Methods of Penicillin Production in Submerged Culture on a Pilot-Plant Scale

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SUMMARY: This paper gives details of a 50 gal. fermentation vessel designed for investigating the formation of antibiotics (or other metabolic products) by micro-organisms grown in submerged culture. This vessel has been used for investigating the submerged culture production of penicillin by *Penicillium chrysogenum* X1612 and Q176, and certain results relating to the size of the inoculum and the yields obtainable from these strains in synthetic and other media have been obtained. Culture fluids containing from 400 to 500 Oxford units penicillin/ml. have been obtained with cultures of Q176 in a corn-steep liquor medium.

A method of extracting penicillin from the broth has been worked out, based on solvent transfer, the method being applicable on virtually any scale of operation and involving only relatively simple equipment. It has the advantage of reducing the time of contact of penicillin with acid to such a degree that extraction at room temperature is possible, although extraction at still lower temperatures improves the yield. Using this method of extraction we have obtained calcium penicillin with a potency of 940 Oxford units/mg., the overall recovery from the broth being of the order of 35–50%.

The large-scale production of penicillin by deep culture methods became possible largely as a result of the development of suitable strains of the mould by methods such as those described by Raper, Alexander & Coghill (1944) and by Raper & Fennell (1946). The development of corn-steep liquor lactose media by Moyer & Coghill (1946*a*), and of synthetic media by the Pennsylvania State University workers (unpublished work) made feasible the proper utilization of these strains. The technique and biochemistry of penicillin formation in submerged culture has been investigated by Koffler, Emerson, Perlman & Burris (1945), Knight & Frazier (1945), Koffler, Knight, Emerson & Burris (1945), Raper & Fennell (1946), Foster, Woodruff, Perlman, McDaniell, Wilker & Hendlin (1946), Moyer & Coghill (1946*b*), Foster, Woodruff & McDaniell (1946) and Raper *et al.* (1944). These workers have been concerned partly with aspects already discussed in the preceding paper (Grenfell, Legge & White, 1947); with establishing, by shake-flask, aerated bottle, and aerated tank experiments, the optimum conditions for penicillin production; and with investigation of the metabolic changes during the course of the experiments. Stefaniak, Gailey, Brown & Johnson (1946) describe the construction and operation of pilot-plant scale fermentation vessels for experimental investigations, and this is the only published information on this aspect. It is felt therefore, that some account of the techniques used in our laboratories, and of the results obtained, may be of value. These techniques have, of course, been based to some extent on the above-mentioned work,

details of which were made available before publication by the official penicillin information exchange scheme.

Since the results now presented are intended mainly to illustrate the value of the methods and equipment described, it is not proposed to discuss in detail the metabolic investigations covered by the above communications. They provide a basis for understanding the effects of changes in medium composition, and of variation of operating conditions, upon the yield of penicillin. The use of high potency strains under optimum conditions and the addition to the medium of substances specifically stimulating penicillin formation, has resulted in penicillin yields of *c.* 500 Oxford units/ml. culture fluid in 70–80 hr. Yields approaching this level have been obtained in our work on a 200 l. scale although in a slightly longer period.

Developments in the extraction of penicillin have received little attention in the literature. Two main methods are used: (*a*) adsorption of penicillin on carbon, elution with organic solvents, concentration, and purification by solvent transfer; (*b*) solvent transfer alone, usually in the sequence culture fluid, organic solvent, aqueous buffer, organic solvent, aqueous alkali; the last solution being used for freeze-drying. We have developed for solvent transfer a convenient technique which can be used on any desired scale.

EXPERIMENTAL

Methods and equipment

Analytical methods

The course of each fementation was followed by periodic determinations of pH (electrometrically); sugar utilization (method of Schaffer & Hartman, 1920); ammonia content (micro-Kjedahl); and penicillin content (Grenfell *et al.* 1947). Other features could, of course, have been followed. Over a period of time, however, it was found that changes in the above constituents constituted the data of greatest significance and that from consideration of pH, sugar, and ammonia values it was normally possible to predict whether or not a fermentation was proceeding satisfactorily, and the time at which the culture fluid should be processed to obtain the best yield of penicillin. These two aspects are naturally of importance for production.

