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# Effect of UV-Irradiated Nitrogen Gas on Biological Nitrogen Fixation in Continuous Cultures of *Azotobacter vinelandii*

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In continuous cultures of *Azotobacter vinelandii* exposed to air and ultraviolet irradiated nitrogen gas, an increase in nitrogen fixation, compared to that of cultures exposed to non-irradiated nitrogen, has been noted. This increase is due to a decrease in glucose consumption. No change in dry weight or in the amount of nitrogen fixed per gram bacteria could be detected.

An increase in nitrogen fixation in batch cultures of *Azotobacter* supplied with ultraviolet irradiated nitrogen gas, compared to cultures fed with non-irradiated gas, has been shown previously.[1–3]

This increase has also been correlated to an increase in gas ions which are produced in nitrogen gas by ultraviolet irradiation.[3]

Nitrogen fixation is often determined as mg nitrogen fixed per g glucose consumed.[1–3] Any change in this ratio must therefore be due to a change either in the amount of nitrogen fixed or in the amount of glucose consumed.

In the present paper it will be shown that the effect of ultraviolet irradiated nitrogen gas on nitrogen fixation by *Azotobacter* is due to a decrease in glucose consumption.

## MATERIALS AND METHODS

All letters in the following description refer to Fig. 1. The culture vessel, A, was made from a Pyrex glass tube, 310 mm long and 40 mm in diameter. The tube was fitted with a rubber stopper, perforated by three glass tubes. One of these tubes was used as a medium inlet and connected by rubber tubing to a medium bottle, B.

The rubber tubing passed through a hose pump, C, designed by E. Meyer and described by Holme.[4] A second tube perforating the rubber stopper was used as an air inlet, and equipped with a sintered Pyrex glass cylinder, F, 10 × 20 mm, pore size 40–60 $\mu$, Corning Glass Works, New York. The air inlet was connected to a filter,[5] E, and to a Krohne gasrotameter, D. The third glass tube perforating the rubber stopper was made as a pocket into which a contact thermometer, G, could be inserted. A capillary glass tube, H, with an inner diameter of 1 mm, was fused into the side at the bottom of the culture vessel. This capillary tube was connected by a piece of rubber tubing to a stainless steel tube, I, 150 mm long and 3 mm in inner diameter. A tube of stainless steel was chosen in order to be able to charge it electrically positive or negative and so use the tube

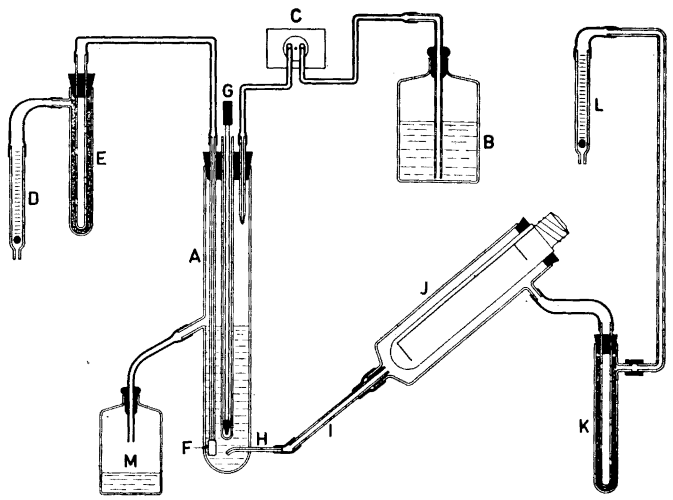


*Fig. 1.* Culture vessel for continuous cultivation of *Azotobacter* under separate supply of air and nitrogen gas.
A = Culture vessel; B = Medium bottle; C = Hose pump; D and L = Gas rotameters; E and K = Filters for sterile filtration of air and nitrogen gas; F = Sintered Pyrex glass cylinder for air dispergence; G = Contact thermometer; H = Capillary glass tube; I = Stainless steel tube; J = Philips' germicidal lamp, TUV 6 W; M = Bottle for collecting outgoing culture.

as a filter for gas ions. The experiments where the stainless steel tube is charged are reported elsewhere.[6] The stainless steel tube was connected to a glass jacketed UV-lamp, J. The glass mantle of the lamp was connected to a gas filter,[5] K, by a piece of rubber tubing. This gas filter was also connected to a Krohne gasrotameter, L. At the side of the culture vessel, 150 mm from the bottom, an overflow glass tube with an inner diameter of 7 mm was fused. This glass tube was connected to a collecting bottle, M, by rubber tubing. The culture vessel was heated by heating tape and the temperature controlled as described by Edebo *et al.*[7] The UV-lamp used for irradiation of the nitrogen gas has been described by Zacharias.[2] The control and the measurement of pH was performed according to Edebo *et al.*[7]

*Organism.* The organism used in this study was *Azotobacter vinelandii*, strain ATCC 7492.

