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STUDIES ON THE ENRICHMENT OF SOIL WITH AZOTOBACTER VINELANDII

MUHAMMAD SAEED, TANWIR-UL-HASSAN, SALAHUDDIN and M. ASLAM

PCSIR Laboratories, Lahore 16

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The production of nitrogen fixing *Azotobacter vinelandii* has been described. Total nitrogen fixed in the fermentation medium was 14.05 mg/100 ml with sucrose and 13 mg/100 ml with molasses as carbon source. Inoculation of the soil with the culture of these bacteria caused a significant increase in the nitrogen concentration of the soil. Nitrogen determinations in the inoculated soil supplied with a dilute solution of molasses as carbon source, showed that the nitrogen increased from an initial value of 30 mg/100g to 160 mg/100 g of the soil. When molasses was replaced with sucrose solution, the increase in the nitrogen up to 260 mg/100 g of soil was recorded. Results on the studies conducted with nitrogen-fixing bacteria in the open fields are also reported.

Nitrogen fixation by certain *Azotobacter* species is well established[1] and its economic importance in the field of agriculture is now beginning to be realised. It has been estimated that about 25 lb of N_2 is fixed per acre annually by the nitrogen-fixing bacteria in the soil.[10] Recently in the soil, use of bacterial fertilizers comprising bacterial inoculants particularly of *Azotobacter* species and those of phosphorus-solubilising organisms, viz. *Bacillus megatherium* var. *Phosphaticum*, has been published principally in the U.S.S.R. and to some extent in other agriculturally advanced countries.[2–9] In some cases, increased fertility of the soil was observed.

This paper reports results on the growth of *Azotobacter vinelandii* together with its nitrogen-fixing activity, both in soil and in liquid cultures.

Fermentation Methods

The culture *Azotobacter vinelandii* (Department of Agri. Chemistry, University of Sydney) is maintained in Winogradsky medium having the composition of KH_2PO_4, 0.5g; $MgSO_4$, 0.0125 g; NaCl, 0.0125 g; $FeSO_4.7H_2O$, 0.005 g; $MnSO_4.4H_2O$, 0.005 g; $Na_2MoO_4.4H_2O$, 0.005 g; and water 1 l. To this salt solution 1% sucrose or 2% molasses is added. The temperature for growth is maintained at 30°C and pH 7.2.

Inoculum is prepared by growing the organism in 100 ml Erlenmeyer flask, containing 25 ml sterilizing medium. After shaking for 24 hr, the culture is transferred to 300 ml Erlenmeyer flask containing 100 ml sterilized medium and shaken for another 24 hr. It is against transferred to 5-l flask, containing 3 l medium and aerated through glass wool column, again for 24 hr. This is finally fed into 40 l medium, contained in a 50-l glass bottle for growing large scale culture. During growth, samples were taken out for microscopic examination and for the determination of total sugar, fixed nitrogen, pH, dry weight and growth rate of cells. The concentration of sugar was maintained at 1% level by periodic addition of the sterilized sucrose or molasses solution, during fermentation.

Nitrogen determination was made by micro-Kjeldahl method[11] and sucrose consumption by iodometric method.[12] Total dry weights were obtained by centrifuging and washing cells from 100 ml of the fermented medium and drying at 95°C to a constant weight. Growth rate was determined by measuring turbidity at 660 μm Unicam Spectrophotometer at 8 hr interval.

Nitrogen Fixation in the Soil.—About 2 kg of soil, moistened with water was placed in three wooden trays, at a temperature around 32°C, in such a way that the thickness of the soil was about 1 in. Fresh cultures of *Azotobacter* were introduced by mixing the cell suspension with the soil, so that, the initial population was 10^8 bacteria/g of the soil. Soil was aerated by stirring daily and nitrogen contents determined after 15 days interval.