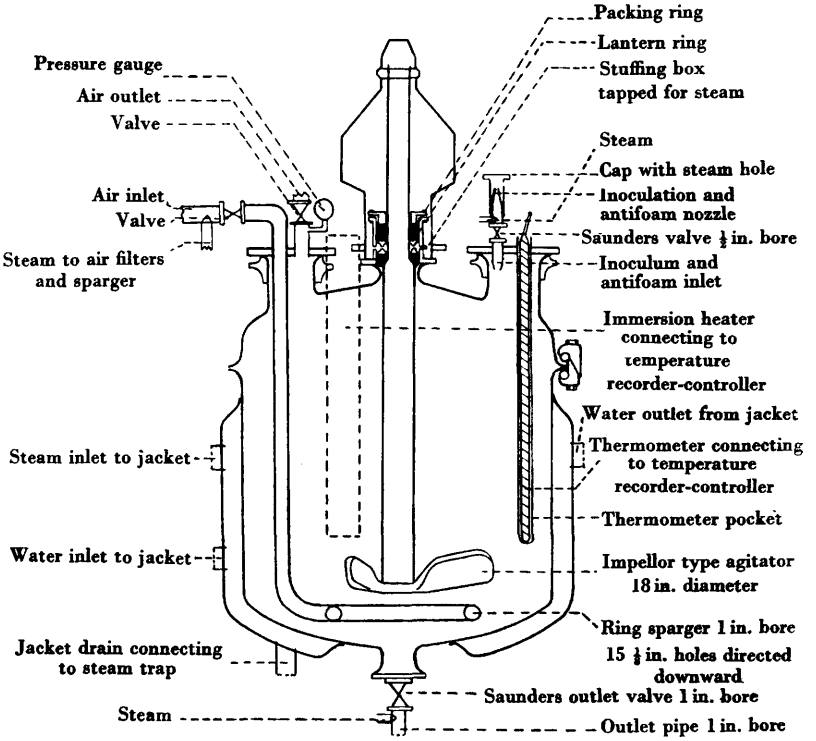
Culture media

Synthetic media No. 22A. This medium was developed for use in penicillin production by the Pennsylvania State University group of workers (unpublished). The composition is: lactose B.P., 15 g.; glucose B.P., 5 g.; acetic acid (glacial), 4 g.; NH_4NO_3, 5 g.; KNO_3, 3·5 g.; KH_2PO_4, 2 g.; $MgSO_4,7H_2O$, 0·5 g.; $FeSO_4,7H_2O$, 0·2 g.; $ZnSO_4,7H_2O$, 0·04 g.; $CuSO_4,5H_2O$, 0·005 g.; phenylacetamide, 0·25 g.; water to 1 l.

Corn-steep liquor medium. The composition of this medium is: corn-steep liquor (Stahley no. 14), 30 ml.; lactose B.P., 40 g.; $CaCO_3$, 10 g.; phenylacetamide, 0·25 g.; water to 1 l. Antifoam (300 ml./200 l. medium) is added before sterilization.

Apparatus for small-scale experiments

Small-scale experimental submerged-culture fermentations were carried out in 10 l. aspirators each containing 7 l. medium. The aspirator assembly and mode of operation are described by Grenfell *et al.* (1947) and, for similar work on tyrothricin, by Appleby, Knowles, McAllister, Pearson & White (1947).



Text-fig. 1. Diagrammatic vertical section of pilot-plant apparatus for penicillin production.

The fermentation vessel

The fermentation vessel used for pilot-scale runs was basically a normal Pfaudler S-type jacketed glass-lined reaction kettle of capacity 50 gal. This was modified slightly and fitted with accessory equipment designed specially for deep culture work on penicillin and tyrothricin. The main details are shown in Text-fig. 1 and Pl. 1. The vessel cover carried five ports, the largest of which was fitted with a cover carrying a light and sight glass and acted as a charge-hole. The central 4 in. port was fitted with a stirrer and the three remaining 4 in. ports carried respectively (*a*) thermometer pocket and nozzle for injecting inoculum and antifoam; (*b*) air sparger and air outlet; (*c*) immersion heater. The chargehole is not shown in Text-fig. 1 and the position of the heater is merely indicated. The bottom of the vessel had a 3 in. opening fitted with

a 1 in. Saunders glass-lined valve plus sampling pipe. Either steam or water could be circulated through the outer jacket, the supply pipes (positions shown in Text-fig. 1) being controlled by valves. The steam exit led to a steam-trap system for draining off condensate.

Agitation. Agitation was by a glass-coated three-bladed stirrer of the 'Impellor' type with extreme blade diameter 18 in. (3 in. greater than the sparger diameter) and revolving at 150 r.p.m. The stirrer blades were sited *c.* 3 in. above the sparger and were angled at 45° from the vertical, thus causing a downthrow of the medium into the air stream. The stuffing-box through which the stirrer was inserted into the fermenter was specially designed to prevent the possible entry of contaminating bacteria. In addition to the normal rings of packing material, the stuffing-box contained a perforated steam lantern ring (Text-fig. 1). The side of the stuffing-box was drilled and tapped at the level of this ring and a steam line inserted into the tapping; a similar tapping at the diametrically opposite point carried a fine steam exit-jet. Steam was passed continuously through the lantern ring throughout operation of the fermenter, the fine exit causing an increase in pressure and thus maintaining a sterilizing zone around the stirrer shaft. As an added precaution, the packing rings were lubricated with grease containing 1% creosote.

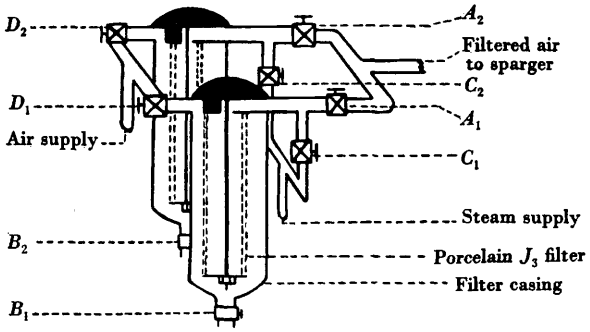
Temperature control. The fermenter was fitted with an immersion heater intended mainly for bacteriological fermentations. Unlike Stefaniak *et al.* (1946), however, we found that heating was required in the early stages of penicillin fermentations and the heater proved advantageous. The heater was linked externally to a 'Temperature Recorder-Controller' (Cambridge Instrument Co.) which was connected also to a metal thermometer inserted in the thermometer pocket of the fermenter. The controller governed the temperature (normally 25°) throughout the fermentation, and also provided a continuous record of the temperature inside the vessel. The immersion heater, being 3 in. in diameter, further served as a baffle aiding adequate air dispersion and preventing clumping of the mycelium.

During rapid mycelial growth the temperature tended to rise above 25°. This was counteracted by running cooling water through the jacket of the fermenter, the automatic action of the heater-controller system preventing the temperature dropping below 25°. Control was secured to 0·5° by these means.

Aeration. Air was provided by a compressor-after cooler-air receiver system of capacity 250 l. free air/min. at 40 lb. pressure. The air passed through a 1 in. pipeline, pressure fluctuations being smoothed out and the pressure reduced to 30 lb. by a reducing valve. After this was a stop-cock and branch pipe for use when adding inoculum or antifoam to the vessel. The main air line then entered a vertical 'Rotameter' calibrated up to 250 l. free air/min. (allowing for subsequent filter resistance, medium resistance, and the pressure drop to that of the atmosphere).

