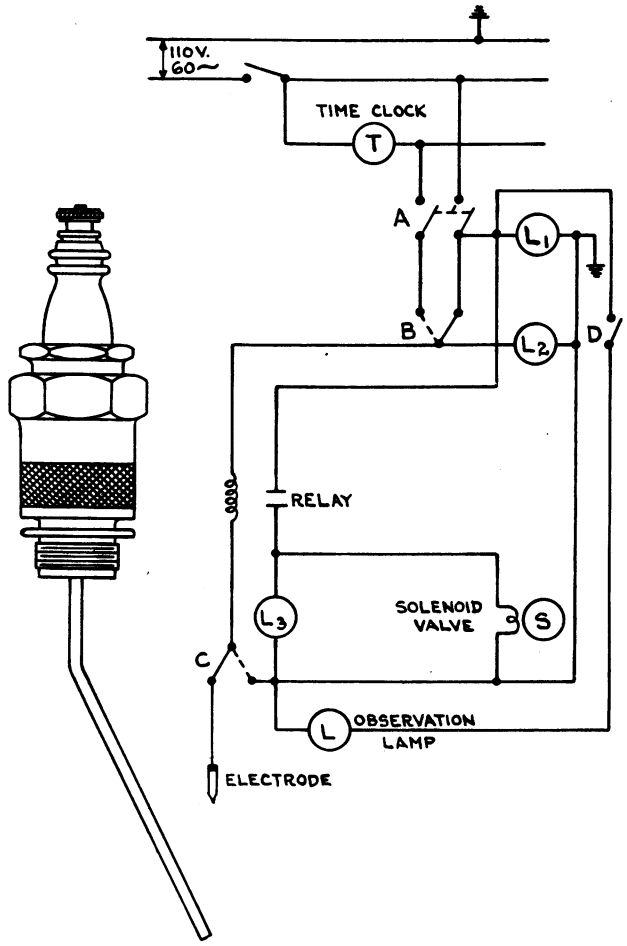

FIG. 4. Electrode and wiring diagram for antifoam system

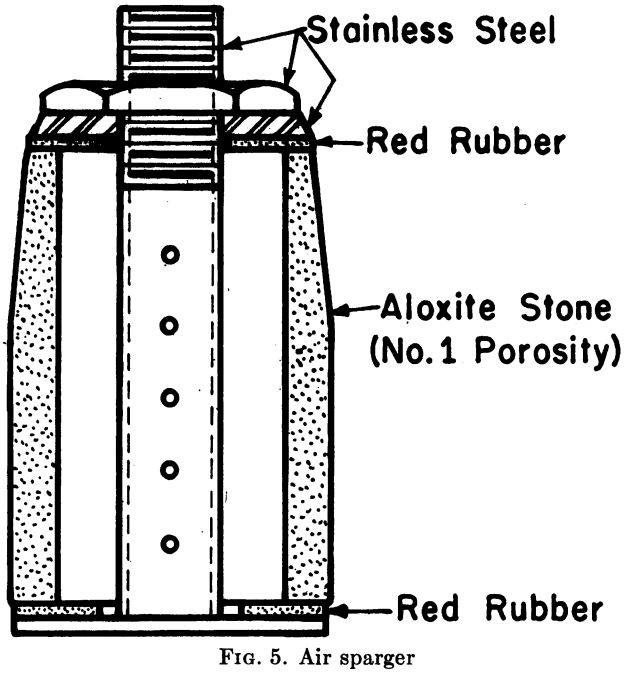

FIG. 5. Air sparger

Compressed air, which is used to aerate the culture media, is received from a reciprocating compressor at

a pressure of 90 psig and is reduced to 30 psig. It is next humidified, reduced to 15 psig, and then sterilized. Humidification is accomplished by passing the air through a standard 40-gallon hot water tank containing approximately 30 gallons of water and about 12 inches of stainless steel shavings to disperse the incoming air. This system does not deliver saturated air to the fermentor but the partial humidification curtails undesirable evaporation of water from the fermentor. For sterilization, the air is passed through a filter consisting of a jacketed 2-inch iron pipe 30 inches long which is packed with 10- to 24-mesh activated carbon. The carbon is held in place by a perforated metal disc welded to the pipe and covered with 4 inches of glass wool. Finally the air is passed through the Aloxite stone into the culture medium. The rate of air flow is regulated by either valve A on the air outlet or valve B before the sparger (figure 2).

