Continuous Addition of Glucose for Evaluation of Penicillin-Producing Cultures[1]

FRED V. SOLTERO AND MARVIN J. JOHNSON

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wisconsin

Received for publication October 6, 1953

It was found previously (Soltero and Johnson, 1953) that considerably higher penicillin yields than those of lactose controls could be obtained in a synthetic medium when glucose or sucrose are continuously added to fermentations which contain no lactose. It was also found that for culture *Penicillium chrysogenum* Q 176 a glucose feed rate of approximately 0.030 per cent sugar per hour was optimal under the conditions employed.

A group of penicillin-producing cultures, developed at the University of Wisconsin as part of a selection program for better producers, was evaluated with this new method of continuous addition of glucose to the fermentations. This paper presents the results obtained from these experiments.

A more reproducible apparatus for the continuous addition of nutrients to shake flask fermentations than the ones previously used in our laboratory was developed. It assures the needed reproducibility of operation for the evaluation of penicillin cultures. Dale *et al.* (1953) described recently a continuous feeding assembly for laboratory fermentations. The arrangement used in the present investigation is simpler, and is believed to be better adapted to aseptic operation. In its present form, however, it does not permit a continuously variable feed rate.

EXPERIMENTAL METHODS

Fermentation techniques. Several strains were used in these experiments. All of them are descendants of culture *Penicillium chrysogenum* Q 176. They were chosen from both good and poor penicillin producers in corn-steep-lactose medium in order to have a more significant test of the new evaluation method.

Inoculations of the fermentation medium were made with 5 ml of vegetative suspension 45–48 hours old grown on synthetic medium containing 4 per cent glucose, 1.3 per cent calcium carbonate, 1.3 per cent ammonium sulfate, and mineral salts in trace amounts. This had been inoculated with 5 ml of a spore suspension which had been grown on the standard spore plate medium described by Gailey *et al.* (1946). All fermentations were run on a rotary-type shaker at a total volume of 100 ml in 500-ml Erlenmeyer flasks. The shaker described a 2-inch circle and operated at 250 rpm. The temperature was 25 C in all runs.

The fermentation medium used for these experiments was as follows (figures represent grams per liter): Lactose, 30 (in controls only); glucose, 10; ammonium acetate, 3.5; ammonium lactate, 6.0; KH_2PO_4, 6.0; $MgSO_4 \cdot 7H_2O$, 0.25; $ZnSO_4 \cdot 7H_2O$, 0.02; $FeSO_4$, 0.02; $MnSO_4$, 0.02; and Na_2SO_4, 1.0. The pH was adjusted to 6.5 before sterilization and the sugar was autoclaved separately. Further additions of sugar were started when the fermentations were 24 hours old. A sugar concentration of 24 per cent (w/v) was used. The feed solution also contained potassium phenylacetate at a 3.3 per cent level (calculated as the acid). The volume of liquid delivered per day to the fermentations was approximately 3 ml.

The mechanical feeders, which had been used before for the continuous addition of sugar to the fermentations (Soltero and Johnson, 1953), did not guarantee sufficiently uniform operation to evaluate a series of different cultures in the same run with exactly equal feed rates. Therefore, a new continuous feeder for shake flask fermentations, based on a different principle, was developed. Actual operational data indicated that the maximum variation between 10 different feeders was about 5 per cent. The variation in feed rate in each individual feeder was not higher than 2–3 per cent throughout the whole fermentation time.

The apparatus is shown in figure 1. The operation is as follows: The sterile sugar solution with precursor is transferred aseptically to a sterile burette connected through a piece of surgical-type gum rubber tubing to a capillary (¼ mm inside diameter nominal size; 0.40 mm actual size) which empties inside the flask. The end of the capillary is bent upward and a reservoir is made near the tip as illustrated in the diagram. This was found to be necessary to prevent air bubbles from entering the rubber tubing. The tubing is taped to a stationary metal bar 4 inches from the top of the flasks which serves as a pivot. The capillary tube is inserted through holes in an aluminum clamp which holds it in place. Two small pieces of rubber tubing

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by grants from Merck and Company Inc., and E. R. Squibb and Sons.

are used as bushings to prevent any movement of the capillary. The clamp and tube are wrapped with cotton and given the form of an ordinary cotton plug. The small cotton plug used to keep the burette sterile is then carefully pushed down and the burette is connected to a delivery tube from the electrolytic cell. The burette should be filled just enough to provide for reasonable air space in order to prevent the cotton plug from getting wet when it is pushed down. The whole system should be completely air tight because it operates under reduced pressure. The electrolytic cell consists of a flask filled with 1 N sulfuric acid. The details of the cell and electrode construction are presented in the diagram. The electrolytic cells are connected in series to a constant current power supply. The circuit diagram is given in figure 2. The current was adjusted to 0.2 milliamperes in these experiments in order to get a delivery rate of close to 3 ml per day. The feed rate can be controlled either by the current output, which controls the amount of gas produced, or by the concentration of sugar solution used.