After leaving the 'Rotameter' the air was freed from micro-organisms by passage through two 'Aerox' air filters fitted with grade 'J3' porcelain filters of pore size 1·5μ. The details of the arrangement are shown in Text-fig. 2;

provision was made at this point for admission of steam to the circuit. The air line led finally into the 1 in. mild steel air sparger of the fermenter, terminating inside the vessel as a horizontal circular air sparger of 15 in. diam., centrally placed about 2 in. above the bottom of the vessel and 3 in. below the stirrer vanes. This circular portion had 15 uniformly spaced downwardly directed holes ($\frac{1}{8}$ in. diam.) which distributed the air evenly into the medium. Air left the tank by a 2 in. exhaust line fitted with a pressure gauge and a steam valve fitment for controlling the pressure inside the fermenter. This was normally 5–10 lb. to minimize the possibility of contaminating organisms entering the fermenter during the runs.



Text-fig. 2. Arrangement of air filter system. A_1, A_2, 1 in. Saunders glass-lined valves; B_1, B_2, filter case drain cocks; C_1, C_2, 1 in. gate valves on steam lines; D_1, D_2, 1 in. gate valves on air lines.

Sterilization of the fermenter, air-supply and medium

Materials sufficient to make up 200 l. medium were added to 180 l. tap water in the fermenter. All openings were then bolted down and the valves closed. The following valves were then opened in sequence (Text-figs. 1, 2): (i) the inoculation nozzle valve; (ii) the air outlet valve of the fermenter; (iii) the steam exit valve leading from the jacket to the steam-trap; (iv) the air line valves A_1, A_2; (v) the drain cocks B_1, B_2 of the filters; (vi) the steam valves C_1, C_2. The last action caused steam at 30 lbs. pressure to pass simultaneously back through both porcelain filters, and forward through the air line and sparger into the medium. The porcelain filters were thus sterilized while the fermenter was being sterilized and thereafter were capable of passing sterile air for tested periods of up to 300 hr.

Steam was next turned on at the jacket to speed up the heating of the medium. When the medium temperature had risen to 100° and steam was passing freely from the inoculation nozzle (*c.* 25 min.) the air outlet valve of the fermenter was closed until the fermenter pressure reached 15 lb. and the valve then adjusted to maintain that pressure. Sterilization was carried out for 30 min. and the inoculation valve then closed, steam being turned on above this valve as described later. Steam was then turned off at the jacket and the steam valves C_1 and C_2. At the same time the valves D_1, D_2 were

opened and the cocks B_1, B_2 closed, thus allowing air to pass through the filters into the fermenter. Adjustment of the air exit valve of the fermenter was then required to maintain the 15 lb. pressure. Normally, to avoid undue evaporation losses, aeration at this stage was limited to 40 l./min., this being sufficient to maintain at least 5 lb. pressure in the fermenter during the cooling process. Cold water was then turned on at the jacket and the medium temperature brought down to 25° in 90 min.; the tank was then ready for inoculation. The temperature control system was then brought into operation and, after inoculation, the air intake adjusted to the desired rate (1 l. air/l. medium/min.). During the sterilizing process, the volume of medium usually rose to the desired 200 l. as a result of steam condensation during the heating stage. The use of 'Aerox' porcelain filters was more convenient than the U.S. practice of using cotton- or glass-wool filters and gave efficient air sterilization without other accessory equipment and without undue mechanical bulk. The two parallel filters were capable, under our conditions, of passing up to 250 l. air/min. with the backing pressure of 30 lb. They were so inserted that either filter could be detached during the course of a fermentation, the porous element changed, steam sterilized, and then brought back into operation without interrupting the fermentation.

Inoculation

The inoculation nozzle of the fermenter served both for the addition of the inoculum and for the subsequent addition of antifoam agent (2% stearyl alcohol in lard oil). Its construction can be seen from Text-fig. 1. Particular attention was paid to the design of this item since contamination was most likely to be introduced at this point. The nozzle consisted of a tube of $\frac{1}{2}$ in. bore entering through a port cover and carrying (as close as possible to the outside of the cover) a $\frac{1}{2}$ in. glass-lined Saunders valve. The portion of the nozzle above the valve had a $\frac{1}{4}$ in. steam line inserted as close as possible to the valve seating: it was threaded to take a cap immediately above this point, and then tapered to a 2 in. long portion of $\frac{3}{8}$ in. bore at the inlet end. This section was covered by a removable threaded winged cap with a lateral $\frac{1}{16}$ in. hole to act as a steam exit.

During sterilization of the fermenter, the inoculation valve was open and the cap of the nozzle screwed on so that steam at sterilizing pressure passed from the tank through the inoculation system and emerged via the lateral hole in the cap. This effectively sterilized the inlet route of the inoculum. Shortly before completion of the sterilization of the fermenter, the inoculation valve was closed and steam turned on at the nozzle steam line to maintain sterile conditions above the valve until the time of inoculation. When the fermenter had been cooled to the operating temperature, this steam was turned off, the winged cap unscrewed and removed, and an aspirator containing germinated spores (Grenfell *et al.* 1947) immediately connected to the nozzle by sterile rubber tubing which was wired in position, both to the nozzle and to the tube normally serving as the aspirator air outlet. The branch air line (vide supra) was

then connected by rubber tubing to the air filter tube of the aspirator and the latter clamped in an inverted position above the fermenter. The fermenter pressure was then lowered to 2 lb. by operating the air exit valve, the inoculation valve opened, and air at 30 lb. pressure passed from the branch air line into the aspirator, thus forcing the inoculum into the fermenter. On completion of this process the air passing into the aspirator was turned off, the inoculation nozzle valve closed, and the aspirator disconnected from the nozzle. The nozzle cap was then refitted and steam turned on at the nozzle steam line to resterilize the system from the valve seating upwards. The fermenter pressure was finally readjusted to 5–10 lb.

A previously sterilized aspirator assembly containing antifoam was then attached to the nozzle and additions of antifoam made when required by the same technique, except that the assembly was left attached to the fermenter throughout the run.

