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The Effect of the Carbohydrate Nutrition on Penicillin Production by *Penicillium chrysogenum Q-176*[1]

FRED V. SOLTERO AND MARVIN J. JOHNSON

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

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Relatively few papers have been published on the carbon nutrition of *Penicillium chrysogenum* and its effect on penicillin yield. Jarvis and Johnson (1947) investigated the relationship between sugar utilization and penicillin formation in detail. They found that optimal penicillin yield is obtained if glucose and lactose are present in such ratios that the proper quantity of mycelium is formed from the readily available glucose before the mold is forced to use the slowly fermented lactose. Cook and Brown (1949–1950) studied the effect of various carbohydrates and salts on penicillin yields and evaluated them for the possible relation to the mechanism of formation.

The work reported here presents the results obtained when lactose is replaced by glucose, or by other carbohydrates, in such a way that there is a controlled growth of the mold during the penicillin production phase. This condition can be established by the slow addition of the sugar to the fermentation by suitable means. This study was done exclusively on a shake-flask scale. Work of a similar nature was conducted in 30-liter fermentors and will be reported elsewhere (Hosler and Johnson, 1952).

EXPERIMENTAL METHODS

Fermentation techniques. Penicillium chrysogenum Q-176 was used exclusively in these experiments. Information about the origin of this strain and previous studies on pelicillin yields by its use have already been published (Gailey *et al.*, 1946).

Inoculations were made with 5 ml of vegetative suspension 45 to 48 hours old grown on standard fermentation medium. 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 operated at 250 rpm and described a 2-inch circle. 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 control 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, 0.5. The pH was adjusted to 6.5 before sterilization. Further additions of sugar were started when the fermentations were 24 hours old and continued thereafter, except for the lactose controls, which received no additional sugar. In the intermittently-fed runs, additions were made in 2 ml portions every 12 hours until penicillin production reached a maximum. Sodium phenylacetate at a 0.05 per cent level (calculated as the acid) was added as precursor to all fermentations every 12 hours (Higuchi *et al.*, 1946).

In the continuously-fed runs, feeding was achieved with the device used as an anti-foam control in tank fermentations (Anderson *et al.*, 1952). Feed rate was controlled by the concentration of the sugar solution

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used (15 to 45 per cent), as well as by the fraction of the total time the device operated. Precursor (sodium phenylacetate) was added with the sugar solution. The flasks were incubated on a rotary shaker.

All fermentations were run in duplicate flasks and the figures reported here are the average of these two flasks. Samples for penicillin assays and chemical analyses were taken under aseptic conditions and handled in the manner described by Gailey *et al.* (1946). Sterilization of solutions was done by autoclaving separately at 15 pounds pressure for 20 minutes. The solutions were then poured aseptically into the burettes.

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 with their reagent 50 containing 5 g of potassium iodide. Titrations were referred to standard curves prepared for each sugar. Sucrose was hydrolyzed in 0.5 N HCl at 100 C for 10 minutes; lactose in 0.5 N HCl in an autoclave at 15 pounds pressure for 15 minutes.

Soluble Kjeldahl nitrogen was determined by the method described by Johnson (1941). The mycelial nitrogen was determined by substracting the soluble nitrogen present at the time of sampling from the soluble nitrogen present at the time of inoculation.

Phosphorus was determined by the modified Fiske and SubbaRow method of Koepsell *et al.*, (1944).

Results and Discussion

It has been realized for some time that a maximum rate of penicillin production is obtained only under fermentation conditions which support a very slow rate of growth.

Although conditions which support a very low mycelial growth rate are desirable for penicillin formation, the growth phase of the fermentation should be characterized by the rapid development of a high concentration of mycelium. Thus, an ideal medium should support two distinct growth rates: a rapid rate throughout the growth phase and a much slower rate during the remainder of the fermentation.

The importance of the pH in penicillin fermentations has been discussed previously in detail by Johnson (1946). Optimum pH values ranging from 4.0 to 6.5 have been established for the growth phase, and from 7.0 to 7.5 for the penicillin production phase. Variables affecting the pH will be discussed later.

A synthetic medium capable of producing this ideal performance had been developed previously by properly choosing and balancing its constituents. Under a given set of conditions, the major factor affecting the rate of fermentation is the nature of the carbohydrate used in the medium. Glucose is fermented at a very rapid rate, and lactose at a much slower rate. Sucrose and galactose are utilized at about the same rate as glucose. As the rate of mycelium production varies directly with the rate of sugar utilization during the growth phase, the use of a mixture of glucose and lactose in the medium will produce suitable rate conditions for the fermentation.