The temperature of each fermentor is controlled individually by a thermoregulator in the discharge line of the water jacket. Hot water is allowed to trickle continuously into the jacket. Cold water is admitted through a solenoid valve as required to control the temperature. A temperature range of approximately 16 to 60 C is possible and fulfills all of our requirements.

A system has been developed whereby the pH of the medium may be followed easily and adjusted manually during fermentation. The accessories required are shown in figure 6. The electrode system (6a) consists of a long stainless steel flanged tube fitted with calomel and glass electrodes mounted in a rubber stopper and held in position by a long brass rod. The electrode leads extend through the tube to a portable pH meter. The electrodes are sterilized by suspending them in 70

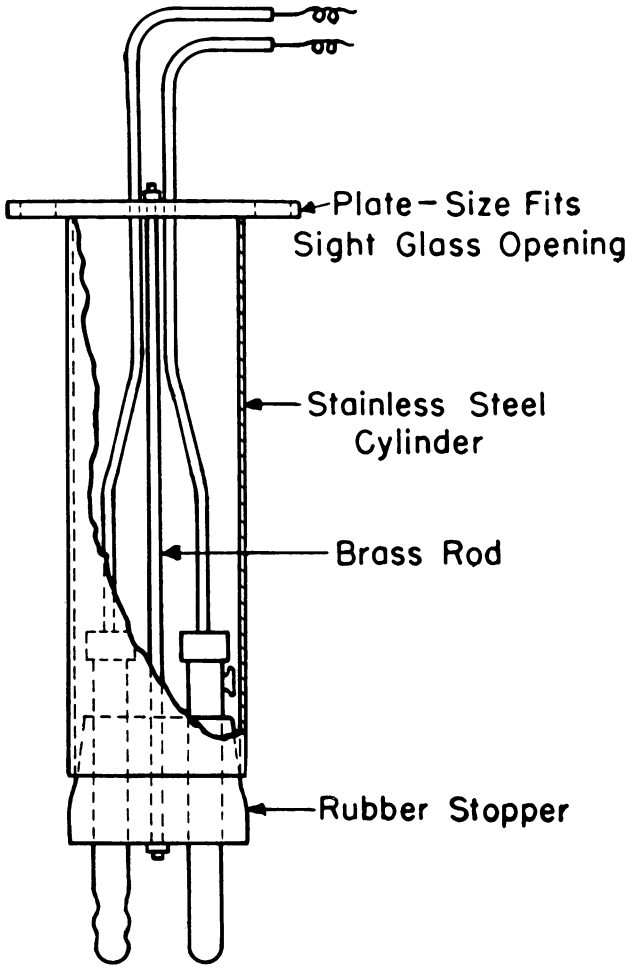

FIG. 6a. The fermentor electrode and assembly used to follow continuously the pH during a fermentation.