Sampling

The 1 in. bottom exit valve of the fermenter terminated in an outlet (3 in. long × 1 in. diam.) with a steam line inserted immediately below the valve seating (Text-fig. 1). The end of the outlet was threaded to take a cap. The outlet and lower face of the valve seating were steamed continuously during fermentations. The steam was turned off immediately before sampling, the outlet valve opened slightly and closed immediately a sufficient sample had been obtained. Enough sample was first run to waste to cool the outlet to a temperature which would not affect the penicillin content of the actual sample used. After sampling the valve was closed at once and steaming recommenced.

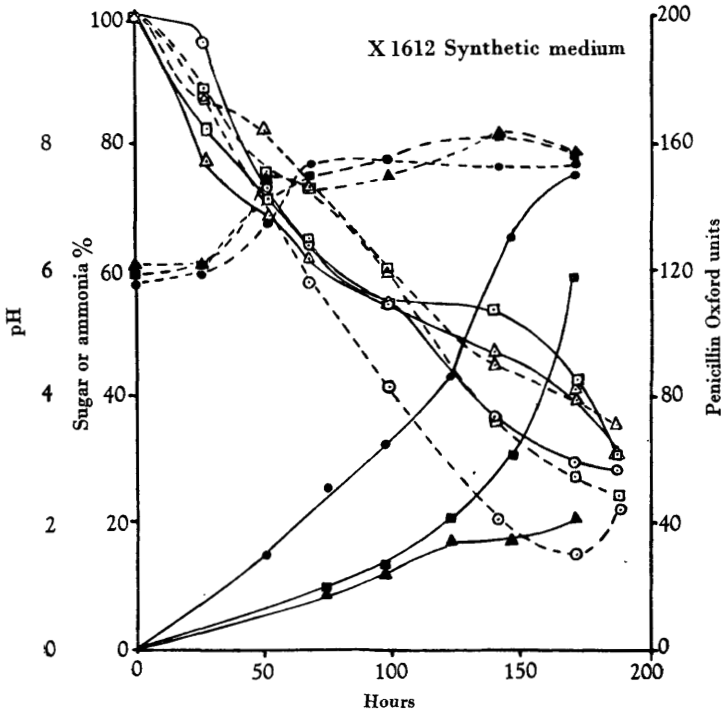
Occasionally the valve became blocked with mycelium. In this case the valve was shut, the outlet pipe capped loosely and steam passed through the outlet pipe via the loose cap for 30 min. Steaming was then stopped, the cap tightened, and the outlet valve opened momentarily while the steam was simultaneously turned full on, thus forcing steam into the fermenter. With quick careful operation this procedure freed the blocked valve without detrimental effect on the fermentation; any other procedure would have involved contamination.

The use of steam sterilization at this point, at the stirrer insert, and at the inoculation nozzle proved highly successful and made it possible to operate without contamination throughout the experiments.

The formation of penicillin

A large number of experiments were carried out both on the aspirator and the tank scale, in which different conditions of fermentation, different media, and different strains were tested. The tank work was done with two U.S. strains (X1612 and Q176) of *P. chrysogenum*. A comparison was made of the behaviour of these two strains in media based on corn-steep liquor, and in synthetic medium. It is proposed to discuss here only certain aspects of the results exemplifying points of importance which have not received previous comment or emphasis.

Text-fig. 3 illustrates the effect of inoculum size on the yield of penicillin (strain X1612, synthetic medium). It has been our practice to inoculate at the final stage with a culture which had already been grown for 48 hr. One of us (T. W.) had found in the U.S.A. considerable disagreement as to the volume of inoculum that should be added, figures given ranging from 0·5–10% of the volume of medium. The variability of spore viability from strain to strain

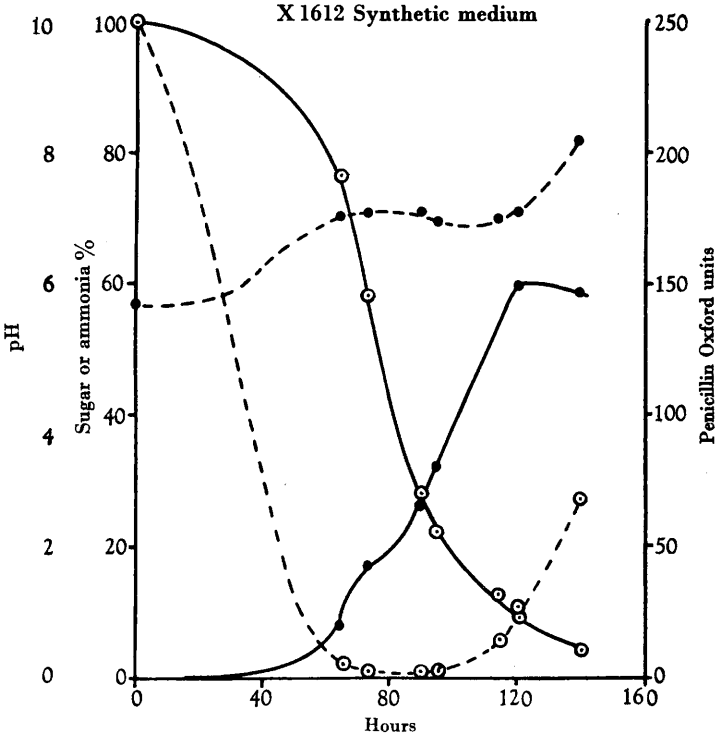


Text-fig. 3. Effect of inoculum size on the metabolism of *P. chrysogenum* X1612 in synthetic medium on an aspirator scale. Inoculum (germinated spores): 5×10^5, ⊙ ●; 5×10^6, ⊡ ■; 10^7, △ ▲. Actual volumes of inoculum were 25, 250 and 500 ml. respectively. Sugar utilization (%), —⊙— etc.; ammonia utilization (%), -- ⊙ -- etc.; pH, -- ● -- etc.; penicillin (O.u./ml.), —●— etc.

