IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Mitigation Of Inhibitory Effect Of Ammonia On Nitrogenase By Increasing C:N Ratio In *Azotobacter*

[1]Dr Sadhan Kumar Das, [2]Tarun Kanti Das
[1]Program Officer, [2]Technical Officer
[1,2] Vivekananda Institute of Biotechnology
Sri Ramkrishna Ashram Nimpith
P.O. Nimpith Ashram, Dist South 24 Parganas, West Bengal, India

Abstract: The inhibitory effect of combined nitrogen on nitrogenase, the enzyme for nitrogen fixation, creates a problem in nitrogen fixation by *Azotobacter* in soil. The present study deals with mitigation of the inhibitory effect of ammonia through increasing the C:N ratio in culture medium. It was found that increase in C:N ratio by increasing glucose level or by decreasing ammonia level in ammonia containing culture medium increases the nitrogenase activity in *Azotobacter chroococcum* (Isolate No. VIBAZOTO-03). Two probable causes of this effect of glucose were suggested. First, increase in respiration rate and thus depleting the cellular oxygen level which protects nitrogenase from oxygen toxicity, and secondly, increase the alginate level in medium which is positively correlated with nitrogen fixation. The growth rate of this bacteria, however, was not affected significantly by ammonia, while increase in glucose level increased the growth rate.

Key words – *Azotobacter chroococcum*, Nitrogenase, Ammonia inhibition, , C:N ratio, Mitigation.

I. INTRODUCTION

Biological nitrogen fixation (BNF) is of special importance from ecological as well as economical view point. It is estimated that annually 1.95 x 10^{11} Kg to 2.5 x 10^{11} Kg of N-NH_3 is produced through BNF on global basis (Galloway *et al*., 2004; Cheng, 2008). *Azotobacter* is one of the most important heterotrophic diazotrophs for its ability to fix atmospheric nitrogen in free living condition within rhizosphere region or outside rhizosphere where adequate organic carbon is available. It is reported that this bacterium can contribute nitrogen to soil annually 0.3 to 60 Kg /h depending upon the soil condition (Saha, *et al.*, 2017; Bhattacharya and Jha, 2012).

The reports that growth and activities of *Azotobacter* is depended on soil environment, especially different nutrients, dates back to 1919 when E. R. Allen reported that the growth and activities of this diazotrophic bacteria is highly affected by presence and absence of different salts of iron, phosphate *etc.* Diazotrophic bacteria can fulfil their nitrogen requirement through access of fixed nitrogen or combined nitrogen already present in the environment, but prefer combined nitrogen for better energy efficiency, faster growth rate and reduced oxidative stress (Reed *et al.*, 2011). Thus, a cheaper source of nitrogen is offered by ammonia and nitrate for nitrogen fixing organisms and presence of these compounds inhibits the nitrogenase activities. (Rittmann and McCarty, 2001; Laane *et al.*, 1980; Dixon and Kahn., 2004).

Combined nitrogen, nitrate, is reported to inhibit nitrogenase in different diazotrophs including *Azotobacter* species (Burns and Bulen, 1965; Lee and Wilson, 1943; Zoonad, 1926). It is detected that increase in cellular concentration of ammonia in diazotrophs reduces the electron potential in cell which, in turn, inhibits the reducing equivalent of nitrogenase, the nitrogen fixing enzyme (Lanne *et al.*, 1980).

This deleterious effect of ammonia on nitrogenase can may be mitigated by increasing the availability of carbon sources in medium. The rate of nitrogen fixation by heterotrophic diazotrophic bacteria, like *Azotobacter*, was studied by Buhler *et al*. (1987) as a function of organic carbon and nitrogen compound content. An increase of nitrogen fixation rate was shown with the increase in C: N ration at a given oxygen concentration.

The present study deals with the growth rate (as colony forming unit per ml of culture) and nitrogen fixation rate (by Acetylene Reduction Assay) of *Azotobacter chroococcum* (Isolate No. VIBAZOTO-03) in different C:N Ratio with varied glucose and ammonia concentrations.

2. MATERIALS AND METHOD

Azotobacter chroococcum (Isolate No. VIBAZOTO-03) was taken as the experimental material and Jensen's medium (Jensen, 1954) was taken as basic medium. Two types of modification were done for experimental purpose. In the first set different glucose concentration was prepared as, 20 g/L, 15 mg/L and 10 mg/L. Adequate quantity of ammonium phosphate was added to each of Jensen's medium containing 20 g/L, 15 mg/L and 10 mg/L glucose to make C:N ratio as 4:1, 8:1, 12:1, 16:1 and 20:1. In an another set of experiment Jensen's medium was prepared adding different concentration of ammonium phosphate as, 100 mg/L, 200 mg/L, 400 mg/L and 500 mg/L. For each ammonium phosphate containing medium, adequate quantity of glucose was added to make the C:N ratio 4:1, 8:1, 12:1, 16:1 and 20:1. The experiment was prepared as triplicate and the average data of three replications were considered for further analysis. The inoculated medium was placed for bacterial growth in rotary shaker within incubator at 28 $\pm$ 2 ^{o}C for 72 h. After 72 h of incubation, the growth of the bacteria was studied following typical dilution plating technique and was expressed as Colony Forming Unit (CFU) per ml of culture.

