STIMULATING THE GROWTH OF AZOTOBACTER BY AERATION [1]

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INTRODUCTION

Numerous theories and methods have been advanced for increasing the growth of the Azotobacter cell. Much attention has been directed toward the improvement of a medium, the dominating thought being that the common media used for azofication purposes are deficient in those elements which are necessary for the prompt development of the Azotobacter organism.

Söhngen (*4*)[2] states that in Beijerinck's medium only nitrogen and oxygen are lacking for the luxuriant growth of Azotobacter. A vigorous growth of these organisms could be induced by the addition of his (Söhngen's) colloids to this medium, the colloids causing a direct contact between the Azotobacter, oxygen, and nitrogen.

It is not doubted that the medium commonly employed in the cultivation of these organisms could be improved; there are few bacteriological media which could not be improved. On the other hand, it is not assumed that the medium exerts the greatest influence upon the tardy development of the Azotobacter.

The theory of Söhngen that the Beijerinck medium lacks only nitrogen and oxygen is here supported. Observations prompt one to believe that both these elements can be supplied by aerating the culture medium and that thereby a rapid and vigorous growth of Azotobacter can be promoted.

The influence of aeration upon nitrogen fixation by Azotobacter is not new. Its influence is taken advantage of in studying the azofication ability of the soil or pure cultures by inoculating media of shallow depth and large surface exposure. This Lipman (*1*) noticed in the cultivation of *Azotobacter vinelandi* Lipman, for the rate of nitrogen fixation increased with the increased surface exposed.

The relative importance of aeration upon the growth of Azotobacter can be surmised from some of its physiological activities. Its obligate aerobic characteristics as well as nitrogen requirements for the synthesis of cell protoplasm are significant. Inasmuch as nitrogen is lacking in the media, the utilization of atmospheric nitrogen is necessitated. The energy required for this assimilating process is supplied by the oxidation of a carbohydrate. The abundance of air therefore offers two highly essential elements, oxygen and nitrogen, both of which are required for the efficient metabolism of the cell.

Observations noted from the growth of Azotobacter cultures on dextrose-Ashby agar slants would not lead one to suspect the media as

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[2] Reference is made by number (italic) to "Literature cited," p. 677.

incapable of supporting rapid cell development. Such cultures have invariably shown a vigorous and heavy growth in 24 to 48 hours, a growth comparable to that exhibited by the common metatrophic forms growing on a nutrient medium. This rapid growth of Azotobacter on such agar slopes is attributed to maximum aeration.

The purpose of the present investigation was to develop a method for inducing a prompt growth of Azotobacter in liquid media. The importance of aeration in this respect is indicated by the following data.

METHOD OF AERATION

Hoffman (*3*) suggested the sand slope cultural method for supplying a maximum surface exposure for aeration. Allen (*7*) used the mechanical agitation method employed by Bonazzi (*6*) in his nitrification experiments and reported variable results with this method of aeration. He attributed the greatest influence to the chemical composition of the media.

The activity of organisms in many fermentation industries has been materially increased by aeration. Probably in most cases the interest has been focused upon the fermentation processes rather than on any direct interest in cell multiplication. However, within the last few years aeration has been employed commercially for the dirct purpose of increasing the growth and thereby the yield of microorganisms. The cultivation of baker's yeast and the production of yeast protein in Germany by the fermentation of factory wastes is dependent upon this principle.

Aeration was accomplished in the following experiments by bubbling air vigorously and continually through the culture medium. The air was passed through the culture by attaching the culture flask equipped with Folin's aerating tubes to a vacuum system. Contamination and evaporation of the culture was reduced to a minimum by filtering the air through a sterile cotton filter and two or three sterile flasks of water. The air was thus filtered through cotton and rinsed in water before entering the culture flasks.

Several flasks of culture media could be easily aerated at the same time. Usually a cotton filter and wash bottle were placed between each two culture flasks. Little trouble occurred from contamination. A good grade of rubber tubing and tight-fitting connections are necessary, however. Flasks containing from 200 cc. to 250 cc. of media were used. The amount of air passed through the cultures was not measured, but a vigorous bubbling of the liquid was maintained continuously.