(Grenfell *et al.* 1947) may account for this disagreement. Our experiments were designed to determine the best inoculum size in terms of viable spores rather than as volume of inoculum only. The three experiments of Text-fig. 3 were carried out simultaneously and show that the heavier the inoculum, the greater was the initial utilization of sugar and ammonia, and the less was the final yield of penicillin. In the end, however, the smallest inoculum actually gave the greatest utilization of sugar and ammonia, and also the most rapid production of penicillin and the highest yield (155 Oxford units/ml.). The results suggest, in fact, that too large an inoculum uses up so much nutrient for mycelial growth that penicillin formation is badly affected either by prolongation of the initial growth phase or by the creation of unfavourable conditions. This and later findings suggested that for strain X1612 under the

conditions of these experiments the inoculum should be 10^5 germinated spores/l. medium. For different strains, media, and operating conditions the figure would probably be different and the aspect is one which we feel merits closer attention.

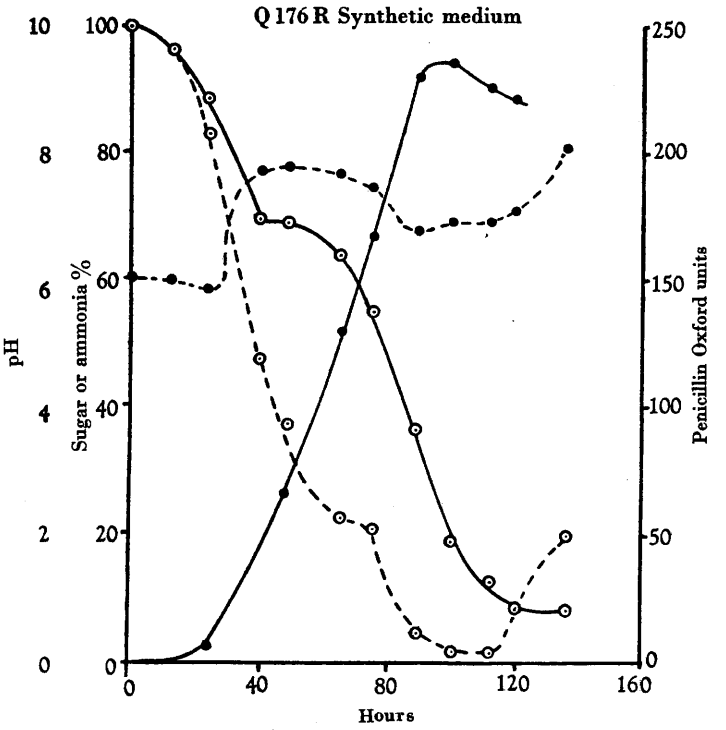
Text-fig. 4 shows the results of a comparable experiment with the same strain and medium in the pilot-plant. A much faster fermentation results



Text-fig. 4. Metabolism of *P. chrysogenum* X 1612 in synthetic medium on a pilot-plant scale. Sugar utilization (%), —⊙—; ammonia utilization (%), -- ⊙ --; pH, -- ● --; penicillin (O.u./ml.), —●—.

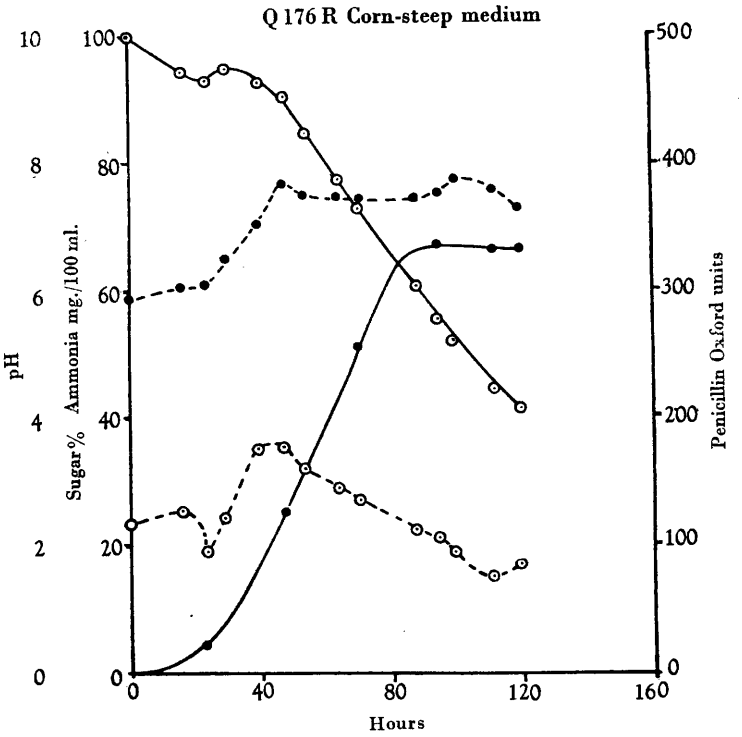
under the more efficient conditions of aeration, agitation, etc., that were possible on this scale. This particular run is chosen because it illustrates clearly the correlation of the time of the peak penicillin yield with the marked final rise of pH and ammonia content of the medium. In practice, this serves as an excellent indication of the time when the cultures in this medium should be processed for optimum yields of penicillin and, since both determinations are more quickly carried out than any penicillin assay yet devised, the point is of some pratical importance. Strain Q176R behaved similarly in this respect in this medium (Text-fig. 5), the peak yield being 245 Oxford units/ml. It is clear also that the advantage of this strain over X1612 lies not in a more complete utilization of nutrients, but in the fact that there is a much shorter lag phase before penicillin formation begins, while the rate of formation is much

faster and the final level higher. There is a clear distinction too in the initial pH changes, strain Q176 showing a more definite and faster initial pH rise than X1612. Our experience with this medium has been that the greater and more rapid this early rise, the higher is the yield of penicillin. The final point of interest in this experiment is the form of the sugar utilization curve. The initial fast rate is due to rapid metabolism of the glucose present, this being followed by a decided lag before lactose is used; this point will receive comment later.