For the evaluation of nitrogenase activity, Acetylene Reduction Assay (ARA) was followed as described by Hardy *et al.* (1968). Same set of Jensen's media that were made semisolid by adding 1.75 g Agar-Agar per Litre were used for ARA analysis. The bacterial isolate was grown in 10 ml medium within 20 ml airtight rubber cap tube for 72 h. The tubes were injected by 10% (v/v) acetylene and incubated for 24 h. Ethelene evolution was measured in Gas Chromatograph (Young Lin, YL6100GC, South Korea). The ethylene production was enumerated as nmol of ethylene produced by 10 ml of culture in 24 h.

3. RESULTS AND DISCUSSION

Bacterial growth was not seen to be affected by increasing concentration of ammonia (as indicated by decrease in C:N ratio), but increase in glucose concentration results on increase in number of Colony Forming Units (CFU) per ml of culture. As seen in Table 1., decrease in C:N ratio did not result in significant decrease in CFU count in same glucose concentration. But in same ammonium phosphate concentration, increase in glucose concentration resulted in significant increase in bacterial growth rate as seen in CFU count.

Table No 1

Colony forming Unit (CFU) / ml (x 10^8) in different C:N ratios in constant glucose concentration and constant ammonium phosphate concentrations

C:N Ratio	Glucose concentration (g/L)			Ammonium phosphate concentration (mg/L)			
	20	15	10	100	200	400	500
20:1	3.5	2.8	1.5	1.5	1.6	1.5	1.7
16:1	3.3	3	1.4	1.8	1.9	1.7	1.9
12:1	3.3	2.7	1.4	2.1	2	2.1	2.2
8:1	3.5	2.6	1.5	2.7	2.8	2.9	2.9
4:1	3.4	2.5	1.4	2.9	3	3.1	3.3

Nitrogenase activity (as indicated by ARA activities, nmol ethylene produced by 10 ml culture in 24 h) was significantly affected by ammonium phosphate. Increase in glucose concentration (as indicated by increased C:N ratio) resulted in increase in ARA activity in each culture containing different concentrations of ammonium phosphate (Fig 1). Increase in ammonium concentration (as indicated by decrease in C:N ratio) resulted in decrease in ARA activity in each culture with different glucose level (Fig 2).

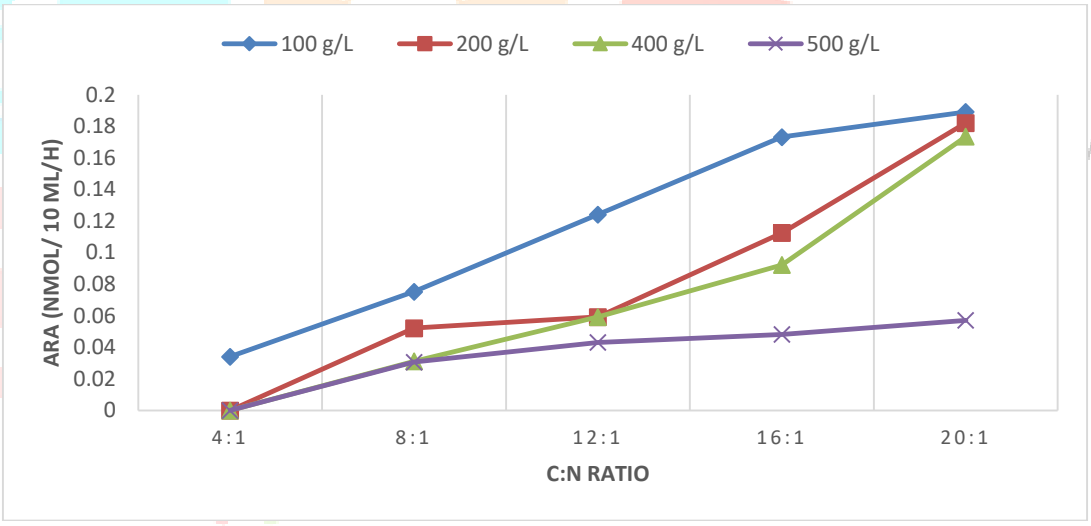

Fig.1. Acetylene Reduction Assay activity (ethylene produced nmol/10ml/h) of *Azotobacter chroococcum* culture at varied glucose level (C:N ratio) in different medium containing varied ammonium phosphate level (100mg/L, 200 mg/L, 400 mg/L and 500 mg/L)

