86. PROTEIN PRODUCTION BY NITROGEN-FIXING BACTERIA UNDER CONDITIONS OF CONTINUOUS AERATION

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SINCE Beijerinck [1901] isolated two species of *Azotobacter* and proved them to be capable of fixing atmospheric nitrogen, many workers have studied this group of organisms and the influence of different factors on their rate of growth and of nitrogen fixation. A suitable medium, such as that used by Ashby [1907], is one containing mannitol and inorganic salts, but no source of nitrogen. The aeration essential for rapid growth is usually ensured by cultivating the organisms in a shallow layer of the medium with a large exposed surface, but good growth also occurs in deeper layers if the medium is aerated by bubbling air through it during the course of incubation. Thus Deo [1920] inoculated such a medium with activated sewage sludge and compared the nitrogen fixation in cultures through which a current of air was passed at room temperature with that in cultures incubated in the quiescent condition at 35°. Fixation of nitrogen during 2–5 weeks was appreciable under both these conditions but the cultures aerated at room temperature gave the higher yield.

The experiments of Ardern & Lockett [1914], who, in collaboration with Fowler, developed the activated sludge process of treatment of sewage, showed that continuous aeration of sewage with a rapid stream of air resulted after some time in the deposition of a sludge containing much of the organic matter, largely in the form of bacterial cells. On allowing the sludge to settle and adding to it a fresh batch of sewage a second period of aeration resulted in the formation of more sludge and a more rapid clarification of the sewage than had occurred previously. Repetition of this process resulted eventually in the formation of a considerable quantity of 'activated' sludge which then clarified sewage, with which it was aerated, in a few hours, compared with the period of several weeks which had been found necessary in the first instance.

It seemed likely that this principle might be applied to a suitable medium containing nitrogen-fixing bacteria, the object being to obtain continuous fixation of nitrogen with the periodic removal in the form of sludge of the bacterial protein which had been formed.

EXPERIMENTAL

Mannitol medium

Preliminary experiments were made with a N-free solution of the following composition: mannitol 20 g.; K_2HPO_4 0.2 g., $MgSO_4$ 0.2 g., NaCl 0.2 g., $CaSO_4$ 0.1 g., $FeCl_3$ 0.006 g., water 1000 ml. The solution was neutralized with NaOH to phenolphthalein and was not sterilized.

A Winchester quart bottle, containing 2 l. of this solution, was fitted with a rubber bung and glass leading tubes to enable a current of air to be passed

continuously through the liquid by suction from a water pump. The lower end of the inlet tube was blown into the form of a bulb perforated with pin holes to break up the current of air into small bubbles. By interposing a tap between the suction pump and the bottle and connecting the inlet tube to a rotameter the flow of air was adjusted and maintained, in this and in subsequent experiments, at 5 cu. ft. an hour. The bottle was immersed in a thermostat at 25°.

In order to prevent developed acidity from inhibiting the growth of N-fixing bacteria, 10 g. of CaCO_3 were added to the solution. The inoculum consisted of 25 ml. of thick activated sludge produced in the laboratory from domestic sewage. This provided the source of N-fixing organisms and probably acted also as a nucleus for the formation of more sludge.

The N content of the mixed suspension was determined, using the semi-micro-Kjeldahl method described by Rowland [1938], on duplicate quantities of 25 ml., and the level of the remaining liquid was marked on the bottle. Aeration was then begun and at suitable intervals the liquid lost by evaporation was replaced by addition of water; further samples were then abstracted for determination of nitrogen. A preliminary experiment, in which the liquid was aerated for 18 days, showed that the N content increased from 2.53 to 9.46 mg./100 ml.

In the second experiment samples were taken more frequently to measure the rate at which N fixation occurred and to find how long was required in each period of aeration to reach the maximum. During the first period of aeration the N content increased from 2.95 to 10.28 mg./100 ml. in 7 days. After 5 more days there was no further increase; in fact there was a slight decrease. On the 8th day 100 ml. of the liquid were poured into a measuring cylinder and allowed to settle. The chalk settled quickly and was soon surmounted by some lighter, cream-coloured flocules. The supernatant liquid was dark brown, very turbid and showed no signs of clarifying. After 12 days' aeration the colour of the liquid was brownish black and the suspended matter still did not settle readily. Microscopic examination of the supernatant liquor showed an apparently almost pure culture of a large, oval or round organism, while dilutions of the liquid plated on mannitol-agar gave rise to a predominance of large, colourless, mucoid, surface colonies. These observations suggested that the organisms responsible for fixation of N in the aerated liquid were species of *Azotobacter*. Since the amount of activated sludge originally added as an inoculum was insufficient to colour the liquid appreciably, the dark brown colour after several days' aeration was probably due to *Azotobacter* pigment. Other workers (e.g. Ashby [1907]) have noted the appearance of a dark brown colour in ageing cultures of this organism.