*Media.* The bacteria were grown in a nitrogen free medium described by Nicholas *et al.*[8] and kept on slants containing the same medium with an addition of 2 % agar. The analysis of the nitrogen used and the amount and treatment of the air and the nitrogen gas used in this investigation, have been described previously.[2,3]

*Calculations.* The flow rate, F, was determined as ml culture fluid collected per hour. The volume, V, of the culture in the vessel was measured at different gas flows. The dilution rate, D, equal to F/V, was calculated. The nitrogen yield, $Y_N$ (the amount of bacteria propagated from 1 g nitrogen) was calculated from the analysis. The glucose yield, $Y_G$ (the amount of bacteria propagated from 1 g glucose), was calculated in the same way. The nitrogen fixation (here called N/G) and the per cent increase in nitrogen fixation were calculated as described previously.[1,2]

*Sterilization and assembling.* The empty culture vessel equipped with the rubber tubing leading to the bottle, M (Fig. 1), and the stainless steel tube L (Fig. 1) were sterilized as one unit. The rubber stopper with the three perforating tubes, and the medium bottle B (Fig. 1) equipped with the rubber feeding tube were sterilized separately. The gas filters E and K (Fig. 1) with the rubber tubings leading to the culture vessel and the

UV-lamp, respectively, were also sterilized separately. The UV-lamp was sterilized by being left burning for an hour before assembling the apparatus. The different parts were then put together aseptically.

*Culturing.* The nitrogen gas supply was opened and regulated to a preset value. A preculture of *Azotobacter vinelandii*, strain ATCC 7492, prepared as described previously[2] was diluted with media to contain $10^8$ bacteria per ml. Of this suspension, 250 ml were aseptically poured into the culture vessel. The air supply was then opened and regulated to a preset value. The heating equipment was then switched on. The UV-lamp was covered with aluminium foil to shield the culture from direct irradiation. After about 18 h run as a batch culture, growth was heavy and the hose pump was started. The culture was then run as a continuous culture at a constant dilution rate and at preset constant air and nitrogen flow rates. After about two days, when the culture was estimated to be in a steady state, the first sample was taken. The UV-lamp could then be turned on or off, so that the culture was alternatively gassed with non-irradiated and irradiated nitrogen gas as well as with ordinary air.

## RESULTS

Fig. 2 shows the effect of variance in pH on a continuous culture of *Azotobacter vinelandii*, strain ATCC 7492. The optimal pH for growth is 7.0—7.2 under the conditions employed in these experiments. If no pH-control is used, the pH increases to about 7.7. The glucose yield 0.391 g bacteria per g glucose consumed, and the nitrogen yield, 22.4 g bacteria per g nitrogen fixed, did not change with variance in pH.

A cultivation system without pH-control was chosen for further work. The reasons for this choice are given in the section headed "Discussion". At a constant oxygen transfer rate and a constant dilution rate, the nitrogen fixation as well as the increase in nitrogen fixation, due to irradiation, remain unchanged while the nitrogen flow rate increases (Table 1, Section A).

When the nitrogen flow rate and the dilution rate are kept constant, the nitrogen fixation increases with an increase in oxygen transfer rate. The per cent increase in nitrogen fixation rises as the oxygen transfer rate increases from 150 mM $O_2$/l/h to 300 mM $O_2$/l/h, but does not change when the oxygen transfer rate is further increased (Table 1, section B.). The nitrogen fixation as well as the per cent increase in nitrogen fixation does not vary with variance