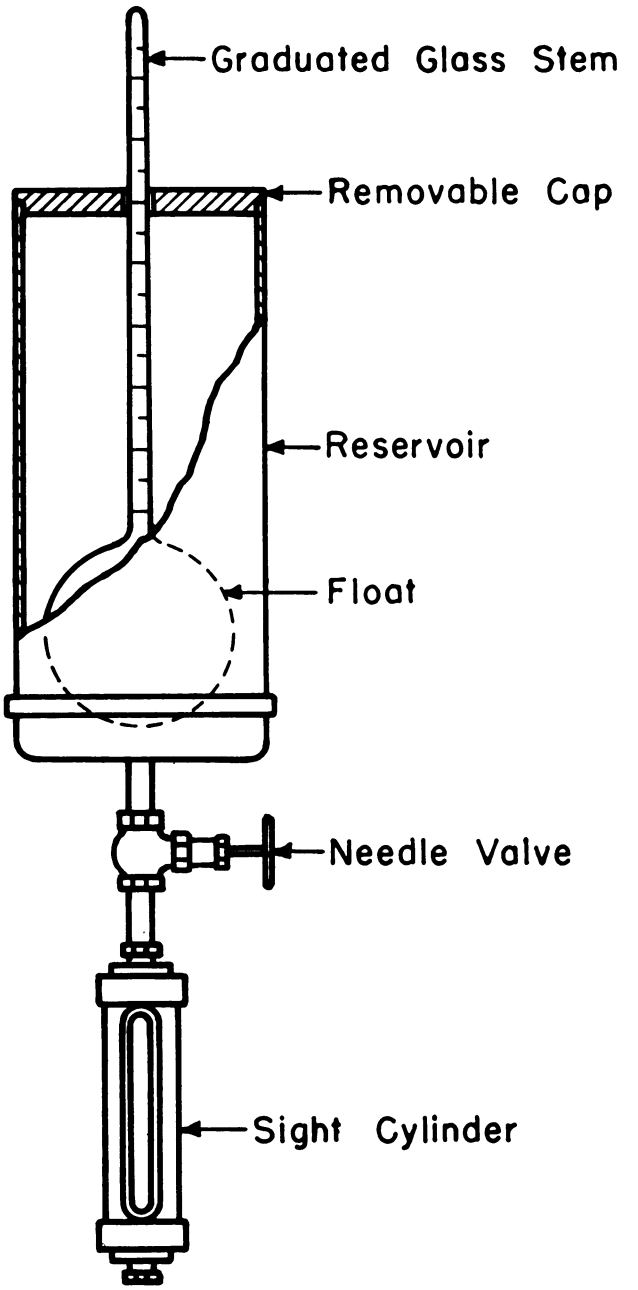

FIG. 6b. The alkali reservoir used in connection with the fermentor electrode for the manual control of pH during a fermentation.

per cent alcohol. After sterilization of the fermentor and its contents, the rear view port glass is removed and the sterile electrode assembly is fastened in its place. During this operation, air is allowed to flow through the fermentor to maintain a slight positive pressure and thereby lessen the danger of contamination. The asymmetry potential of the electrodes is corrected by drawing out a sample of the medium and ascertaining its pH with another meter. Proper adjustments to compensate for the electrodes in the fermentors can then be made.

The assembly for the addition of liquid chemicals, such as NaOH, is shown in figure 6b. It consists of a sight glass secured to the inoculation port, a needle valve, a reservoir made from a 2-inch iron pipe, a glass float with a calibrated stem (1-ml pipette), and a flat plate to cover the reservoir. The rate of addition of the alkali is determined visually through the sight glass while the total amounts added are read from the graduated stem of the float. The flow may easily be controlled with the needle valve. The value of the accessories for determining pH during the fermentation is emphasized later in the discussion of the production of the enzyme, dextransucrase.

The port in the bottom of the fermentor is used to drain the fermentor, to admit steam during the sterilization step, and to draw samples of the fermenting medium. A steam seal below the drain valve prevents contamination of the fittings, thus permitting the sampling to be done under aseptic conditions.

Operation of Fermentor

Aerobic fermentations have been made in the fermentors described above for the production of antibiotics, vitamins, enzymes, and acids. The operation of the fermentors in all cases is essentially the same.

A volume of water equal to five-sixths of the amount desired in the final sterilized medium is introduced into the clean fermentor. After the constituents of the medium are added, they are thoroughly mixed by means of the agitator. Steam is turned into the jacket of the fermentor. The temperature of the medium is raised to 100 C in about 5 to 7 minutes. Then steam at 15 psig is injected directly into the medium through the air sparger and through the drain to raise the temperature to 121 C in 5 minutes. The agitator is allowed to run throughout the sterilization. The increase in the volume of the medium due to the condensation of steam can be controlled by the manipulation of steam in the jacket and in the medium. For example, if the volume appears to be low, the steam to the jacket is closed resulting in greater condensation and vice versa.

During the sterilization of the medium, the air lines, air filter, air filter jacket, inoculation and sampling cocks, and antifoam system are sterilized with steam at 15 psig. The entire system is steamed as a unit to prevent the formation of pockets or dead ends which may not otherwise be sterilized.

After the usual 30 minutes at 121 C, the steam is turned off and the pressure on the system is maintained at all times with sterile air to prevent contamination through the packing gland and valves which may not be seated perfectly. Cold water in the jacket is used to cool the medium to fermentation temperatures in approximately 10 to 12 minutes.

Recently an alternate method of sterilizing the fermentation medium has been developed which involves higher temperatures and pressures, but less time. The constituents of the medium are mixed in water equal to one-fifth to two-fifths of the final volume desired. Steam of 35 psig is injected directly into the medium through the air sparger and drain to raise the temperature to 135 C in about 3 minutes. Steam in the jacket of the fermentor is optional but is generally used to force the water out of the jacket and is turned off after the temperature of the medium reaches 135 C. The time of sterilization is varied from 5 to 15 minutes depending on the types of medium to be sterilized. The medium is cooled as previously described in about 4 minutes.