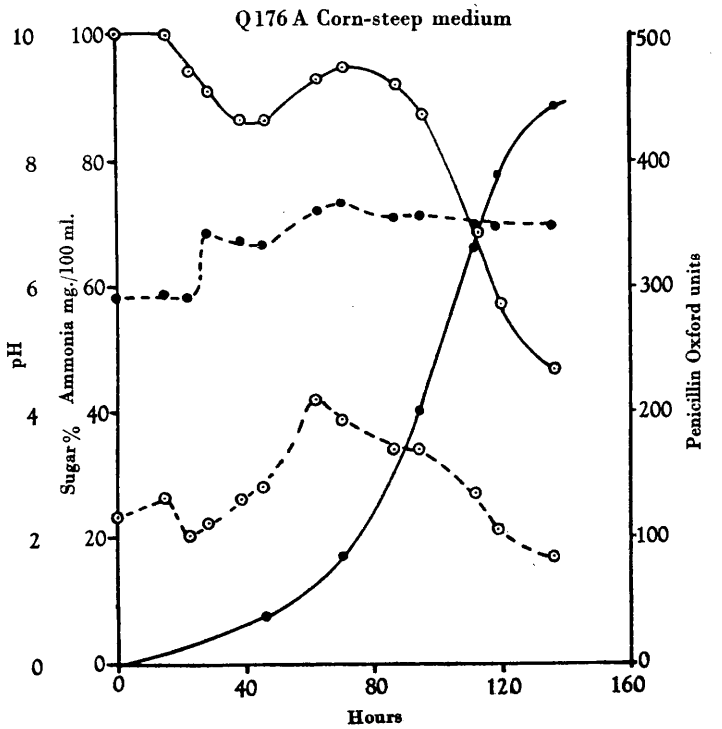


Text-fig. 5. Metabolism of *P. chrysogenum* Q 176 R in synthetic medium on a pilot-plant scale. Symbols as in Text-fig. 4.

The results of experiments in which corn-steep liquor medium was used in the pilot-plant are shown in Text-fig. 6 (strain Q176R) and Text-fig. 7 (strain Q176A). These two strains were separate isolates from the same original stock. The general similarity between the fermentations is evident, particularly as regards the changes in pH and ammonia content. These curves are very different from the corresponding curves for the synthetic medium. The pH reached an early peak value and then remained approximately constant at a value eminently suitable for penicillin formation, while the ammonia values showed an initial drop (not recorded by other workers) followed by a marked rise and then an equally marked fall with a tendency to rise shortly after the peak penicillin value had been attained. This medium was of particular value not only because it gave high yields (400–500 Oxford units/ml.) but also because



Text-fig. 6. Metabolism of *P. chrysogenum* Q176R in corn-steep liquor medium. Symbols as in Text-fig. 4, except that ammonia values are given as mg./100 ml. medium.



Text-fig. 7. Metabolism of *P. chrysogenum* Q176A in corn-steep liquor medium. Symbols as in Text-fig. 6.

the yields did not fall off very rapidly after the peak—a point of decided value in processing and probably due to the pH remaining constant at a value at which penicillin can persist.

The main difference between the two strains Q176R and Q176A was in their metabolism of sugar and their yield of penicillin. Initially both attack the corn-steep liquor carbohydrate, and this is followed by a lag before lactose metabolism begins. The lag is much more pronounced with Q176A, and penicillin formation is correspondingly delayed. This strain A incidentally shows the same lactose lag in synthetic media. On the other hand the peak penicillin yield is significantly higher with Q176A than with Q176R, which ran consistently to 330 Oxford units/ml. despite slight medium modifications. Foster, Woodruff, Perlman, McDaniell, Wilker & Hendlin (1946), who have provided the only other comparative data on X1612 and Q176, have also commented on the lag in lactose metabolism and advocated adaptation of the strains to lactose to obtain maximum effectiveness. We found also that crude lactose prolonged the lag still more, but increase of quality above the B.P. standard did not affect the lag, nor did the addition of inorganic salts to the medium.

The extraction of penicillin

The extraction of penicillin by laboratory scale methods becomes rather difficult with large volumes of medium. Batchwise extraction is undesirable from several points of view and we have therefore developed a method whereby the transfer of penicillin from aqueous solution to organic solvent is carried out by a continuous extraction process in which the time of contact of penicillin with acid is decreased to such a minimum that operation without refrigeration is possible even with volumes of 200 l. The general procedure is shown in the Chart on p. 199. As an example of the use of this method of extraction the following details based on the treatment of a batch of Q176 broth of penicillin content *c.* 400 Oxford units/ml. may be considered:

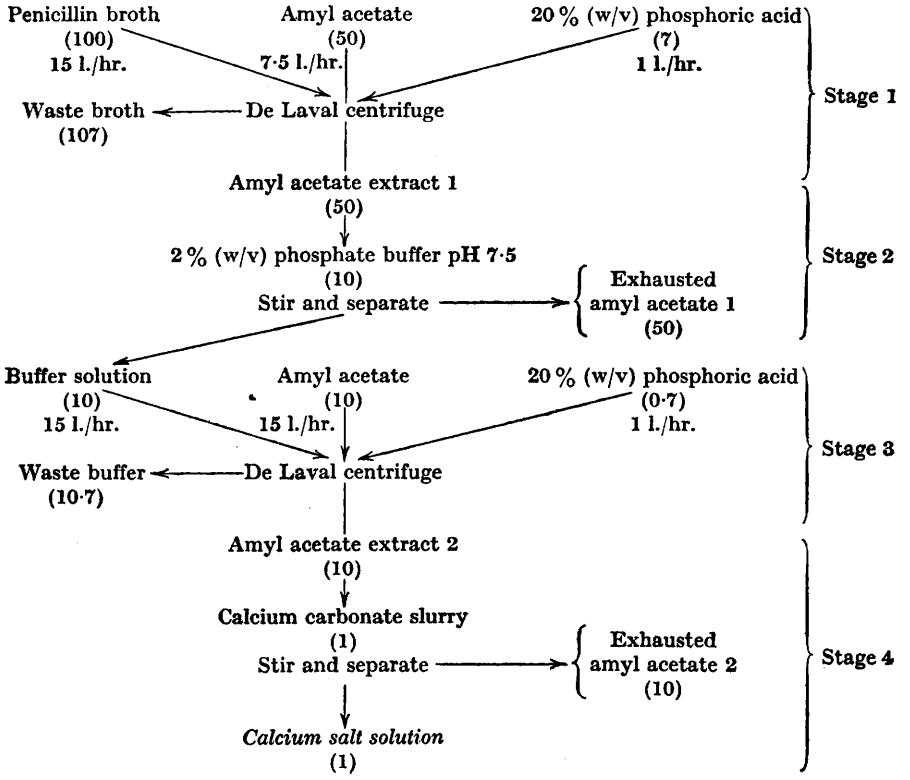
Stages 1 *and* 2. Penicillin broth, amyl acetate, and 20% (w/v) phosphoric acid were each placed in Pyrex aspirators fitted with stoppers carrying an open glass tube extending to the bottom of the aspirators to provide a constant head of liquid. The lower aspirator outlet had a stopper carrying a delivery tube fitted with an adjustable screw clip; this, in conjunction with the constant head of liquid, enabled constant set rates of flow to be obtained. The broth flow was set at 15 l./hr., the acetate flow at half that value and the acid flow at that figure (predetermined by titration) which would bring the broth to pH 2·0–2·1 during the extraction.

A De Laval no. 1200 laboratory-type centrifuge was set up with the bowl assembled as shown in Text-fig. 8, i.e. with a lower clarifying disk but an upper disk arrangement as for separation—a combination of the two methods of assembly normally used with this machine. With this arrangement, two (or more) liquids run in simultaneously are first thoroughly mixed and emulsified, and then separated as the emulsion passes up through the bowl. The normal clarifier assembly would carry out the first function but not the second, while

the normal separator assembly would perform the second function but would not mix and emulsify efficiently.

The bowl was primed with broth, the centrifuge started, and broth, solvent, and acid fed simultaneously into the machine without prior mixing. The acid flow was then finally adjusted until the pH of the discharging broth was 2·0–2·1,

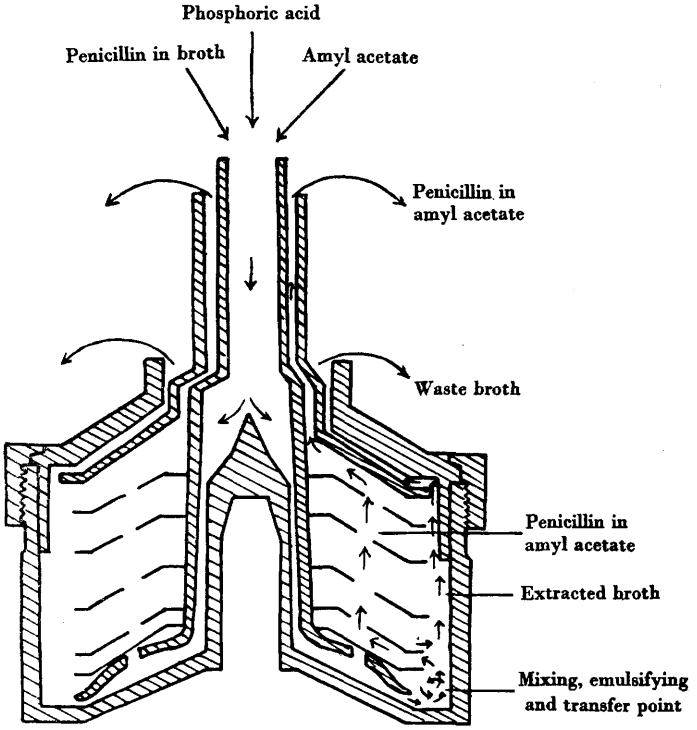
Chart showing extraction procedure.



(The numbers in brackets indicate volumes in l.)

the machine being then run continuously until the first stage of extraction was complete. The process resulted in efficient transfer of penicillin from the broth to the amyl acetate, the waste broth now containing only *c.* I Oxford unit/ml. penicillin. The amyl acetate was discharged into a stainless steel vessel containing 2% (w/v) phosphate buffer pH 7·5 (one-tenth of the volume of the original broth). This solution was stirred continuously while the flow of amyl acetate continued, thus securing immediate transfer of the penicillin from the organic solvent phase to a neutral aqueous phase where its stability was high. The pH of the buffer was tested periodically during the addition of the amyl acetate solution; it should not be allowed to fall below 7·1, and more buffer should be added if necessary. The solution of penicillin in buffer was finally run off through a cock at the bottom of the apparatus.

The method had the advantage that the time of contact of penicillin with acid conditions (both in aqueous and organic solvent phases) was a maximum of 1 min.; the time of contact with the acid aqueous phase was only *c.* 10 sec. In consequence it was possible to conduct the process at room temperature without appreciable acid decomposition of the penicillin, the recovery to the buffer stage being of the order of 65–70% even at 20–25°. A typical broth containing 440 Oxford units/ml. (13 Oxford units/mg.) gave a buffer solution of concentration 3000 Oxford units/ml. (150 Oxford units/mg.)—a twelvefold initial purification and tenfold concentration.



Text-fig. 8. Special method of assembly of the bowl of the De Laval centrifuge.