Field experiments were conducted in three experimental fields of the dimensions 4 ft × 10 ft. Field No. 1 was inoculated with the freshly grown culture of *Azotobacter vinelandii* and weekly sprayed with 2% solution of molasses for a period of 8 weeks (about 8 kg). Field No. 2 was weekly sprayed with 2% molasses but no culture was introduced. Field No. 3 was weekly sprayed with water only, and acted as control. Soil was always stirred before spraying culture molasses or water. Field studies were conducted from September to November 1969.

Results and Discussion

Table 1 shows that in the growth medium, with sucrose as carbon source, after 16 hr of growth, 7.9 mg of nitrogen was fixed per 100 ml of the medium. The fixation was 10.2 mg/100 ml after 24 hr and after 32 hr, 14.105 mg of nitrogen/100 ml was fixed. Beyond 32 hr there was no increase in the nitrogen fixation.

The value of 14.05 mg N/100 ml is considerably lower than that of 32 mg N/ml reported by Sylvan and Burris[14] with their strain of *Azotobacter vinelandii*. In a large number of experiments carried

TABLE 1.—EFFICIENCY OF CELL PRODUCTION AND NITROGEN FIXATION IN LIQUID CULTURE.

Growth time (hr)	Nitrogen fixed mg/100 ml	Dry wt % mg/100 ml	Sugar [a] consumed mg/100 ml	pH[b]
8	3.2	13.3	181	7.2
16	7.9	41.9	226	7.29
24	10.2	66.8	345	7.0
32	14.05	79.8	522	7.09
40	9.2	51.7	401	7.4

[a] The concentration of sugar was maintained at 1% level throughout the growth period by periodic addition of the sterilised sugar solution

[b] The pH of the medium was maintained at 7-7.4 by adding 0.2% $CaCO_3$ in the beginning.

out by us under vigorous aeration, a maximum of 14 mg N/100 ml was fixed. By replacing sucrose with molasses, as carbon source, the fixation was 13 mg of N/100 ml indicating that molasses served as efficient a carbon source as sugar. Further addition of sugar or molasses to the growth medium after 32 hr of growth, instead of increasing the nitrogen fixation, rather decreased it from 14.5 mg/100 ml to 9.2 mg/100 ml. There was a corresponding decrease in the dry weight of the cells.

Table 2 shows results on the bacterial inoculation in the soil. It is seen that the nitrogen of the soil was raised from 30 mg to 260 mg/100 g in the case of sucrose and to 160 mg/100 g in the case of molasses as carbon source. The increase in the nitrogen in the case of molasses was five-fold whereas in the case of sucrose it was nine-fold.

These studies lead to the conclusion that bacteria continue to fix nitrogen even for 8 weeks on the soil. This is in contrast to the liquid culture experiment, where the nitrogen fixation ceased after 32 hr of growth. Also the nitrogen fixed in 100 g soil is considerably greater than that fixed per 100 ml of the liquid culture (Tables 1 and 2). This continuous nitrogen fixation by the bacteria in the soil may be explained by the fact that soil allows the removal of toxic end products of bacterial metabolism by subsequent de-composition and absorption, whereas this is not possible in liquid cultures.

In the field experiments where soil was inoculated with Azotobactor vinelandii, increase in the nitrogen contents was also recorded from 60 mg to 110 mg/100 g of soil after 8 weeks but it was much lower than that obtained in the laboratory experiments described above. This is apparently due to better aeration, temperature and moisture control possible in small scale tray experiments

TABLE 2

Time (weeks)	Nitrogen in soil (control) %	Nitrogen in soil after culture spray with sucrose as carbon source %	Nitrogen in soil after culture spray with molasses as carbon source %
0	0.030	0.042	0.039
2	0.031	0.070	0.059
4	0.032	0.110	0.100
6	0.032	0.220	0.130
8	0.032	0.260	0.160

Experiments are now in progress by actually growing the selected crops in the inoculated fields to assess the true impact of the nitrogen fixation in the agricultural economy. Other sources of energy, such as sulphite waste-liquor and saw dust are under investigation with respect to their usefulness for the growth of nitrogen-fixing bacteria.

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