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CONTROLLING THE pH OF CULTURES OF *PENICILLIUM NOTATUM* THROUGH ITS CARBON AND NITROGEN NUTRITION¹

Albert E. Dimond and George L. Peltier

A SURVEY of published material reveals relatively little information concerning the nutritional physiology of *Penicillium notatum* Westl. on synthetic media. Metabolism studies of *P. notatum* on various media have appeared to be incidental to the more pressing problem of producing penicillin quickly and in quantity. Fleming (1929) originally used a nutrient broth non-synthetic in nature. The Oxford group (Abraham *et al.*, 1941) then adopted a modification of the so-called Czapek-Dox synthetic medium which supplies nitrogen as sodium nitrate and glucose as carbohydrate. Hobby, Meyer, and Chaffee (1942) suggested that brown sugar be substituted for glucose in this nutrient, with resultant higher yields and decreased time for maximum production of penicillin. Apparently a variety of carbon and nitrogen sources have been employed in liquid nutrients (Foster, 1943; Foster, Woodruff and McDaniel, 1943; McKee and Rake, 1942; Kocholaty, 1942; Waksman and Horning, 1943); but specific data have not been offered. At the present time corn steep liquor appears to be an important component of the medium used.

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It is the purpose of the present paper to discuss the relation between the period of incubation and the pH trend in nutrient solutions, as influenced by the composition of the nutrient on which *P. notatum* is grown. As these studies will show, the pH developed in liquid culture can be well regulated by supplying the organism with appropriate sources of carbon and nitrogen. Such a possibility would seem of value in the production of penicillin, in view of the fact that the pH of culture media must be kept in the range of 5.5 to 7.5 for maximum production (Abraham *et al.*, 1941).

EXPERIMENTAL.—In this study a "surface" strain of *P. notatum* was grown in wide-mouth gallon jars having an internal diameter of 15 cm. The maximum depth of the medium was 10 cm. so that the surface: volume ratio was 0.1. Two liter quantities of media were added to each jar, sterilized, and inoculated with spore suspensions from several cultures of *P. notatum*. The incubation temperature varied from 25° to 28°C. Two days after inoculation, the surface mycelium was usually sufficiently heavy so that the culture medium could be gently and continuously stirred below by means of an aseptic stirring device

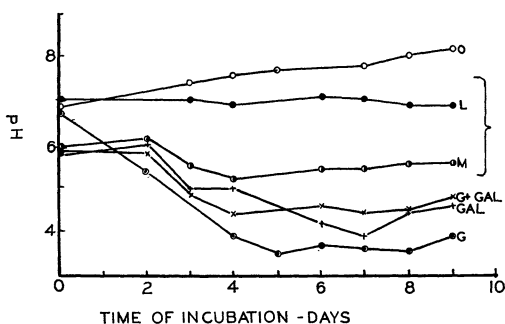
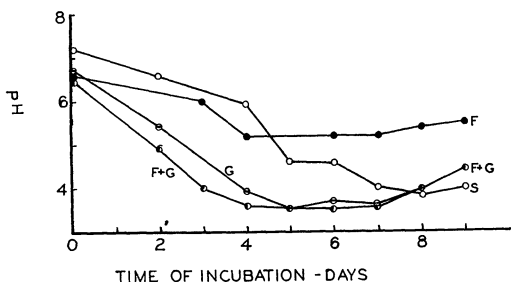
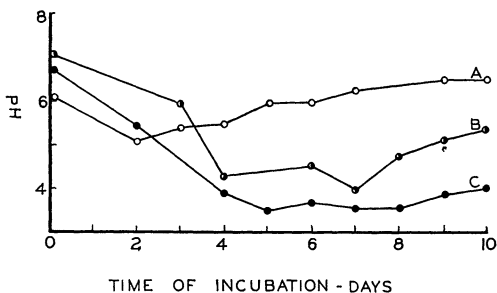
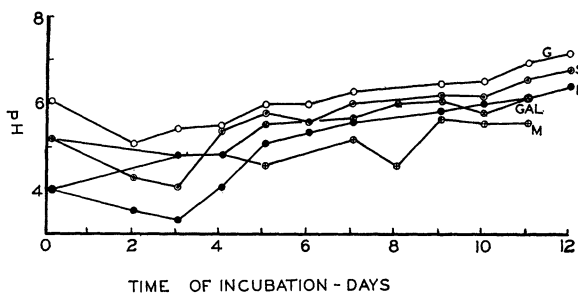