Stages 3 *and* 4. The technique used for these two stages was similar to that used in stages 1 and 2 except that the buffer solution from stage 2 replaced the original broth, and the amyl acetate flow was the same as the buffer flow. The amyl acetate was discharged continuously into a stirred slurry of calcium carbonate (20 g. carbonate plus 500 ml. water/100 l. initial broth). After all the amyl acetate had been run in, the mixture was stirred for a further 10 min. at 1500 r.p.m. and the solution of calcium salt separated off; the pH of the latter was normally 6·1. The amyl acetate solution was then treated as above with two further lots of calcium carbonate slurry (20 g. carbonate in 300 ml. water/100 l. initial broth; 20 g. carbonate in 200 ml. water/100 l. initial broth). The second calcium salt solution was usually pH 7·0, and the third pH 7·2. The

distribution of penicillin between the three fractions, and the potency of each is shown in Table 1. The greatest part of the recovered penicillin was in the first solution. Recoveries of the order of 60–70% were again obtained at this stage, the overall recovery for the whole process being 35–50%. Pooling of all three calcium salts gave a final solution of concentration *c.* 15,000 Oxford units/ml., and potency 800 Oxford units/mg. This final solution was suitable for experimental freeze-drying and gave a penicillin of higher potency than has been used for clinical work until recently.

Table 1. *Details of calcium salts obtained at the final stage of penicillin extraction*

Calcium salt fraction	Penicillin content		
	O.u./ml. solution	O.u./mg. dry wt.	% of total penicillin
1	20,000	940	68
2	12,000	600	23
3	6,000	780	9

The isolation of penicillin of this degree of purity on a laboratory scale and without refrigeration proves the value of the simple extraction method used above. Other experiments showed that the use of refrigeration at the two acid transfer stages markedly improved the yield and this, of course, is in accord with large-scale practice. On the small scale, however, the above procedure provides a simple and convenient technique for investigating the effects of variations in the extraction procedure. The continuous flow method could have been applied also to the transfer of penicillin from organic solvent to aqueous neutral buffer. This was not usually done, however, since the transfer can be carried out at temperatures up to 25° without loss of penicillin beyond that due to its distribution coefficient between the two solvents.

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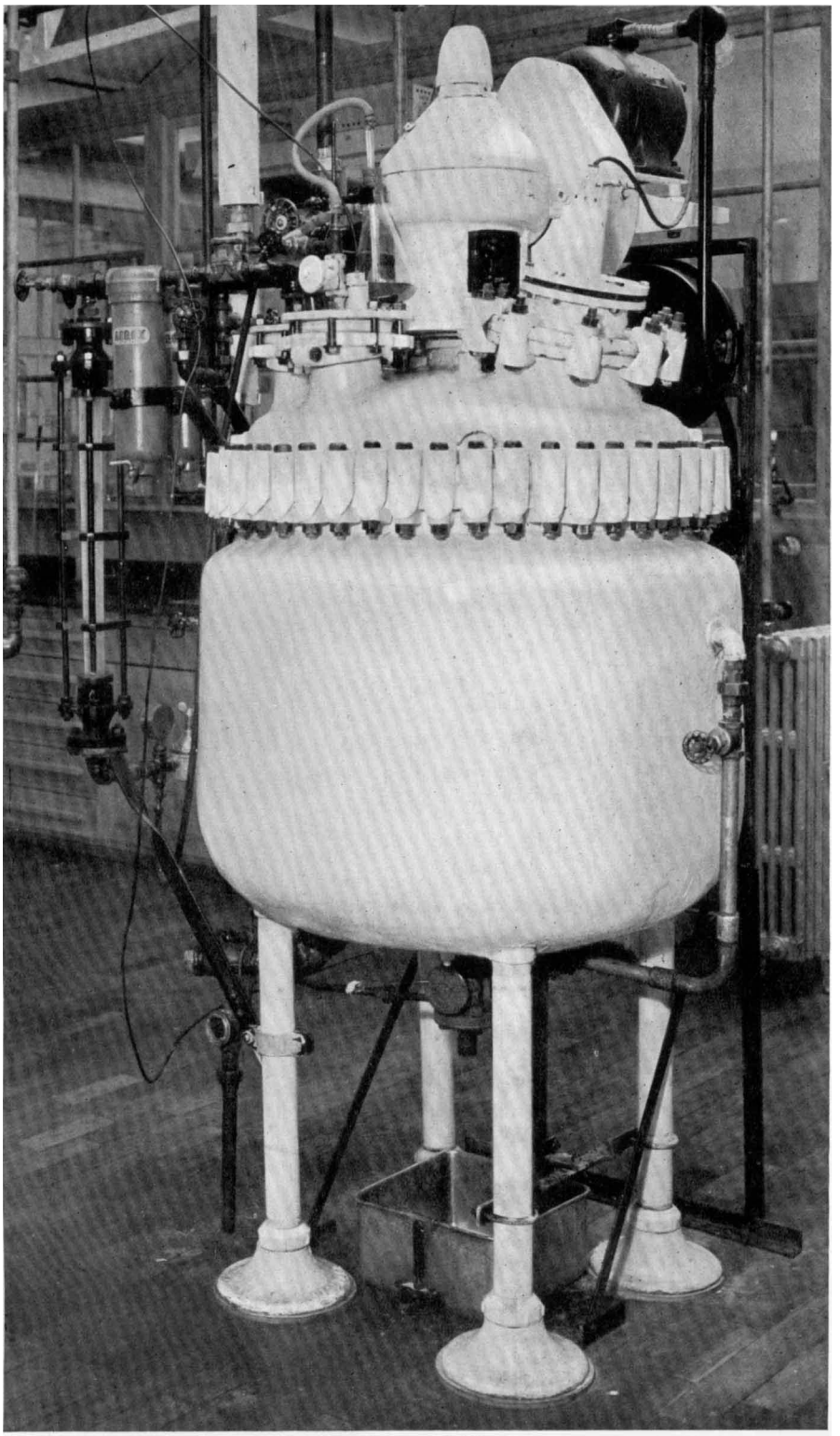

50 gall. pilot-plant penicillin fermenter.

J. J. Gordon, E. Grenfell, E. Knowles, B. J. Legge, R. C. A. McAllister and T. White—Methods of penicillin production in submerged culture on a pilot-plant scale. Plate 1