The whole setup was placed over the shaker after sterilization and allowed to equilibrate for 5 minutes. Empty flasks were used for the sterilization and equilibration processes so that the liquid delivered because of the pressure differential was not added to the fermentation medium. Once equilibrium was attained the cotton-wrapped clamp was transferred carefully to a flask containing the 24-hour-old fermentation.

Sampling was done with sterile pipettes without removing the flasks from the shaker. It was found that

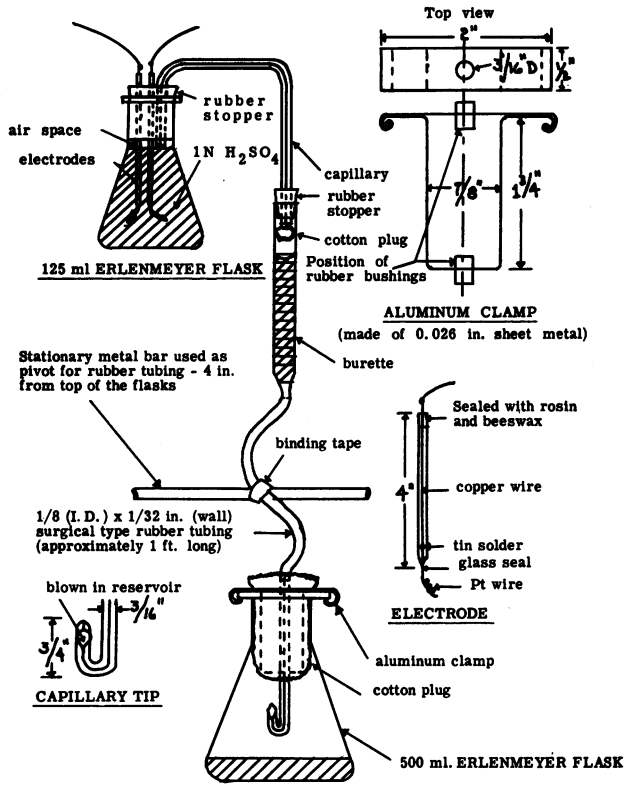

FIG. 1. Schematic diagram of slow-feeding apparatus (not drawn to scale).

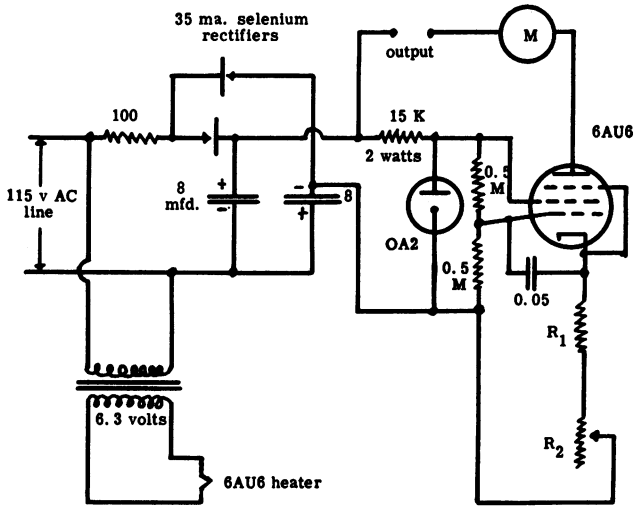

FIG. 2. Circuit diagram for constant current power supply. R_1 = 5,000 ohms minimum. R_2—The output current is inversely proportional to $(R_1 + R_2)$. Roughly, the product of $(R_1 + R_2)$ and the output amperes is equal to 55. Thus, if R_1 is 10,000 and R_2 is 500,000 ohms, the output current will be adjustable from 0.11 to 5.5 milliamperes, approximately.