Intermittent feed. Lactose was known to be the best carbohydrate for penicillin production. This superiority was attributed to its slow rate of fermentation. It was thought that a fermentation to which a rapidly utilized carbohydrate was slowly added to simulate the slow utilization of lactose, would give good penicillin yields.

Table 1. *Penicillin yields on various carbon sources*

Substrate	Amount added every 12 hours	Maximum yield	Time of maximum yield
	g/l	*u/ml*	*hr*
D-glucose control	30.0*	140	119
Lactose control†	30.0	500–600	120
D-arabinose‡	5.0	88	71
L-arabinose	5.0	440	95
L-inositol	5.0	236	95
L-sorbose	5.0	745	143
D-xylose	5.0	770	143
D-glucose	5.0	620	119
D-galactose	5.0	520	96
D-sucrose	5.0	618	119
Starch§	4.0	520	119

Substrates added to mycelium grown in synthetic medium containing 1 per cent glucose. Additions started when the fermentations were 24 hours old. Sodium phenylacetate was added as precursor to every flask.

* Added at zero hours.

† Average of several separate runs; added at zero hours.

‡ Apparently not fermented.

§ Separate run.

The method first employed was intermittent feeding of the carbohydrate every 12 hours, starting after the fermentation was 24 hours old, when the mycelial growth was almost complete. In table 1 are shown the results obtained on the synthetic medium when a variety of carbon sources were fed intermittently. The glucose control, in which all the sugar was added at the beginning, illustrates the results obtained when a rapidly fermenting sugar is used as a simple replacement for lactose.

Figure 1 gives data on sugar concentrations in a fermentation to which glucose was intermittently added. It is apparent that under the feed schedule employed, periods of sugar excess alternated with periods of starvation. It will be noted that the time required for sugar exhaustion increased as the fermentation became older. A special fermentation, in which the feed rate was progressively decreased to compensate for this, gave a somewhat longer penicillin-producing phase with accompanying increase in yield. No

direct correlation could be established between the actual penicillin production and the sugar starvation periods when the intermittent feeding system was employed.

Experiments were then designed to determine the optimum rate of feed. Figure 2 presents the information obtained from such runs. It will be seen that approximately 0.4 per cent glucose every 12 hours was found optimal. When sucrose was fed to a synthetic medium the optimum values appeared to be less sharp. Figure 3 presents the data from the sucrose fermentations. High sugar feed rates lead to a rapid drop in pH and very heavy growth, followed by sporulation because of depletion of nutrients other than sugar. Low feed rates lead to eventual autolysis because of sugar starvation.

Continuous feed. The success obtained with the intermittent feed method led to the assumption that continuously fed fermentations might give an additional improvement. It was found that with the feeding arrangement used, the feed rate varied about 10 per cent during the fermentation. The average feed rate was used in all calculations.

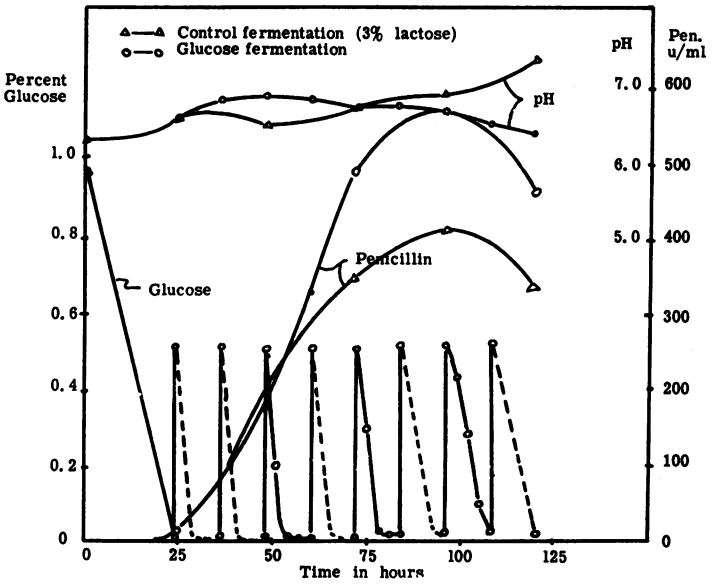

FIG. 1. Penicillin production from glucose. Average of 3 flasks on synthetic medium. 0.5 per cent glucose added every 12 hours beginning at 24 hours.