On the 13th day the aeration was stopped and the mixture was left overnight. A greyish white sludge appeared at the bottom, beneath an opaque, dark brown liquor. This supernatant liquor contained a large proportion of the total N of the culture. Its N content was 6.59 mg./100 ml., compared with 10.05 mg./100 ml. in the whole culture. The NH_3 content of the supernatant liquor was extremely small so that most of the N presumably represented proteins or their decomposition products. Centrifuging for 30 min. at 2000 r.p.m. did not clarify the liquor and caused the separation of only a small amount of brown deposit.

The greyish white sludge which had settled at the bottom of the mixture, and the deposit obtained after centrifuging the supernatant liquor, were transferred to a second Winchester bottle, a fresh quantity of mannitol medium was added, and a second period of aeration commenced. The N content then increased from 5.47 to 16.27 mg./100 ml. in 5 days. After 7 days aeration was stopped. The liquor was now light brown in colour and the sludge separated on centri-

fuging more easily than at the end of the first aeration. The settled sludge and the deposit after centrifuging were again transferred to a fresh bottle of mannitol medium and a third period of aeration was begun. The N content on this occasion increased in 6 days from 12.63 to 18.88 mg./100 ml. The rate of fixation was slower than in the previous aeration and the amount of N fixed was appreciably less. The liquor and sludge at the end of the period were light coffee colour.

The figures for N content of the liquid at various stages in each of the periods of aeration are shown in Table 1. The experiments showed that considerable

Table 1. *Nitrogen fixation in aerated mannitol solution*

	(mg. N/100 ml. culture)								
Time (days)	0	1	2	4	5	6	7	12	13
1st Aeration	2.95	—	—	—	—	—	10.28	10.05	Stop
2nd Aeration	5.47	9.04	11.33	14.23	16.27	—	Stop	—	—
3rd Aeration	12.63	13.93	16.39	18.63	—	18.88	—	—	—

fixation of N occurred under the conditions described and that the deposit or sludge formed during one period of aeration could be collected and added to a fresh batch of medium in order to carry on the process in the next period of aeration. If the process is to be satisfactory, however, conditions must be found in which the sludge settles easily so that the supernatant liquor can be siphoned away, and the N lost in the discarded fluid must be relatively small.

Glucose medium

A similar series of experiments was carried out in which the nutrient medium contained glucose instead of mannitol but was otherwise of the same composition as that given previously.

The figures for N content of the liquid at various stages during four successive aerations are shown in Table 2. It may be seen that there is a marked lag phase

Table 2. *Nitrogen fixation in aerated glucose solution*

	(mg. N/100 ml. culture)											
Time (days)	0	1	2	3	4	5	6	7	8	9	10	12
1st Aeration	2.43	—	—	2.68	—	5.09	—	—	—	14.61	—	Stop
2nd Aeration	6.54	9.38	13.91	—	20.41	—	20.06	—	Stop	—	—	—
3rd Aeration	14.90	16.49	18.92	20.30	—	22.95	—	24.64	—	—	Stop	—
4th Aeration	5.58	—	10.77	—	13.36	—	18.58	—	19.04	—	—	—

at the beginning of the first aeration, corresponding presumably to the time required to establish active growth of the relatively small number of N-fixing bacteria present in the inoculum of sewage sludge. In the subsequent aerations, when there are large numbers of N-fixing bacteria at the commencement, there is no lag phase.

During the first period of aeration colour changes in the medium were observed which were similar to those noted in the experiments with the mannitol medium. For several days the deposit was white or cream in colour, and the supernatant liquid was colourless. Towards the end of the aeration period of 12 days the colour darkened and was finally very dark brown. The sludge deposited on allowing the mixture to stand and the deposit remaining after centrifuging the supernatant fluid were transferred to a fresh bottle of glucose medium for the second period of aeration.