AZOTOBACTER CULTURES

Cultures of Azotobacter were isolated from samples of soil received from various parts of Kansas, Colorado, California, Iowa, and Mississippi. Flasks containing dextrose or mannite Ashby media were inoculated with the soil samples. Upon the formation of the characteristic surface growth Ashby agar plates were streaked. Dextrose-Ashby-agar slants were inoculated from well-isolated colonies, and repeated streakings of the cultures upon Ashby agar plates were made. A large number of the cultures were streaked consecutively from 1 to 12 generations. The utmost care was exercised in attempting to obtain and maintain pure cultures. Much difficulty was experienced in satisfying one's self of the absolute purity of a culture. This is accounted for by several justifiable reasons.

The purity of the culture is dependent practically entirely upon morphological considerations. The evident complex life cycle of Azotobacter, with its varied and fluctuating forms, makes a morphological differentiation indefinite, especially since our present knowledge pertaining to this life cycle, and its relative importance to the physiological activities of the cell is limited.

In all, 16 cultures of Azotobacter were used in these experiments. No attempt has been made to classify them as to species. In general, cultures 232, 6A, 12B, 9B, 8B, 1B, 10B, and 19 were all typically Azotobacter organisms producing a brown to black pigment. Culture 3A, 213, 2B, 5B, 11B, 9A, 222, and 3B were likewise typical, but pigment production by them was absent or doubtful. Culture 6A and 9B were isolated from Mississippi soil, 10B from California soil, 5B from Colorado soil, and 3A from Iowa soil. The remaining cultures were isolated from soil obtained from various parts of Kansas.

The inoculum used for seeding media was prepared by adding a portion of the emulsion from a young dextrose-Ashby-agar culture to a flask of dextrose-Ashby broth. This was aerated for two to four days. The amount of this starter usually used for inoculating the experimental medium was 0.05 per cent to 1 per cent of the medium seeded. In all cases a morphological analysis of the starter was made as a test for purity before using. The temperature for incubation was 30° C.

MEDIUM

The medium used in the following experiments, unless otherwise noted, was:

	Grams.
Tap water	1,000.0
Potassium phosphate (K_2HPO_4)	.5
Magnesium sulphate ($MgSO_4$)	.2
Sodium chlorid (NaCl)	.2
Dextrose	10.0

This solution was neutralized, filtered if necessary, and the required amounts placed in flasks and sterilized in the autoclav at 20 pounds pressure for 30 minutes. A pinch of calcium carbonate ($CaCO_3$) was added to each flask before sterilization.

CHEMICAL ANALYSIS

Total nitrogen determinations and sugar analyses of the Azotobacter cultures were made at frequent intervals. In all cases the total nitrogen was determined by the usual standard methods. Unless otherwise noted, 50-cc. portions of the media were used in duplicate. The figures referred to in the tables denote the average of the duplicate analyses. Nitrogen is recorded in all cases unless stated as net gain in milligrams of nitrogen per 100 cc. of media. This, in other words, refers to the amount of nitrogen fixed per gram of dextrose, as 100 cc. of media contains this amount of sugar.

Sugar determinations were made according to the method proposed by Shaffer (5). The copper resulting from the reduced Fehlings was determined by colorimeter readings. Duplicate readings were made each time, and the average of these was recorded. As a general rule, 50 cc. of the media were used. The protein was precipitated and removed, the filtrate was diluted to 100 cc., and 20 cc. of this filtrate were used for reduction. The figures cited, unless so stated, refer to grams of dextrose per 100 cc. of medium.

EFFECT OF AERATION UPON NITROGEN FIXATION

Flasks containing 250 cc. of media were inoculated with six Azotobacter cultures. Altogether, 36 flasks of media were used. Six flasks were inoculated with each respective culture. Three of the flasks for each organism were aerated and three were not. Examinations were made on the second, fourth, and sixth day.

The invigorating action of aeration is conclusively shown by these data, given in Table I.

An average of the figures from the six cultures give a net gain of 5.02 mgm. nitrogen per 100 cc. of the aerated media in two days as compared with 0.48 mgm. nitrogen in the nonaerated cultures. In four days the comparison indicates an average gain of 8.5 mgm. nitrogen in the aerated cultures to 1.24 mgm. nitrogen in the nonaerated cultures. The average net gain of the aerated flasks for six days was 10.35 mgm. nitrogen to 3.25 mgm. nitrogen for the nonaerated cultures.