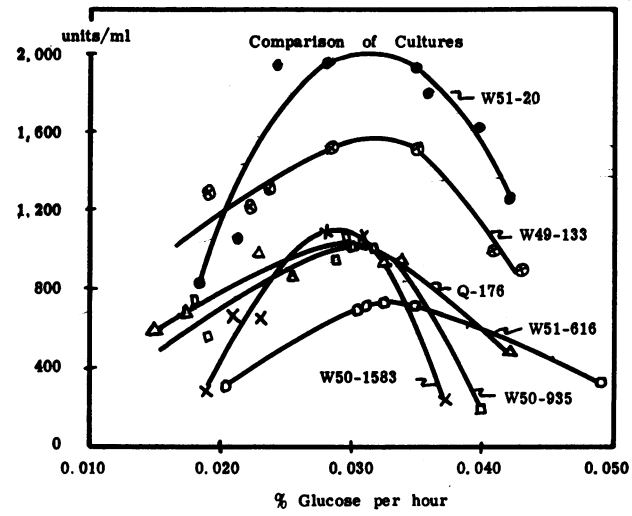

FIG. 3. Comparison of penicillin yields of different strains in synthetic medium with precursor.

TABLE 1. *Comparison of penicillin yields of several cultures in synthetic medium*

CULTURE	MAX YIELD 3 PER CENT LACTOSE	TIME OF MAX YIELD	MAX YIELD CONTINUOUS FEED GLUCOSE	TIME OF MAX YIELD	RATE OF LACTOSE FERMENTATION
	μ/ml	*hours*	*μ/ml*	*hours*	*per cent/hr*
W51-20	425	144	1,920	165	0.022
W49-133	345	144	1,545	144	0.038
W50-1583	790	144	1,200	192	0.033
W50-935	375	144	1,140	192	0.014
Q 176	550	120	1,080	144	0.022
W51-616	750	96	712	165	0.033

this could be done aseptically by lifting the plugs just enough to insert the pipette and take the sample. So far, no contamination problems have appeared.

Analytical procedures. Penicillin was assayed by the Oxford cup method with the use of *Micrococcus pyogenes* var. *aureus* as the test organism, and penicillin G as the standard.

The pH of each sample was determined immediately after removal, by means of a glass electrode.

All sugars were determined by the Shaffer and Somogyi (1933) method. Titrations were referred to standard curves prepared for each sugar. Lactose was hydrolyzed before the determination in 0.5 N HCl in an autoclave at 15 pounds pressure for 15 minutes.

Results and Discussion

It was found previously that a glucose feed rate of approximately 0.030 per cent sugar per hour was optimal for penicillin production for culture *Penicillium chrysogenum* Q 176. The optimum glucose feed rate was determined for several new cultures under the same experimental conditions and it was found that the value of 0.030 per cent sugar per hour was again the best one for penicillin production for all the cultures tested. The experimental data are given in figure 3.

The same cultures were also compared in a 3 per cent lactose-1 per cent glucose synthetic medium. Table 1 presents a summary of data obtained on both media. It can be seen that an increase in yield of over 4 times the lactose control is obtained in the best producer, culture W51-20, under the conditions employed (a yield of over 1900 units/ml as compared to one of 425 units/ml in the lactose medium). With the exception of culture W51-616, which gave the same yield in both cases, all the cultures tested showed a marked increase in penicillin yield when the continuous-feeding method was used. As all the cultures tested had approximately the same optimal glucose feed rate, namely 0.030 per cent sugar per hour, a more significant comparison of the penicillin-producing ability of different cultures can be readily made by running the fermentations at this glucose feed rate instead of utilizing lactose in the medium.

The marked difference in yield between these two types of fermentations can be attributed in part to the rate at which the cultures utilize the lactose. The last column of table 1 shows the average rate at which lactose is fermented in the interval between 24 hours and the time of maximum yield. It can be observed that the best producers in the lactose medium, cultures W50-1583 and W51-616, have a fermentation rate of 0.033 per cent sugar per hour, which is slightly higher than the experimentally found optimum glucose feed rate. The other cultures utilize the lactose much faster (W49-133) or much slower than the optimum value. This behavior causes undesired changes in the pH of the fermentation, which is one of the reasons for comparatively low yields.