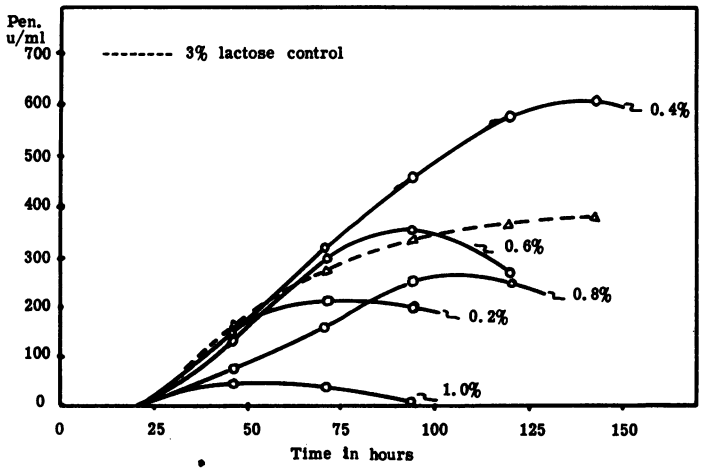

FIG. 2. Penicillin production on synthetic medium at various glucose feed rates. Percentage figures represent amount of glucose added every 12 hours starting at 24 hours. Control contained 3 per cent lactose added at zero time. No further sugar additions were made to the control.

By continuously feeding glucose or sucrose solutions to shake flasks fermentations, it was possible to obtain penicillin yields sometimes twice as high as those obtained from 3 per cent lactose controls. The summary of fermentations at different sugar feed rates is presented in figure 4. The optimum sugar feed rate under the conditions employed here lies close to 0.030 per cent sugar per hour when glucose is the carbohydrate utilized. In the case of sucrose the curve appears more flattened and the feed rate is less critical than with glucose.

Optimal values appear to be close to 0.036 per cent sucrose added per hour.

Figure 5 represents a typical fermentation showing the chemical changes occurring when the continuous feeding system is employed. It can be seen that penicillin production increases very slowly after 120 hours. pletion did not occur; 66 per cent of the original phosphorus was found present after 120 hours. In more recent work it has been possible to extend the penicillin production to 196 hours at a more or less constant rate by the addition of ammonium acetate mixed with the sugar solution. This evidence strongly supports the

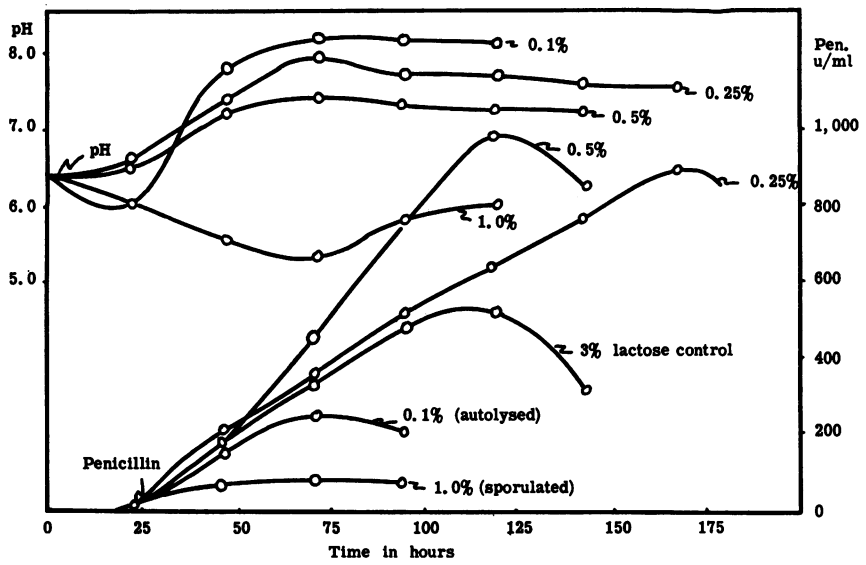

FIG. 3. Penicillin production in synthetic medium at various sucrose feed rates. Percentage figures represent amount of sucrose added every 12 hours starting at 24 hours. Control contained 3 per cent lactose added at zero time. No further sugar additions were made to the control.