TABLE I.—*Effect of aeration upon nitrogen fixation*

Culture.	Milligrams of nitrogen fixed.					
	Aerated cultures.			Nonaerated cultures.		
	2 days.	4 days.	6 days.	2 days.	4 days.	6 days.
1 BM	5.3	8.9	11.5	0	2.0	6.6
3 BM	4.9	14.2	15.9	0.34	1.2	6.0
222 M	6.0	8.0	11.0	.68	2.1	6.0
10 BP	.....	7.1	8.1	.2	.2	.2
12 BE	4.3	5.6	8.4	.7	.7	.7
232 E	4.6	7.2	7.2	0	.....	.6
Average	5.02	8.5	10.35	.48	1.24	3.25

It is to be assumed that on account of insufficient surface exposure in the nonaerated cultures azofication would be tardy. Nevertheless the experiment demonstrates the stimulating effect of aeration and the possibility of cultivating the Azotobacter rapidly in large volumes of media.

To compare the relative efficiency of this method of aeration with shallow depth culture media the following experiment was performed. Eighteen 300-cc. flasks containing 50 cc. of dextrose media were inoculated with 0.5 per cent of inoculum of culture 19. These cultures were incubated and three flasks were removed for analysis each day for six days. Nitrogen determinations were made upon the contents of each of two flasks. The third flask was used for sugar analysis. Six 300-cc. flasks containing 250 cc. of the same media were inoculated with a similar proportion of culture and treated to forced aeration. One flask was removed each day for nitrogen and sugar determinations. This media contained no $CaCO_3$. The reaction of the medium was readjusted to a P_H of 7.0 to 7.4. The results are shown in Table II.

TABLE II.—*Effect of aeration upon nitrogen fixation in culture 19*

	1 day.			2 days.			3 days.		
	Gm. dextrose.	Mgm. N.	Reaction.	Gm. dextrose.	Mgm. N.	Reaction.	Gm. dextrose.	Mgm. N.	Reaction.
Aerated	0.67	5.60	Alkalin..	0.06	10.20	Alkalin..	0	12.50	Alkalin.
Nonaerated	.848	0	...do.....	.60	4.50	...do.....	Trace.	11.26	Do.

	4 days.			5 days.			6 days.		
	Gm. dextrose.	Mgm. N.	Reaction.	Gm. dextrose.	Mgm. N.	Reaction.	Gm. dextrose.	Mgm. N.	Reaction.
Aerated	0	12.90	Alkalin..	0	10.80	Alkalin..	0	10.80	Alkalin.
Nonaerated	0	12.06	...do....	0	10.80	...do.....	0	10.80	Do.

The effect of the forced aeration is noticed in the first three days of incubation by both the nitrogen fixed and the dextrose fermented. The amount of nitrogen finally fixed in the shallow culture flask was practically as great as in the aerated culture. This would naturally be expected, however, as such cultures offered a maximum surface exposure. However, nitrogen fixation and dextrose consumption was more prompt in the aerated cultures.

The daily reaction of the cultures was alkaline, with possibly one exception which was questionable. This was the nonaerated culture on the fourth day of incubation. The P_H for the cultures reported alkaline averaged between 7.0 and 7.4.

EFFECT OF AERATION UPON DEXTROSE FERMENTATION

The ability of Azotobacter to fix nitrogen is dependent upon the energy derived from carbohydrate fermentation. The effect of aeration upon dextrose fermentation and the corresponding nitrogen fixation is demonstrated by the data in Table III.

TABLE III.—*Effect of aeration upon dextrose fermentation and nitrogen fixation*

Culture.	Aerated cultures.											
	1 day.		2 days.		3 days.		4 days.		5 days.		6 days.	
	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.
12 BE	0.54	1.3	0.26	4.3	0.07	6.1	0.04	5.6	0.04	.....	0.03	8.4
232 H	.61	4.6	.09	4.6	.04	.....	.03	7.2	.03	.....	.03	7.2
Average	.57	2.9	.17	4.4	.05	6.1	.035	6.4	.035	.....	.03	7.8

TABLE III.—*Effect of aeration upon dextrose fermentation and nitrogen fixation*—Con.

Culture.	Nonaerated cultures.											
	1 day.		2 days.		3 days.		4 days.		5 days.		6 days.	
	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.
12 BE.....	1	0.7	0.8	0.7	0.8	0.7	0.8	0.7	0.8	0.7	0.8	0.7
232 H.....	1	0	.8	0	.8	.....	.7	.....	.7	.6	.7	.6
Average.....	1	.35	.8	.35	.8	.7	.75	.7	.75	.65	.75	.65

Flasks containing 250 cc. of media were inoculated and incubated under aerating and nonaerating conditions. An aerated and nonaerated culture flask was analyzed each day for six days. The daily increase of nitrogen fixed as well as the daily consumption of dextrose in the aerated and nonaerated cultures is shown.