In view of the fact that selection of cultures has always been done in a lactose-corn-steep-liquor medium, it was decided to test a group of cultures representative of both good and poor penicillin producers in the lactose-steep liquor medium with this new evaluation method. Fifteen cultures were tested with 3 per cent lactose and with glucose continuously fed to synthetic medium at a rate of approximately 0.030 per cent sugar per hour. The results were then compared to the ones obtained from the lactose-steep liquor medium. A summary of the data is presented in table 2. It can be seen that in almost all cases a significant increase in yield over both the synthetic and the steep-liquor lactose media can be obtained when glucose is con-

Table 2. *Summary of data*

Culture	Yield 3 per cent lactose	pH plateau	Yield slow feed glucose	Feed rate	pH plateau	Time max. yield	Lactose-steep liquor medium*	Steep liquor rating	Lactose rating	Glucose rating	Increase glucose/steep liquor
	μ/ml		*μ/ml*	*percent/hour*		*hours*	*μ/ml*				
W51-20A	1075	7.2–7.5	2225	0.030	6.9–7.4	168	1500	2	1	1	1.49
W51-20B	770	6.5–7.5	2060	0.029	6.9–7.4	168	2000	1	3	2	1.03
W49-133	550	6.1–7.3	1260	0.029	7.3–7.6	120	1200	3	9	3	1.05
W49-482	760	6.6–7.9	1090	0.029	7.3–7.4	168	650	7	4	4	1.67
W50-935	460	6.2–7.5	1040	0.030	6.7–7.3	144	900	5	12	5	1.16
W52-700	560	7.2–7.3	980	0.029	6.9–7.3	168	350	10	7	6	2.80
W49-2429	525	6.8–7.0	835	0.029	7.3–7.5	144	400	9	10	7	2.08
W50-1247	800	6.7–7.6	830	0.029	6.8–7.4	120	625	8	2	8	1.33
W47-762	550	7.1–7.4	830	0.028	7.0–7.4	168	250	14	8	9	3.30
W51-1113	750	7.0–7.5	800	0.029	7.2–7.7	144	1200	4	5	10	1.50
W47-638	630	7.2–7.3	800	0.029	7.4–7.5	120	275	13	6	11	2.90
WQ176-A827	510	7.6–7.3	750	0.033	7.6–7.2	120	300	11	11	12	2.50
W49-411	205	5.2–7.8	740	0.029	7.4–7.1	120	700	6	14	13	1.06
WQ176-A8	420	7.6–7.4	540	0.028	6.9–7.4	120	300	12	13	14	1.80
W49-408	25	5.6–7.1	100	0.029	7.4–7.6	120	0	15	15	15	∞

* Data obtained from Botany Department.

tinuously fed at the optimum rate. However, the relative rating of the cultures does not change significantly when the lactose-steep liquor medium is compared to glucose slowly fed to synthetic medium.

The feed rates obtained from the new feeders were completely satisfactory as can be seen from the table. The pH plateaus (average of pH values from 48 hours to time of peak yield) corresponding to those feed rates were also satisfactory for the penicillin-producing phase. In the case of synthetic lactose medium a wide variation in pH can be observed in some cases due to the different rates at which the cultures ferment the lactose.

The best penicillin producers of the cultures tested were two variants of culture W51-20 (A and B). They were selected from good sporulating colonies of the parent culture in an attempt to obtain better sporulation from the normally poor W51-20. The yields obtained from these two cultures were 2225 and 2060 units per ml, respectively. They appear to be the most promising ones now at hand and will be the subject of further study.

The problem of selecting cultures for high production is a difficult one. Within certain limitations, no matter which method or medium is used for making the comparison, it is obvious that the selection will fall upon the culture to which the test fermentation conditions are best suited for penicillin production. There is probably no condition in which it can be said that optimum performance of all the cultures tested is achieved. The best that can be done is to try to make the evaluation under the best conditions known to give the maximum penicillin yields. For this reason, the continuous-feeding method seems to be an improvement in that direction. No systematic work has been done with glucose-fed corn-steep-liquor media in shake flasks, and it remains to be seen whether or not the same improvement could be obtained in such media.

Summary

A glucose feed rate of approximately 0.030 per cent sugar per hour was found to be optimal for penicillin production for several cultures tested under the conditions employed. Further evaluation of cultures was done with this optimum feed rate for all the cultures.

A group of penicillin cultures was evaluated with 3 per cent lactose and with glucose continuously added to synthetic medium. Yields increased considerably with the latter method but the relative rating of the cultures did not change significantly when a corn steep liquor-lactose medium was compared with glucose slowly fed to synthetic medium. Cultures W51-20 A and B were the best producers of the group tested. They gave penicillin yields of 2225 and 2060 units per ml in the synthetic medium used.

A new continuous feeder for shake flask fermentations has been developed and used successfully in penicillin fermentations. It assures more reliable and reproducible operation than the metering valves used previously for this purpose.

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