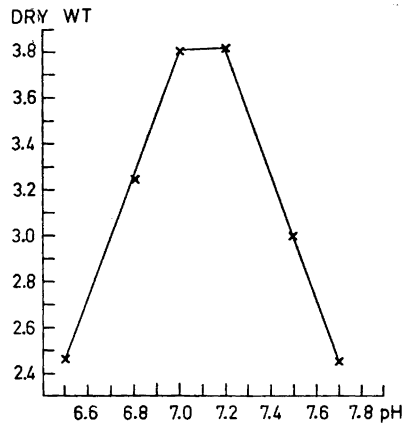


*Fig. 2.* Influence of pH on growth of *Azotobacter vinelandii*, strain ATCC 7492, in continuous culture. Dry wt = dry weight expressed in g bacteria/litre. Oxygen transfer rate 300 mM $O_2$/l/h. Nitrogen gas flow rate = 0.5 l/min. Dilution rate = 0.325 ± 0.007 $h^{-1}$.

*Table 1.* The effect of variance in nitrogen flow rate, A, oxygen transfer rate, B, and dilution rate, C, on nitrogen fixation by *Azotobacter vinelandii*, strain ATCC 7492 grown in continuous culture.

In each experiment the culture was fed with non-irradiated nitrogen gas for seven days, N.I. and with ultraviolet irradiated nitrogen gas for another seven days, I. The ultraviolet lamp used was a Philips TUV 6W germicidal lamp. O.T.R. = oxygen transfer rate, expressed in mM $O_2$/l/h. N.F.R. = nitrogen flow rate expressed in l/h. D = dilution rate expressed in $h^{-1}$. N.I. = non-irradiated nitrogen gas. I = irradiated nitrogen gas. N/G = nitrogen fixation expressed in mg nitrogen fixed/g glucose consumed.

| Section | Expt. No. | O.T.R. | N.F.R. | D. | N.I. N/G | I. N/G | % Increase |
|---|---|---|---|---|---|---|---|
| | 1 | 150 | 0.23 | 0.29 | 15.6 | 17.7 | 13.5 |
| A | 2 | 150 | 0.50 | 0.30 | 15.9 | 18.0 | 13.4 |
| | 3 | 150 | 0.75 | 0.30 | 15.8 | 17.9 | 13.3 |
| | 2 | 150 | 0.50 | 0.30 | 15.9 | 18.0 | 13.4 |
| B | 4 | 300 | 0.50 | 0.31 | 17.2 | 21.5 | 23.6 |
| | 5 | 500 | 0.50 | 0.32 | 19.4 | 23.9 | 23.2 |
| | 6 | 300 | 0.50 | 0.19 | 17.5 | 21.8 | 24.6 |
| C | 4 | 300 | 0.50 | 0.31 | 17.2 | 21.5 | 23.3 |
| | 7 | 300 | 0.50 | 0.43 | 18.0 | 22.3 | 23.9 |

in dilution rate at a constant oxygen transfer rate and nitrogen flow rate (Table 1, section C).

An oxygen transfer rate of 300 mM $O_2$/l/h, a nitrogen flow rate of 0.5 l/h and a dilution rate of about 0.3 $h^{-1}$ were chosen for further work. These conditions gave a satisfactory nitrogen fixation and per cent increase in nitrogen fixation without much foaming.

It can be seen from Fig. 3 that the effect of ultraviolet irradiated nitrogen gas on a culture of *Azotobacter vinelandii*, strain ATCC 7492, is one of an increase in glucose yield, indicating a decrease in glucose consumption. Table 2 gives the mean values and sample standard errors of the data for dry weight, nitrogen and glucose yield and nitrogen fixation given in Fig. 3.

## DISCUSSION

The working principles of the apparatus used here for continuous cultivation of *Azotobacter* have been given by Monod [9] and Novick and Szilard.[10] It is operated through the control of the flow-rate of media. This system is self-regulating: that means that if the flow of medium is kept constant and suitable the system will adjust itself to a steady state, in which all variables remain constant. The growth-rate in such a system is controlled by a limiting medium-nutrient, essential for growth. At optimal pH for growth in the system used here, a steady state was reached at a bacterial density of 3.8 g per litre dry weight. Analysis showed that the glucose was in excess. No analyses were performed to find out whether any of the salts were deficient or not, but when the medium used was modified to contain 30 g glucose per litre, it supported growth of 15.9 g of *Azotobacter* per litre in batch culture.[7] From these results