The marked effect of aeration upon Azotobacter is again indicated by these figures. In the aerated cultures the consumption of dextrose is very rapid. An average of 95 per cent of the original amount of dextrose disappeared within three days. Such is not the case in the nonaerated cultures. The average of the two cultures indicates that only 20 per cent of the dextrose was consumed within six days in the absence of aeration.

The comparative effect of aeration and nonaeration upon azofication and dextrose consumption is more clearly illustrated in figure 1 by the curves made from these data.

The striking effect of aeration is thus plainly evident, both in the nitrogen fixed and in the dextrose consumed. A close relationship is evident between the dextrose fermentation and azofication.

RATE OF NITROGEN FIXATION AND DEXTROSE FERMENTATION

Observation was made upon the comparative rate of nitrogen fixation and dextrose consumption in aerated cultures.

Allen (*8*) contributes data from one experiment and concludes that the results show that the rate of carbohydrate (dextrose) consumption by Azotobacter does not proceed in a manner similar to the rate of increase in cell numbers. Some of his cultures were agitated by mechanical means. This agitation appeared to hasten dextrose consumption as compared with those cultures which were not agitated.

The results obtained from six cultures of Azotobacter are shown in Table IV. The organisms were inoculated into a bottle holding 1,500 cc. of 1 per cent dextrose medium. The cultures were aerated two, four, six, and eight days. Total nitrogen and sugar determinations were made at the end of these periods by removing aseptically 150 cc. of the culture from the bottle. Curves plotted from the average of these results in figure 2, demonstrate a correlation between the rate of nitrogen fixation and dextrose fermentation. In general the results are similar to those indicated in figure 1.

The data representing the general average of the figures in the table are recapitulated in Table V. It will be noticed that the rate of azofication per gram of dextrose for each period of two days, is practically the same, namely, 6.90, 9.37, 8.34, and 9.74 gm. of nitrogen.