After the temperature of the medium is adjusted, the inoculum and occasionally other materials, which through necessity are sterilized separately, are added. Sterile water is also added to bring the medium to the desired volume which is indicated by a small collar on the agitator shaft. The contents are thoroughly mixed and samples for initial analyses are withdrawn.

As mentioned previously, air flows may be controlled by valves on the air inlet or on the fermentor outlet. This provides the alternatives of running the fermentation at either atmospheric pressure or increased pressures as high as 30 psig. In general, a pressure of 5 psig is initially employed in studies of new fermentations, and therefore the air flow is regulated at the air outlet. The amount of aeration is ascertained by measuring the air leaving the fermentor with a flowmeter.

After the aeration of the fermentor has been adjusted and the antifoam agent, which is sterilized in an autoclave, is transferred aseptically to the sterile reservoir, the antifoam system is turned on by master switch A, a double pole single throw switch, shown in the wiring diagram of figure 4. Foam, when it occurs, will rise until it makes contact with the electrode which activates the solenoid valve controlling the addition of agent. In our system, however, an interval timer further regulates the time of addition to an interval of 3 seconds in a 3-minute period. During this short interval, approximately 5 ml of agent can be added, unless otherwise throttled by the needle valve C, figure 2. The arrangement is adequate to control all types of foaming we have thus far encountered. The time clock may be bypassed by turning switch B,

a single pole double throw switch, to the position of the broken line and the system is converted to one which adds agent as long as the foam makes contact with the electrode. If desired, the electrode may then be bypassed by turning switch C, a single pole double throw switch, to its alternate position which permits one to add the agents manually. Switch D controls a light source used to view the contents of the fermentor.

Typical Fermentations

Dextransucrase. The culture conditions for the rapid elaboration by *Leuconostoc mesenteroides* NRRL B-512 of dextransucrase, an enzyme used for the production of the blood plasma extender, dextran, have been reported by investigators of the Northern Branch (Tsuchiya *et al.*, 1952). Some of these data were obtained from fermentations conducted in the 20-liter stainless steel fermentors. Subsequently, numerous runs were made in a 300-gallon fermentor, which is equipped with a continuous pH controller.

The adjustment of the medium between pH 6.5 to 7.0 during the fermentation was found to be a factor critically affecting enzyme yield. Maximum enzyme activity was obtained in 8 hours in cases where the reaction was controlled at pH 6.7. Dextransucrase is relatively unstable at this pH but it is possible to stabilize the enzyme solution by lowering the pH to 5.0 to 5.2 at the time of maximum yield. The manipulation in the 20-liter fermentors was accomplished with a minimum of effort by the adaptation of the accessories shown in figures 6a and 6b.

The inoculum for this fermentation is developed by transferring the cells of *L. mesenteroides* NRRL B-512 from an agar slant to liquid cultures containing, per liter: Sucrose, 20 g; corn steep solids, 20 g; KH_2PO_4, 20 g; and R salts,[3] 5 ml. The medium is adjusted to a pH of 7.4 and sterilized for 15 minutes at 121 C. The cultures are transferred after 24 hours of agitation on a reciprocal shaker at 25 C from 100 ml of medium in a 500-ml Erlenmeyer flask to 500 ml in a Fernbach flask, and finally to 10 liters in an aerated carboy. A volume equal to 2 per cent of the fermentation mash is used to inoculate both the 20-liter and the 300-gallon fermentors.

The medium used in the plant fermentors is the same as the one given above with the exception that the KH_2PO_4 concentration is reduced to 0.1 per cent. Batch sterilization is employed with the 20-liter fermentors whereas continuous sterilization at 135 C for 3 minutes is used for the 300-gallon fermentor. All fermentors were aerated at the rate of 0.1 volume of air per volume of medium per minute and were agitated at the rate of 100 rpm.

Table 1 gives data from the dextransucrase fermentation when the pH of 10 liters of medium is varied in the 20-liter fermentor and, also, data obtained from 150 gallons of medium in the 300-gallon fermentor.

[3] R salts include $MgSO_4 \cdot 7H_2O$, 10 g; NaF, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.5 g; and $MnSO_4 \cdot 7H_2O$, 0.5 g per 250 ml distilled water.