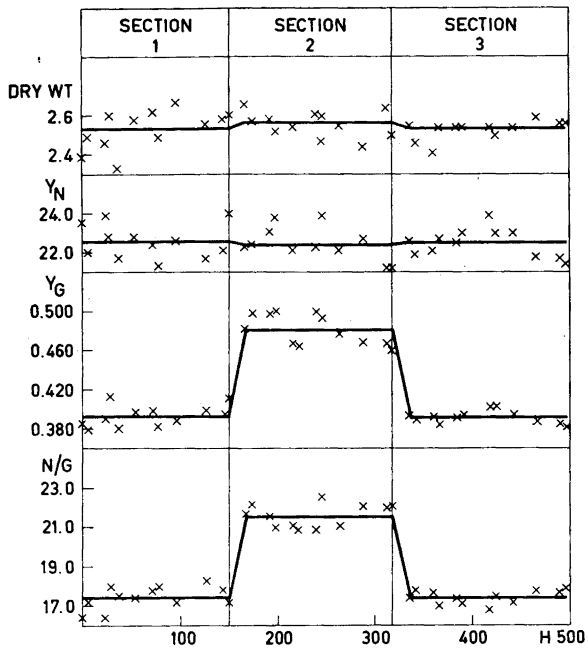


*Fig. 3.* The effect of ultraviolet irradiated nitrogen gas, Section 2, on *Azotobacter vinelandii* strain ATCC 7492, compared to organisms grown on non-irradiated gas, Sections 1 and 3. Each mark is a mean value of four samples taken from two different experiments.
Dry weight expressed in g bacteria per litre culture.
$Y_N$ = nitrogen yield expressed in g bacteria/g nitrogen
$Y_G$ = glucose yield expressed in g bacteria/g glucose
N/G = nitrogen fixation expressed in mg nitrogen fixed/g glucose consumed.
Dilution rate = 0.326 ± 0.009 $h^{-1}$. Air = 300 mM $O_2$/l/h. $N_2$ = 0.5 l/min. The ultraviolet source used was a Philips' TUV 6W germicidal lamp.

*Table 2.* Mean value of the results presented in Fig. 3. The ± sign indicates sample standard error.
Dry weight is expressed in g bacteria/litre.
$Y_G$ is expressed in g bacteria/g glucose consumed.
$Y_N$ is expressed in g bacteria/g nitrogen fixed.
N/G is expressed in mg nitrogen fixed/g glucose consumed.

| Section | Dry weight | $Y_G$ | $Y_N$ | N/G |
|---|---|---|---|---|
| 1 | 2.53 ± 0.29 | 0.393 ± 0.003 | 22.6 ± 0.3 | 17.4 ± 0.2 |
| 2 | 2.56 ± 0.19 | 0.481 ± 0.005 | 22.4 ± 0.3 | 21.5 ± 0.2 |
| 3 | 2.53 ± 0.14 | 0.391 ± 0.002 | 22.5 ± 0.2 | 17.4 ± 0.1 |

the conclusion was drawn that at optimal pH for growth the limiting nutrient was in all probability oxygen.

At values of pH lower and higher than 7.0—7.2 the growth density is less, which means that in comparison to optimal conditions, the total oxygen demand was reduced. If no pH-control is used in this system, it regulates itself to a pH of 7.7 with a cell density of about 2.4 g/litre dry weight. In a system without pH-control all medium nutrients and oxygen are in excess. Growth must then be limited by some other means. The increased pH might influence the dissociation of nutrients, the intracellular pH or the permeability of the cell membranes.

The only medium ingredients whose dissociations are influenced by pH are acetate and citrate. Neither of these is necessary for growth as the bacteria grow equally well on Burk's nitrogen-free medium [11] as on the one used here.

A change of intracellular pH in living cells induced by a change in external pH seems unlikely.[12]

The surface layers of a bacterial cell are of a complex structure and some of the ingredients such as proteins are sensitive to pH-changes. Such a change could influence the permeability or transport mechanisms of the cell.[13]

Taking all factors into consideration, the growth-limiting factor at a pH of 7.7 is most probably caused by a decreased rate of permeation of some nutrient or nutrients essential for growth through the surface layers of the cells.

In this system where growth is indirectly limited by pH, the nitrogen fixation and the glucose yield are increased when the *Azotobacter* cells are supplied with UV-irradiated nitrogen gas, compared to cells fed with non-irradiated gas. The dry weight and the nitrogen yield did not change throughout the experiment.

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