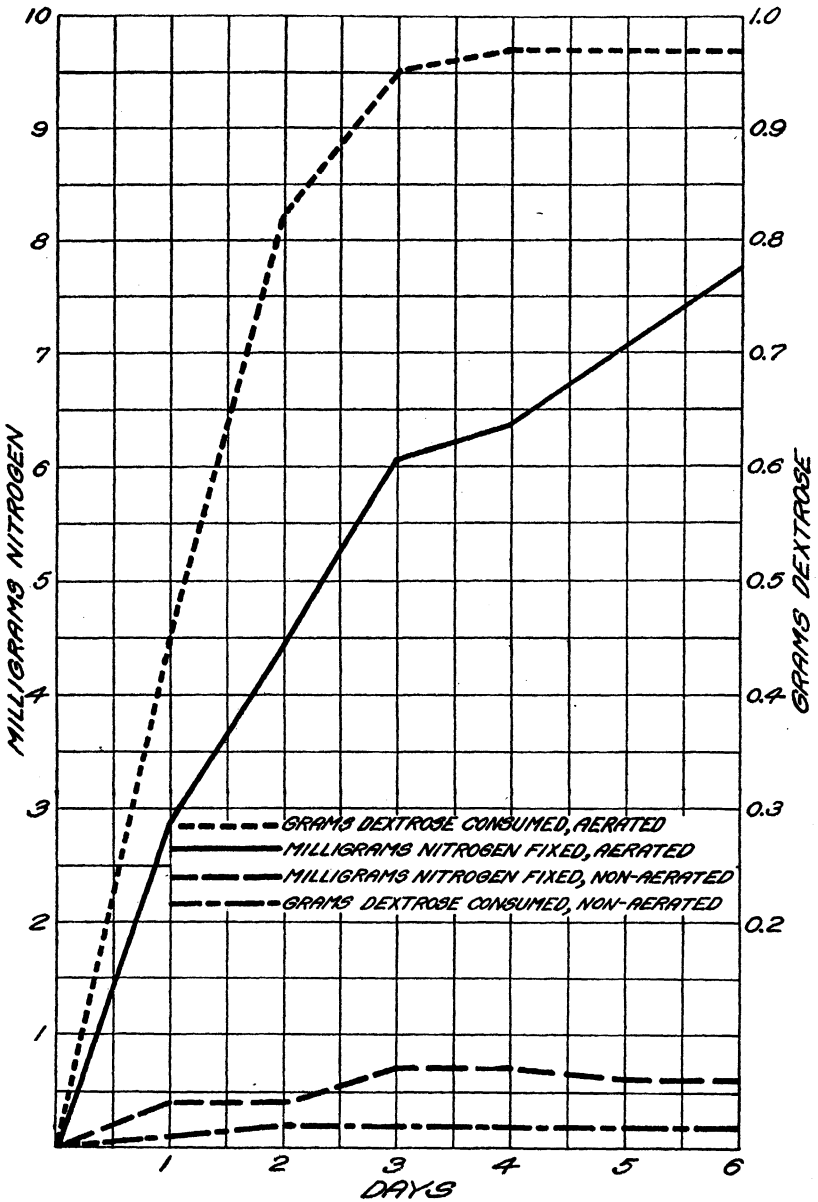

FIG. 1.—Effect of aeration on nitrogen fixation and dextrose fermentation.

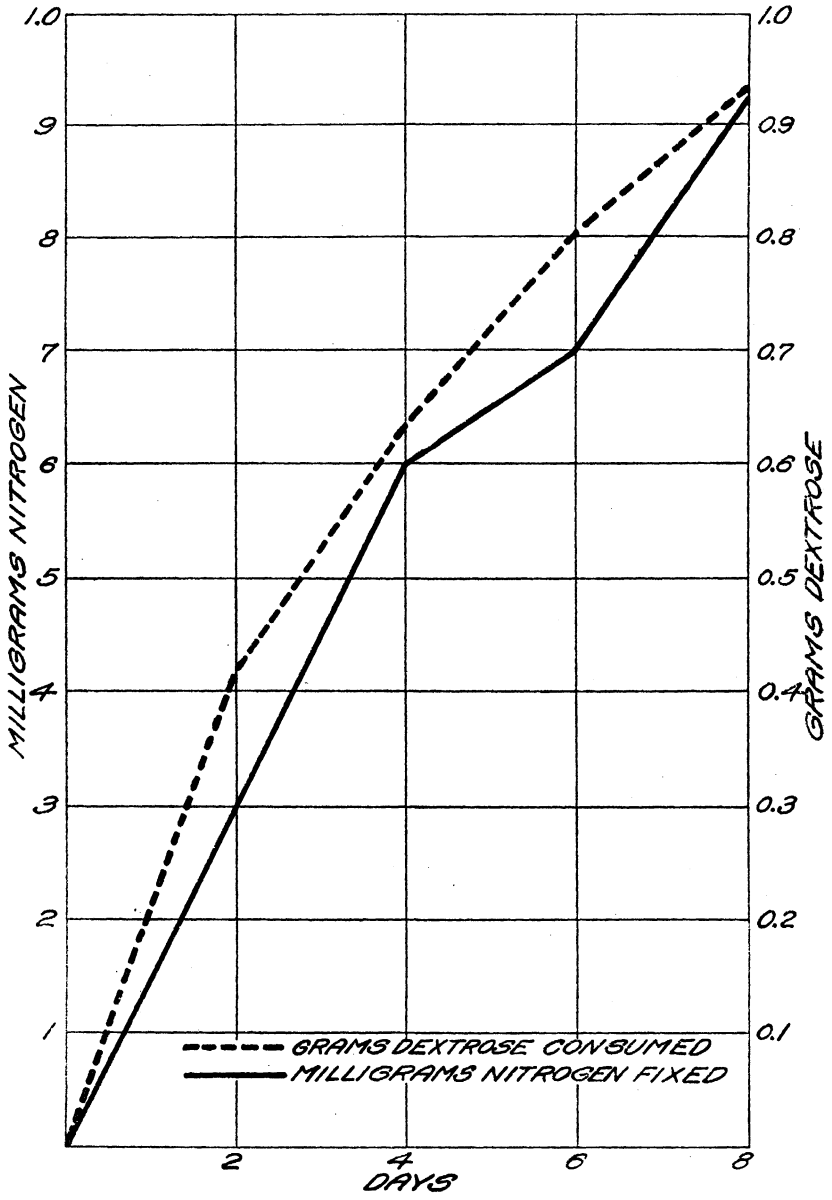

FIG. 2.—Rate of nitrogen fixed to dextrose consumed.