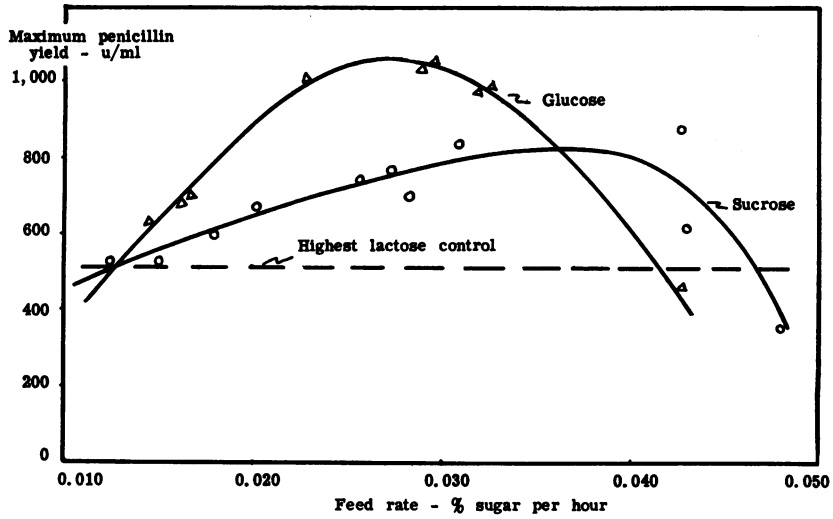

FIG. 4. Effect of feed rate on penicillin yield. Sugar additions started after 24 hours, with feed rates as indicated. Synthetic medium used in all fermentations.

There is the possibility that this could be due to an almost complete depletion of nitrogen in the medium. In many instances the figures obtained for soluble nitrogen in the medium at that time showed only 10 to 15 per cent of the original remaining, and it should be remembered that this soluble nitrogen might include any secretion products from the mycelium which were unavailable for nutrition of the mold. Phosphorus depletion did not occur; 66 per cent of the original phosphorus was found present after 120 hours. In more recent work it has been possible to extend the penicillin production to 196 hours at a more or less constant rate by the addition of ammonium acetate mixed with the sugar solution. This evidence strongly supports the belief that the depletion of ammonia nitrogen is the first limiting factor in the production of penicillin when the synthetic medium is used.

As can be expected, no autolysis of the mycelium occurred as long as sugar was being added, and the pH tended to drop as the fermentation grew older. Practically no sugar was detectable at any time during the fermentation when glucose was used. In the case of

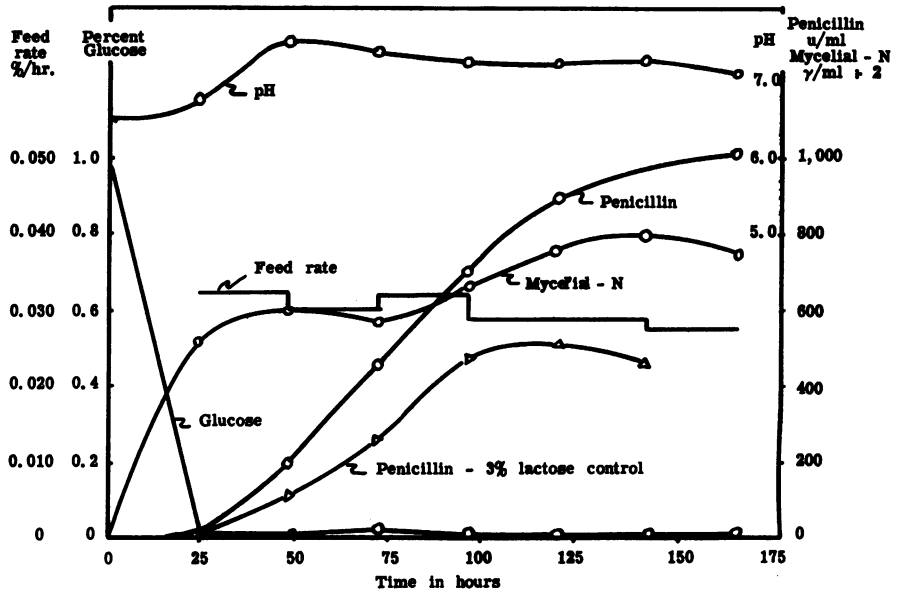

FIG. 5. Chemical changes in a continuously fed glucose fermentation. Overall feed rate—0.029 per cent glucose per hour. Total sugar added—5.08 per cent in 168 hours.

sucrose, considerable amounts (up to 0.7 per cent) could be detected after 48 hours when high feed rates were employed (over 0.050 per cent per hour).

Effect of pH. When the slow addition of sugar is begun the pH becomes a function of the feed rate employed. Low sugar feed rates lead to operation at above optimum values while high sugar feed rates have the opposite effect. These facts can be explained on the basis of differences in rates of ammonium intake by the mycelium, of utilization of organic anions from the medium, or of controlled liberation of basic substances from the mycelium. It was observed that the pH plateau (defined as the average of pH values from 48 to 120 hours) can be fixed at will at any reasonable desired value if the proper feed rate is employed. When the pH plateau values are plotted against the feed rate used a straight line relationship is obtained. Operation at a feed rate of 0.042 per cent sugar per hour gave a pH plateau of 6.7, while operation at 0.015 per cent sugar per hour gave a pH plateau of 7.8. It is apparent that one effect of high feed rates is the establishment of a pH plateau below the optimal value, as previously determined (Singh and Johnson, 1948). It must be clearly established that no attempt was made to keep the pH at optimum values in any case by using external control.