Fig. 1-4.—Fig. 1 (uppermost). Relation of time of incubation to pH developed in liquid culture by *P. notatum* when NaNO_3 is the source of nitrogen and different sugars are supplied. Curve G is curve for glucose, S for sucrose, L for lactose, GAL for galactose and M for maltose.—Fig. 2. Relation of time of incubation to pH developed in liquid culture by *P. notatum* when glucose is the sugar supplied and varying sources of nitrogen are used. Curve A is obtained when NaNO_3 is supplied, B when a mixture of NaNO_3 and the amino acids tryptophane, asparagine, and cystine are available, and C when these amino acids only are present.—Fig. 3. Relation of time

without hindering further growth of the fungus pad. Except for media containing lactose, subsequent growth was on the surface of the medium. Stirring was effective in hastening penicillin production, so that the peak was reached in eight to nine days on all media in which penicillin was produced. Through the cotton plug in the mouth of the culture jar were placed Y tubes of such construction that 10 ml. samples of culture medium would be withdrawn for use in measuring pH of the culture medium without contaminating the culture (fig. 3 in Beckord, 1943).

All chemicals employed were of reagent grade. Two types of media were employed for the work, the first being a slight modification of the medium commonly used (Hobby, Meyer, and Chaffee, 1942) in experiments reported in the literature, consisting of 3.5 g. NaNO_3 , 1.5 g. KH_2PO_4 , 0.5 g. KCl, 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 g. of pure sugar and trace elements (Cu, Zn, Mo, Mn, Fe, B). These salts were diluted with a liter of distilled water. The second medium (Burkholder and McVeigh, 1942) was similar except that NaNO_3 was omitted and in its place 2.6 g. asparagine, 0.1 g. tryptophane, and 0.05 g. cystine were substituted.

In some instances media were prepared in duplicate within a single experiment; in others single batches of media were employed for each experiment. All results reported are based on at least three independent tests. The results from test to test were always consistent.

RESULTS.—*Nitrogen sources and change in pH.*—The pH trend with time in all cultures which contained NaNO_3 as a source of nitrogen was upward from an initially acid reaction (see fig. 1). Under these conditions the pH slowly rose from a value as low as 4.0 toward the range favorable for penicillin production, arriving in this neighborhood at least by the eighth day. Thus, when nitrate was the source of nitrogen, the trend in pH was apparently determined by use of nitrate ion at a more rapid rate than sodium ion, while the effect of sugar metabolism on pH was secondary (fig. 1 and 2).

With the amino acids, the trend in pH with growth of the fungus was in the opposite direction, i.e., downward, when glucose and sucrose were the sugars supplied (fig. 3, curves G and S). The downward of incubation to pH developed in liquid culture by *P. notatum* when the source of nitrogen is a mixture of the amino acids tryptophane, asparagine, and cystine, and when different sugars are employed. Curve F is for fructose, curve F + G is for a mixture of equal quantities of fructose and glucose, curve G is for glucose, and curve S is for sucrose.—Fig. 4 (lowermost). Relation of time of incubation to pH developed in liquid culture by *P. notatum* when the source of nitrogen is a mixture of the amino acids tryptophane, asparagine and cystine, and when the sugar supplied was varied. Curve O was obtained when no sugar was supplied. Curve L is for lactose, M for maltose, G + GAL for a mixture of equal quantities of glucose and galactose, GAL for galactose alone, and G for glucose alone. The region enclosed within the brackets is the region most favorable to production of penicillin.

ward trend of pH with growth was more pronounced for the glucose medium than for sucrose.

Figure 2 contrasts nitrates and amino acids as nitrogen sources in liquid media, with glucose as the sugar source. Similar results were obtained when sucrose was used. In figure 2, curve A was obtained with nitrate as the sole N source, while curve C was obtained when amino acids were supplied. Inasmuch as the pH trends are opposite in direction in these two instances, it seemed of interest to test whether or not an intermediate type of curve might be obtained when amino acids and nitrate ion were supplied together in the medium. Curve B of figure 2 is the typical curve noted for this situation, nitrate being present at the rate of 2 gms. per liter. In such a case the drop in pH is initially almost as rapid as for a medium supplying nitrate only. However, the pH does not finally drop so low, and starts to rise sooner than the curve for nitrates. Under these conditions the pH apparently remains low until most of the amino acids present are utilized.

Carbon sources and change in pH.—Various carbohydrates were used as constituents of the nitrate medium for *P. notatum*. In all cases, the trend was that already noted for glucose and sucrose. In figure 1 are given the trends of pH with time when the sugars making up the medium were respectively glucose, sucrose, lactose, galactose, and maltose. Similar changes were observed for fructose and brown sugar.

Figures 3 and 4 indicate the results of typical experiments for all sugars tested on the amino acid medium except brown sugar. Brown sugar and sucrose showed the same trend in pH, though growth was somewhat more rapid on brown sugar.