Examination also reveals that 42 per cent of the original amount of dextrose was consumed within two days, 22 per cent within four days, 17 per cent within six days, and 13 per cent within the eighth-day period, while 6 per cent was not fermented.

It also appears from these data that 31.6 per cent of the total nitrogen is fixed within two days, 33.8 per cent within the second period, 10.9 per cent within the third period, and 23.5 per cent within the fourth period. The amount of dextrose consumed in four days was 64 per cent of the sugar originally present, while the amount of nitrogen fixed within the same period was 65 per cent of the total nitrogen fixed. This periodical rate of nitrogen fixation and dextrose fermentation is noted in figure 3 by the curves plotted from these data.

Resummarizing the data in Table III as noted in Table VI shows that the rate of azofication per gram of dextrose per day is very uniform.

On the sixth day the amount of nitrogen recorded is greater than the amount actually fixed for this period as the nitrogen determinations were lost on the fifth day. The figures, therefore, in reality give the amount of nitrogen fixed between the fourth and sixth days.

During the first three days a fixation of 78 per cent of the total nitrogen occurred. The amount of dextrose fermented in the same period was 95 per cent of the original amount present.

The curves in figure 4, plotted from these data, exhibit curves similar to those noted in figure 3.

TABLE IV.—*Rate of nitrogen fixation and dextrose fermentation*

Culture.	2 days.		4 days.		6 days.		8 days.	
	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.	Gm. dextrose.	Mgm. N.
9 B	0.70	.....	0.49	6.6	0.28	6.7	0.10	7.0
2 B	.70	1.5	.49	8.6	.34	7.0	.13	7.4
9 A	.54	2.8	.24	8.0	.08	.....	0	11.6
5 B	.61	1.5	.49	5.6	.30	6.8	.2	9.8
11 B	.70	3.2	.49	4.9	6.21	5.3	0	10.0
8 B	.54	3.8	.49	4.9	.34	8.4	.3	.....
3 A	.50	2.9	.18	5.3	.04	7.7	.....	.....
213	.63	5.2	.32	5.6	.18	8.0	.....	.....
6 A	.38	5.3	.05	4.9	0	6.1	0	.....
Average	.58	2.9	.36	6.0	.19	7.0	.06	9.16

TABLE V.—*Rearrangement of data from Table IV*

	2 days.	4 days.	6 days.	8 days.
Total grams dextrose consumed	0.42	0.64	0.81	0.94
Total milligrams nitrogen fixed	2.90	6.00	7.00	9.16
Grams dextrose consumed each 2 days	.42	.22	.17	.13
Milligrams nitrogen fixed each 2 days	2.90	3.10	1.00	2.16
Rate of azofication per gram dextrose each 2 days	6.90	9.37	8.64	9.74