SUMMARY

Glucose, or a number of other rapidly used carbohydrates, intermittently fed to shake flask fermentations, has given penicillin yields on synthetic medium equal to, or better than, those obtained under the same conditions with lactose. Under the conditions used, with a 12-hour interval between additions, an addition rate of 0.4 per cent glucose per 12 hours was found optimal.

A method for slow continuous addition of nutrients to shake flask fermentations is described.

Penicillin yields of twice those of lactose controls have been obtained in synthetic medium when glucose or sucrose are continuously added to the fermentations. Under the conditions employed, a sugar feed rate of approximately 0.030 per cent sugar per hour has been found optimal for glucose and one of 0.036 per cent per hour for sucrose.

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A Microbiological Study of Lymph Nodes, Bone Marrow and Muscle Tissue Obtained from Slaughtered Cattle

B. C. LEPOVETSKY, H. H. WEISER AND F. E. DEATHERAGE

Departments of Bacteriology and Agricultural Biochemistry, The Ohio State University, Columbus, Ohio

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The etiology of the deep spoilage that sometimes occurs in improperly handled pork and beef carcasses is not well understood. It has been postulated by some workers that the causative organisms gain access to the affected tissues as a result of post-mortem invasion from the intestinal tract, while others believe that they may be introduced into the carcass by way of the stick wound and distributed through the circulatory system. Another possibility, and one to be considered here, is that these spoilage organisms may be the inherent flora of the involved tissues.

Amako (1910), Bierotti and Machilda (1910), Brewer (1925), Conradi (1909), Hauser (1886) and Von Fodor (1886) examined tissues taken from living and dead animals of various kinds. The results of these studies were conflicting, some indicating that bacteria are commonly found in normal muscle tissue and others seeming to demonstrate that such tissue is generally sterile. More recently Reith (1926) concluded that bacteria could be found in the musculature of living hogs. On the other hand, the work of Jensen and Hess (1941) led them to believe that the muscle tissue and bone marrow of living hogs are generally sterile, but that many kinds of bacteria can be isolated from these sites after the animals have been slaughtered. It was also shown that organisms placed on stick knives prior to the slaughtering process could be recovered later from the muscle tissue and bone marrow of the carcasses. While not undertaken specifically with the subject of meat bacteriology in mind, a study conducted by Adamson (1949) should be of interest to those working in this field. His extensive study revealed that a large proportion of 804 lymph nodes removed from human corpses contained a variety of bacteria, with coliforms, micrococci and streptococci predominating. It was suggested that the bacteria found in the nodes might have gained access to the body through skin abrasions, localized in the nodes and survived there for some time.

EXPERIMENTAL METHODS

In the course of this investigation samples from 11 chucks and 12 rounds of beef were examined. These were secured from 23 cattle which had been slaughtered at the Ohio State University Meats Laboratory, or at commercial establishments in Indianapolis and Chicago. The prescapular lymph node, the humerus and muscle tissue bordering this bone were removed from each chuck; while the popliteal lymph node, the femur and neighboring muscle tissue were taken from each round.

Samples could not be taken aseptically from the carcasses since doing so would decrease their commercial value. However, it was possible to secure lymph nodes completely encased in masses of fat. A procedure was used which allowed the aseptic removal of the nodes from these fat capsules by repeatedly searing a surface of the fat capsule with a large red-hot spatula, and then removing the node aseptically. The node was dipped in 95 per cent ethyl alcohol and then held in the flame of a Fisher burner until the alcohol was burned off. This procedure was repeated twice. A similar method was used to remove smaller samples from the large portions of muscle. However, this procedure was modified for the aseptic removal of marrow from bone. Each bone was first cut transversely with a meat saw. Then the cut ends were seared with a Fisher burner. The charred marrow was scraped away and the sample removed with a sterile spatula, approximately one inch from the cut end of the bone. Preliminary studies have indicated that these methods were sufficient to destroy cultures of *Bacillus subtilis* placed on the surface of fat capsules, bones, and strips of muscle tissue, although cultures of non-spore forming *Serratia marcescens* inoculated into the nodes, marrow and muscle tissue were not destroyed.

As each sample was removed, it was placed in a sterile aluminum Waring blendor cup containing 200