Table 1. *Dextransucrase fermentations*

Age of fermentation	Volume fermented				
	10 Liters				150 Gallons
	pH				
	6.1	6.4	6.7	7.0	6.7
Hrs	*units/ml*	*units/ml*	*units/ml*	*units/ml*	*units/ml*
2	6.9	4.8	4.4	3.3	1.3
4	26.0	17.0	11.0	9.0	4.5
6	30.0	44.0	44.0	38.0	18.0
8	24.0	44.0	54.0	42.0	40.0
10	18.0	37.0	44.0	35.0	44.0

Calcium 2-ketogluconate. The calcium salt of 2-ketogluconic acid may be converted in good yield to *d*-araboascorbic acid (isovitamin C) which has been suggested as an antioxidant in foods and other products and as a developing agent in photography (Stubbs, 1940). Owing to its importance, numerous fermentations have been made both in the 20-liter and 300-gallon fermentor.

A culture of *Pseudomonas fluorescens*, NRRL B-334, is transferred from an agar slant into a liquid medium containing 100 g glucose and 5 g yeast extract per liter, and is then transferred into an aeration bottle containing the medium used in the large-scale experiments. These cultures are incubated for 48 hours at 32 C. A volume of inoculum equal to 3 per cent of the fermentation mash is used to inoculate the 10-liter and 150-gallon fermentations.

The medium used in both scale fermentors contains per liter: Glucose, 100 g; corn steep solids, 2.5 g; KH_2PO_4, 0.68 g; and $MgSO_4 \cdot 7H_2O$, 0.43 g. The medium is adjusted to pH 5.0 and sterilized as previously described for 30 minutes at 121 C. A 20 per cent solution of urea and a 20 per cent slurry of $CaCO_3$ are sterilized separately and are added to the cool sterilized medium at a rate of 2 g urea and 27 g $CaCO_3$ per liter.

The fermentors were operated at 33 C and 10 psig with one-half per cent octadecyl alcohol dissolved in ethanol as antifoam agent. Air flow of 1.5 and 0.75 volumes of air per volume of medium per minute and agitator rates of 220 and 190 rpm were employed for the small and large fermentors, respectively.

Data from two typical fermentations are given in table 2.

Riboflavin. Production of riboflavin with *Ashbya gossypii* NRRL Y-1056 (Wickerham *et al.*, 1946) has proved to be a valuable method for manufacture of this vitamin for feeds. The yields of riboflavin obtained in the 20-liter stainless steel fermentors agree well with the yields obtained in 50-liter aluminum, 300-gallon stainless steel, and 800-gallon copper fermentors. The

TABLE 2. *2-Ketogluconic acid fermentations*

AGE OF FERMENTATION	VOLUME FERMENTED					
	12 Liters			150 Gallons		
	Glucose	2-keto-gluconic acid	Yield*	Glucose	2-keto-gluconic acid	Yield*
hrs	*g/100 ml*	*g/100 ml*	*per cent*	*g/100 ml*	*g/100 ml*	*per cent*
0	9.1	—	—	9.4	—	—
18	3.0	2.6	—	2.9	4.4	—
24	0.9	5.7	—	0.7	6.7	—
28	0.1	—	—	0.0	8.1	80.2
48	0.0	7.9	80.8			

* Based on glucose available.

50-liter aluminum tanks were similar in design to the 20-liter fermentors described in this paper. Investigations of various factors which influence biosynthesis of the vitamin have been described by Pfeifer *et al.* (1950), Pridham *et al.* (1950), and Tanner *et al.* (1949).

DISCUSSION

The comparative results presented above show that it is possible with the 20-liter fermentors essentially to establish conditions and anticipate yields for fermentations operated on a larger scale. Nevertheless, the problem of scale-up in industrial aerobic fermentations is difficult and extremely important. Recent publications (Wegrich and Shurter, 1953; Karow, *et al.*, 1953; and Wise, 1951) stress that agitation, aeration, and the geometric characteristics of fermentors are essential factors in the transition from pilot plant to production-scale fermentors. Some variation in results may be accounted for by the inherent differences between the fermentors. The 300-gallon fermentor, for example, is 2 feet in diameter and 13 feet high, with a liquid depth of 6.7 feet for 150 gallons, while the 20-liter fermentors, as previously described, are 8.75 inches (0.75 feet) in diameter and 20 inches (1.67 feet) high with a liquid depth of 11 inches (0.92 feet) for 10 liters (2.64 gallons), thus making the geometric characteristics of the two fermentors different. In addition, the large fermentor has an agitator which revolves in a vertical plane, causing a churning of the liquid unlike the rotary motion in the 20-liter fermentor. The medium for the large equipment is generally sterilized by continuous rather than the batch sterilization method. This is advantageous, especially if the medium contains nutrients which react unfavorably (Pfeifer and Vojnovich, 1952). In spite of these differences, the ease with which cultural conditions can be ascertained for plant operations has been demonstrated.