The contrast in the pH curves for various sugars is striking. Variation ranged from practically no change in pH on incubation (lactose) to a rapid drop in pH and gradual recovery to pH 4 (glucose). Nutrients containing maltose and fructose became slightly more acid as growth of *P. notatum* went on, while solutions containing galactose and sucrose as well as those containing equal parts of fructose and glucose, glucose and galactose became acid more rapidly.

In figure 4 are plotted the curves for lactose, maltose, glucose, galactose, and a mixture of glucose and galactose. In the mixture each sugar is added at half the normal concentration. Considering the family of curves consisting of lactose, galactose, glucose and the mixture of galactose and glucose, one can see that these curves form three separate groups. These groups are (1) lactose alone, (2) glucose alone, and (3) galactose alone and glucose plus galactose. No change in pH occurred during the normal course of metabolism on lactose. Curve G for glucose contrasts with this, for the pH changed from the neighborhood of neutrality to pH 3.5 in about five days. The curves for galactose and for glucose plus galactose are similar to one another. For these the pH tends to drop more slowly than for

glucose alone and drops only to pH 4.5 in the same period.

DISCUSSION.—*Nitrogen sources and change in pH.*—The striking point about the curves relating pH of the culture medium to time of incubation for different sources of nitrogen (fig. 1, 2, and 3) is that the pH of the medium can be controlled by supplying the appropriate nutrients. By supplying graded quantities of nitrate and amino acids, any desired pH for a particular stage of the growth period can be obtained when a sugar is used which is metabolized to organic acids. Reasons for such behavior are not hard to find. Foster, Woodruff, and McDaniel (1943) have ascribed the initial drop in pH in nitrate media to formation of gluconic acid from glucose or sucrose, and the gradual trend back toward neutrality to the absorption and metabolism of nitrate ion without a correspondingly rapid utilization of the sodium ion. The latter presumably becomes associated with hydroxyl ion from water in the nutrient and pH rises as these accumulate. This trend of pH with time is characteristic of nitrate metabolism in plants (Nightingale, 1937). The trend toward decreasing pH values with amino acids might be similarly considered as due to the accumulation of organic acids resulting from the deamination of amino acids and from formation of gluconic acid when glucose is the source of carbon. Thus, one might expect a more rapid and greater drop in pH when amino acids are the nitrogen source than when nitrates are supplied.

The trend for the intermediate curve might well depend on which form of nitrogen was most readily used by the fungus. In general, amino acids appear to be used selectively when both NO_3 ion and amino acids are available. The organic acids from amino acids may be used as the carbon source more rapidly than glucose, after the glucose concentration has been reduced somewhat by metabolism with the result that pH remains stable. All the curves in figure 4 indicate that the pH does remain stable from the fifth day onward. Much more rapid growth occurred on the amino acid medium than on the nitrate medium.

Carbon sources and change in pH.—A comparison of the various curves in figures 3 and 4 indicates something as to the metabolism of the sugars involved. In figure 3 are compared the pH trends for sucrose, fructose, glucose, and a mixture of equal parts of glucose and fructose. The last nutrient contained each sugar at half the usual concentration, and would be the mixture obtained if sucrose were hydrolyzed initially to its constituent monosaccharides. The pH trend for sucrose is somewhat different from that for the mixture of the two monosaccharides. The curve for sucrose is distinct from that for fructose. Each differs from that of glucose alone or of glucose and fructose in equal quantity. In the mixture of glucose and fructose, glucose appears to be the principal sugar metabolized, whereas the fructose is used slightly if at all. This is indi-

cated by the similarity of curve F + G and curve G and the contrast between these and curve F as shown in figure 3. Other aspects of the behavior of *P. notatum* growing on these nutrients give evidence for the relative unavailability of fructose. The growth of *P. notatum* on nutrients containing fructose alone is slow and the nutrient becomes gradually colored by a black pigment. No such pigmentation occurs when the nutrient contains a mixture of fructose and glucose and growth is almost as rapid as in a nutrient containing glucose alone.

It appears as though sucrose is gradually hydrolyzed to fructose and glucose, and the glucose then proceeds to the gluconic acid stage as a subsequent step. This picture of sucrose metabolism would account for the gradual drop in pH with time as contrasted with the more rapid drop in media containing glucose and fructose together or glucose only.