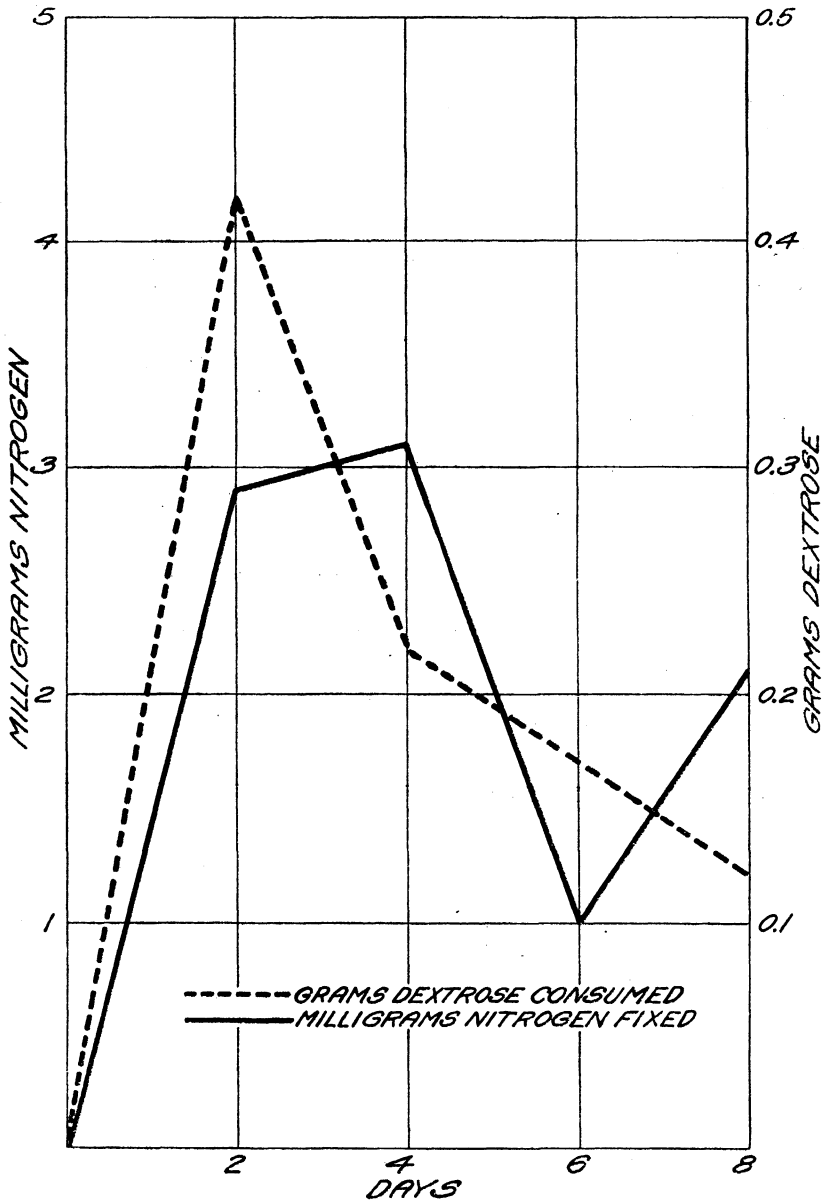

FIG. 3.—Daily amount of nitrogen fixed and dextrose consumed.

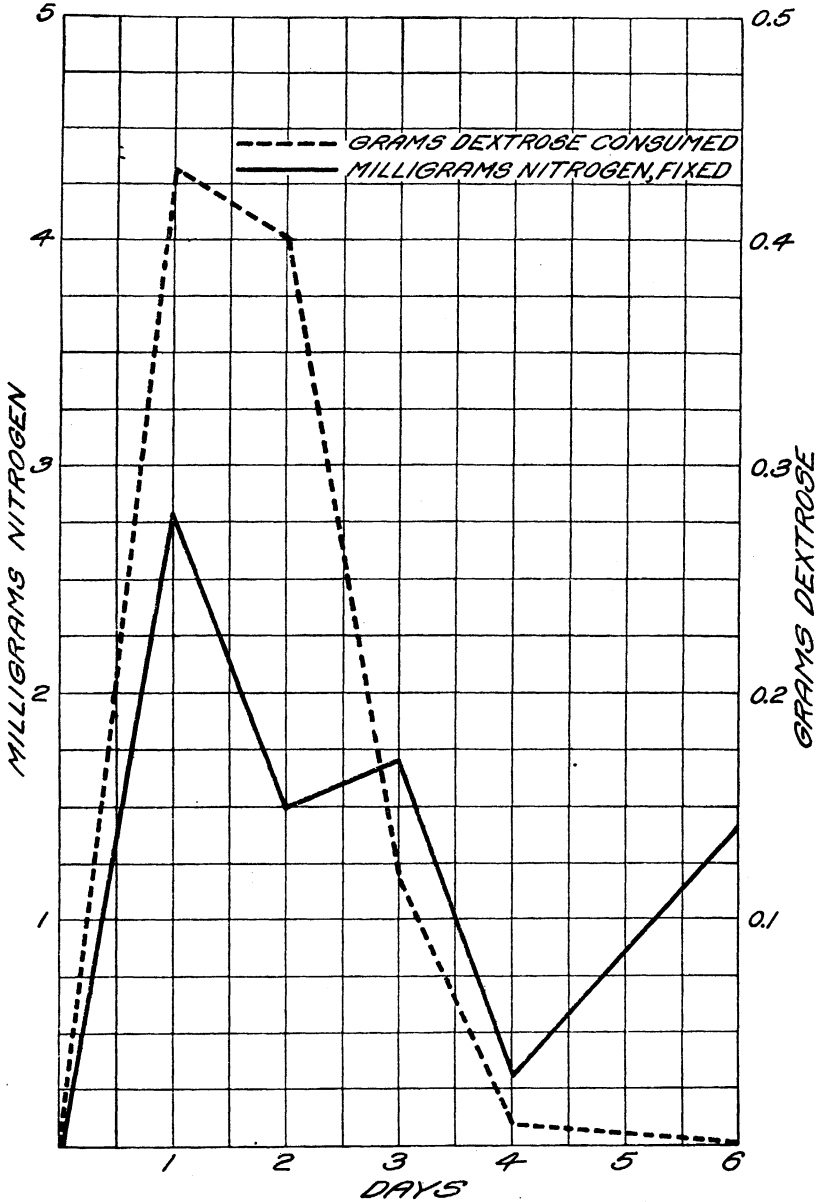

FIG. 4.—Daily amount of nitrogen fixed and dextrose consumed.