In the sodium gluconate fermentation, for example, after a number of trials in the small fermentors, it was found that maximum rate of gluconate production was obtained when agitation, fermentation pressure and aeration were maintained as high as possible. The rate at which oxygen is supplied from the gas to the liquid phase is known to be an essential factor in scaling-up geometrically similar, fully baffled fermentors. The specific oxygen absorption coefficients [(g moles oxygen/(ml) (atm) (hr)) $\times 10^4$] for the 20-liter fermentors as determined in a copper-catalyzed sulfite-water system (Cooper *et al.*, 1944) are adequate for scale-up studies. Depending upon agitation and pressure, the values obtained in tanks, equipped with four 10 to 18 per cent baffles, vary from 0.35 to 13.5 for the Aloxite stone spargers, and from 0.15 to 10 for pipe spargers having six $\frac{5}{64}$-inch holes.

The aeration and rapid agitation of certain media, especially those containing proteinaceous materials, result in excessive foaming and the need for appropriate control measures. Soybean oil, lard oil, lard oil containing 1 per cent octadecyl alcohol, ethyl alcohol containing 0.5 or 1 per cent octadecyl alcohol and others have been used successfully as antifoam agents. Improper selection or large amounts of these agents have been shown to affect fermentation rates and efficiencies (Blom *et al.*, 1952, and Pfeifer, *et al.*, 1952). When foaming occurs, the time clock in our antifoam system permits the addition of limited amounts of the agent at definite intervals. In this manner, full advantage is taken of the agent's ability to break foam, especially when a stable or persistent one is formed, by allowing sufficient time for it to act on the foam. Under these conditions, only relatively small amounts are required to control all types of foaming we have encountered.

The antifoam agents are added to the individual fermentors as required, but since some agents are toxic to the organisms, it is necessary to keep the concentrations comparable. The electrodes may be wired in series, permitting the agent to be added to all fermentors each time the system reacts to a single signal. This feature removes the variable arising from the addition of various amounts of the antifoam agent and is especially desirable during aeration studies when foaming is most likely to occur in the fermentors with the highest aeration rate.

Evaporation due to aeration and sampling losses are problems common to fermentors having small working capacities. In an attempt to prevent evaporation, the air is first passed through a humidifier as described in preceding paragraphs. It has been found that the evaporation losses from 10 liters of medium held at temperatures of 24, 28, 32, and 38 C and aerated at the rate of 0.5 volume of air per volume of medium per minute of humidified air are 90, 120, 180, and 230 ml, respectively, per day. When the air by-passes the humidifier, the evaporation losses are 120, 160, 230, and 300 ml, respectively, per day. Since most of the fermentation processes are held at about 30 C for 2 to 3 days, losses in volume of approximately 5 per cent due to aeration can be expected. The losses in volume

due to sampling are minimized by placing the drain valve as close to the bottom of the fermentor as possible. In this manner, only 20 to 30 ml of medium are needed to flush the valves before a sample is taken.

The stainless steel construction of the fermentor permits the study of culture conditions when high acidity is required, such as that in the itaconic and citric acid processes, and the use of fermentation pressures of 30 to 50 psig as used in the sodium gluconate fermentation. The individual control of temperatures and agitation during both the sterilization and fermentation steps are features which are generally not offered in many installations of a similar nature.

Acknowledgment

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Summary

The description and operation of a 20-liter stainless steel fermentor similar in design to industrial units are given. The accessories, such as aerators, agitators, antifoam system and electrode, and equipment to facilitate the continuous manual measurement and control of pH during the fermentation are also described.

Comparisons of data obtained from the 20-liter and a larger pilot plant fermentor are given in this report for the production of dextransucrase, calcium 2-keto gluconate, and riboflavin. A battery of these fermentors is most helpful in obtaining quickly and inexpensively data which are readily translated to large-scale equipment.

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