During the second and subsequent aerations this dark colour no longer appeared and the sludge produced was light coffee colour. Moreover, at the end of the second period of aeration the sludge settled quickly, leaving a supernatant liquid which was colourless and almost clear. Thus from 100 ml. of the mixed liquor, which was poured into a measuring cylinder, the sludge which settled occupied 50 ml. after 15 min. and 33 ml. after 2½ hr. The N content of the supernatant liquor was small, being only 2.36 mg./100 ml. when that of the mixed liquid was 20.06 mg.

Microscopic examination of the sludge at this point showed the presence of numerous large round organisms, corresponding in appearance with that of *Azotobacter*, together with considerable numbers of other bacteria, mainly rod forms. When the liquid was plated on mannitol-agar large mucoid surface colonies developed but were accompanied by a large number of much smaller colonies. It is well known that, although a mannitol medium encourages an almost pure culture of *Azotobacter*, the corresponding glucose medium allows the growth of other organisms to a considerable extent. Since many of these contaminants occurred in chains and tangled groups it is possible that they assisted in floccule formation by acting as binding agents for masses of *Azotobacter* cells, thus facilitating the settling of the sludge.

The contents of the bottle were allowed to settle for 1½ hr. at the end of the second aeration, the supernatant liquor was siphoned off and replaced by a fresh batch of glucose medium for the next aeration. It may be seen from Table 2 that during the third period of aeration the rate of N fixation was lower than during the second aeration. At the end of this period the mixture did not settle easily and there was no clear demarcation between sludge and supernatant liquor after several hours. Since the initial N content had been high and the sludge now occupied an appreciable proportion of the total volume of the mixture it seemed likely that ability to settle, and possibly rate of N fixation, became less when the concentration of sludge protein passed a certain point. This possibility was confirmed when, on taking one quarter of the contents of the bottle and transferring it to a fresh bottle of glucose medium for the fourth aeration, it was found that N fixation proceeded rapidly, as can be seen from the figures in Table 2. After 4 days' aeration, when the N content was 13.36 mg./100 ml., the sludge settled rapidly from the standing liquid and left a fairly clear supernatant fluid. After a further 2 days' aeration, when the N content had risen to 18.58 mg./100 ml., the sludge settled very slowly indeed. It was noticed that mixtures containing a high proportion of sludge were much more viscous than those with a lower proportion and the slowness of settling may have been due to this.

These results suggest that in order to end each aeration period with the sludge in a suitable condition for rapid settling, the amount of sludge carried over from the previous aeration should be sufficiently small to ensure that the N content of the liquid does not rise above the critical value towards the end of the period of aeration. On the other hand the quantity of sludge added at the beginning of the aeration should be sufficient to allow of rapid N fixation without a lag period. From the figures in Table 2 it appears that the optimum conditions will be obtained if, after allowing the sludge to settle at the end of one period of aeration, sufficient is added to the next batch of medium to give an initial N content of 5–6 mg./100 ml. of mixture. The residue of sludge not used for carrying on the next aeration can be removed to build up a stock of protein formed by fixation.

CONCLUSIONS

1. When a nitrogen-free solution, containing mannitol or glucose, inorganic salts and sufficient chalk to neutralize acidity, is inoculated with activated sewage sludge and aerated with a rapid current of air, active fixation of nitrogen occurs through the growth of nitrogen-fixing bacteria. The deposit of bacterial sludge which separates on allowing the liquid to stand, may be transferred at the end of one aeration to a fresh batch of medium for the next aeration. In this way the process becomes continuous.

2. The sludge settles quickly and completely from a glucose medium under suitable conditions, leaving a comparatively clear supernatant liquid, in which the nitrogen content is low. If, however, the concentration of sludge in the mixture is allowed to rise above a critical value, settling of the sludge floccules becomes very sluggish, possibly owing to the increased viscosity of the liquid.

3. The rate of nitrogen fixation appears to be highest when the conditions are most suitable for the settling of the sludge.

4. Under optimum conditions in these experiments approximately 7 mg. of nitrogen were fixed for every gram of glucose utilized. It is possible that, by improving the conditions of aeration and experimenting with the constituents of the medium, both the rate and total amount of nitrogen fixation might be increased.

5. The method may be used to build up a stock of bacterial protein accompanied by the other constituents of the bacterial cells.

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