TABLE VI.—*Rearrangement of data from Table III*

	1 day.	2 days.	3 days.	4 days.	5 days.	6 days.
Total grams dextrose consumed	0. 43	0. 83	0. 95	0. 965	0. 965	0. 97
Total milligrams nitrogen fixed	2. 90	4. 40	6. 10	6. 40	.....	7. 80
Grams dextrose consumed per day	. 43	. 40	. 12	. 015	0	. 005
Milligrams nitrogen fixed per day	2. 90	1. 50	1. 70	. 30	.....	1. 40
Rate azofication per gram dextrose per day	6. 51	5. 30	6. 42	6. 56	.....	8. 04

EFFECT OF $CaCO_3$

Calcium carbonate appears to be a universal constituent of the media used for the cultivation of Azotobacter. Lipman (*2*) asserts that calcium carbonate stimulates the growth of Azotobacter by either directly furnishing calcium or indirectly by making available more phosphorus, sulphur, and magnesium. Other investigators attribute its beneficial influence to the maintenance of a neutral reaction. Allen (7) in his studies on the composition of media for Azotobacter found $CaCO_3$ to be essential.

For further investigation with the cultivation of Azotobacter a substance fulfilling all the requirements of a good medium was desired—namely, one that provides the proper food constituents, proper reaction, and is clear and free from sediment. The presence of $CaCO_3$ interfered with the latter qualification. Preliminary experiments with a dextrose solution similar to the medium already mentioned, with and without $CaCO_3$, had furnished evidence supporting the doubtful value of $CaCO_3$ for the growth of Azotobacter in pure culture.

Flasks containing 250 cc. of media with and without $CaCO_3$ were inoculated with pure cultures of Azotobacter and aerated for six days. One flask of media was removed each day, and duplicate determinations of total nitrogen were made. The results are given in Table VII.

The averages of the analyses for two and four days indicate a slight increase in the amount of nitrogen fixed in the $CaCO_3$ medium. However on the sixth day the non-$CaCO_3$ medium shows a slight increase over the $CaCO_3$ solution. The slight increase of nitrogen fixed in one medium over the other is attributed to experimental error rather than to any marked effect of the media. These results are comparable with numerous others obtained in this laboratory. The conclusions are that with pure cultures undergoing a vigorous aeration the presence of $CaCO_3$ in the media is not essential for the prompt growth of the Azotobacter.

It should be stated that the medium used in these experiments and in all other work where $CaCO_3$ is not added was always corrected to a reaction near a P_H of 7.0 to 7.4. The reaction of such a medium inoculated with pure cultures of Azotobacter and aerated will vary but little.

TABLE VII.—*Effect of $CaCO_3$ upon nitrogen fixation*

Culture.	Milligrams nitrogen per gram of dextrose.					
	2 days.		4 days.		6 days.	
	No $CaCO_3$.	$CaCO_3$.	No $CaCO_3$.	$CaCO_3$.	No $CaCO_3$.	$CaCO_3$.
1 BM	4.3	5.3	7.0	8.9	9.1	11.5
3 BM	4.3	4.9	9.4	14.2	14.9	15.9
222 M	4.9	6.0	6.1	8.0	15.9	11.0
10 BP	1.7	.9	5.2	7.1	9.1	8.1
12 BE	.....	4.3	5.6	5.6	10.0	8.4
232 H	6.3	4.6	7.2	7.2	7.2	7.2
Average	4.3	5.0	6.75	8.5	11.03	10.35

SUMMARY

(1) A prompt and vigorous growth of Azotobacter can be induced in large quantities of liquid medium by sufficient aeration.

(2) Aeration stimulates rapid nitrogen fixation by Azotobacter.

(3) There exists a close correlation between the rate of dextrose fermentation and nitrogen fixation.

(4) The presence of calcium carbonate is not essential in a medium used for aerating pure cultures of Azotobacter.

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