From figure 4 it can be inferred that glucose is used rapidly in growth, presumably with formation of gluconic acid. Likewise acid is formed as galactose is used. When a mixture of glucose and galactose is available, the galactose appears to be used first, since the time *vs.* pH curve of the mixture is almost that for galactose alone. The curve for lactose is of interest since there is practically no change in pH with time in this medium. On the lactose-amino acid medium, growth is slower in starting than for other sugars, but penicillin activity, as measured from the crude filtrates in these tests, appeared both earlier and in greater quantity than with any other sugar tested. Figure 4 indicates that pH remains fixed within the optimum range for penicillin formation in this medium. There are several possible interpretations of the difference between the curve for lactose utilization and for curves of the related sugars. Lactose may be utilized very slowly and completely as it is broken down to the monosaccharides without accumulation of sugar acids. A second possibility is that lactose is not hydrolyzed to constituent sugars, or if hydrolyzed, the corresponding sugar acids are not formed in the resulting metabolism. The third possibility is that lactose is not used at all by the fungus, but that the fungus obtains its carbon from the amino acids.

The medium consisting of the mixture of glucose and galactose is approximately the medium which would result from hydrolysis of lactose. If the course of lactose utilization involves hydrolysis, then one might logically expect a gradual drop in pH with time, since a nutrient composed either of glucose, galactose, or a mixture of these two becomes more acid with time. Though the drop in pH with time in a lactose medium might be slower than in a mixture of glucose and galactose owing to gradual breakdown of lactose, there would still be a drop in pH with time. Figure 4 shows that no such accumulation of hydrogen ions occurs and this leads to the conclusion that lactose is not hydrolyzed to constituent sugars in its metabolism.

Slow growth by the fungus on an amino acid-lactose medium suggests that lactose may not be utilized. To test this point, growth of the fungus was observed on a medium containing amino acids without sugar. The resulting curve of pH with time is curve "O" in figure 4. This curve indicates a gradual but definite rise in pH with time, starting with a neutral reaction and ending in excess of pH 8. Evidently, then, lactose is slowly used by the fungus. In the medium lacking sugar, the gradual rise of pH with time is interpreted as being the result of more rapid utilization of the carbon chain of the amino acids than the amino nitrogen of the molecule. Thus, there is a gradual accumulation of basic nitrogen in the nutrient solution, resulting in a rise in pH.

The pair of curves (fig. 4) describing the change of pH with time for nutrients containing maltose on the one hand and glucose on the other presents a similar problem. There is marked contrast between these two curves and little if any accumulation of acid in the medium supplying maltose as a sugar. Since growth is rapid on the medium, maltose must be used by the fungus. Moreover, there is a difference between the pH *vs.* time curve in media containing amino acids with and without maltose (curves M and O). It would seem, again, that, if maltose were hydrolyzed to glucose in metabolism, gluconic acid would accumulate and there would be a more rapid drop in pH than was observed in this medium.

The data presented indicate that pH of the nutrient medium can be regulated without addition of acids or alkalies by merely supplying the fungus with the proper sources of carbon or nitrogen. Addition of acids and alkalies is of questionable value in production of penicillin, for in our tests, no penicillin was produced in media in which pH was controlled by means of this technique.

Growth in the presence of mass inocula is very rapid on an amino-acid sugar medium. The use of yeast extract has been suggested to hasten the production of penicillin (Clifton, 1943). It may be that the organic nitrogen introduced in this process is sufficient to cause an initially rapid growth, which is followed by more leisurely absorption of nitrate, once the fungous pad is established. In this latter phase of growth, pH of the culture medium would be rendered favorable to penicillin production. This hypothesis is strengthened by the work of Kocholaty (1942) which indicates that the usual growth factors are not important in the metabolism of *P. notatum*.

SUMMARY

The present investigation concerned itself with the relation between pH of the nutrient medium and time of incubation, particularly as this relation is affected by varying the source of nitrogen or sugar.

When nitrate nitrogen was supplied, the pH first dropped slightly, then gradually rose to the range

favorable for penicillin production. This was observed for sucrose, lactose, glucose, brown sugar, maltose and galactose.

When amino acids were present, the relation of pH to time of incubation varied with the sugar. For lactose, the pH remained practically constant at pH 7.0. With maltose, the pH dropped slightly to about 6.0 and remained constant. For fructose the same type of curve was obtained. For sucrose, glucose, galactose and mixtures of glucose and fructose as well as glucose and galactose, the pH fell to the range of 3.5 to 4.5 and remained there until autolysis began. The relation of this behavior to sugar metabolism is discussed.

When amino acids and nitrate nitrogen were supplied together, the pH fell somewhat, but neither

to so great an extent as with nitrate alone or amino acids alone and rose again to a level intermediate between that observed for nitrate alone and amino acids alone.

These results suggest that by supplying the proper nutrients to cultures of *P. notatum*, control of the pH of the culture medium may be maintained. The relation of pH to the production of penicillin is by now well known. Such techniques for control of pH, together with the fact that growth becomes rapidly established on amino acid media may find use in production of penicillin.

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