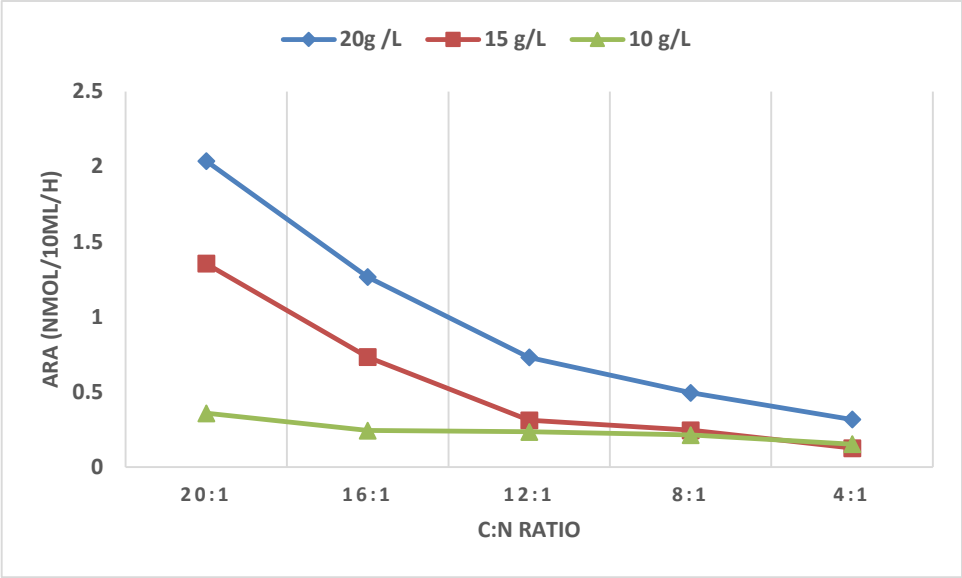

Fig. 2. Acetylene Reduction Assay activity (ethylene produced nmol/10ml/h) of *Azotobacter chroococcum* culture at varied ammonium phosphate level (C:N ratio) in different medium containing varied glucose level (20 g/L, 15 g/L and 10 g/L)

Our observation keep parity with the previous observations by different authors. It is well evident that active nitrogen fixation occurs in nitrogen deficient condition (Vitousek *et al.* 2013) and in higher concentrations of combined nitrogen, as ammonium compounds, the nitrogenase activity is highly affected. But an efficient and constant carbon source may mitigate this situation. Inomura *et al* (2018) suggested that excess sources of organic carbon increase the respiration rate depleting intra-cellular oxygen level enhancing nitrogenase activity. Post *et al.* (1983) reported that increase of sucrose concentration in inflowing medium of *Azotobacter* from 3 g /L to 15 g/L. Thus, from this study it is suggested that nitrogen contribution to agricultural field by *Azotobacter* can be best achieved by maintaining constant organic matter content in field through addition of organic fertilizer and/or through recycling of biomass produced in the field.

ACKNOWLEDGEMENT:

The authors are grateful to Srimat Swami Sadanandaji Maharaj, Chairman of Vivekananda Institute of Biotechnology and Secretary of Sri Ramkrishna Ashram Nimpith, for his constant support and encouragement. The financial support of SEED Division, Department of Science and Technology, Government of India, through STI Hub project, is duly acknowledged.

4. REFERENCES

- Allen, E.R. 1919. Some conditions affecting the growth and activities of *Azotobacter chroococcum.* Annals of Missouri Botanical Garden, 6(1): 1-44.
- Bhattacharya, P. & Jha, D. 2012. Plant growth promoting rhizobacteria (PGPR) emergence in agriculture. World J Microbiol Biotechnol, 28: 1327-1350.
- Buhler, T., Sann, R., Monter, U., Dingier, C., Kuhla, C, & Oelze, J. 1987. Control of dinitrogen fixation in ammonium assimilating cultures of *Azotobacter vinelandii*. Arch Microbiol, 148: 247-251
- Burns, R,C. & Bulen, W.A. 1965. ATP-dependent hydrogen evolution by cell-free preparations of Azotobacter vinelandii. Biochim Biophys Acta, 105(3):437-45
- Cheng, Q. (2008). Perspective s in biological nitrogen fixation research. J Integr Plant Biol, 50: 786-798.
- Chuacharoen, T., Aroonsong, S. & Chysirichote, T. 2022. Alginate production of *Azotobacter vinelandii* using sugarcane juice as the main carbon source in an airlift bioreactor. Industrial & Engineering Chemistry Research, 61(34): 12329- 12337.
- Dixon, R., & Kahn, D. 2004. Genetic regulation of Biological nitrogen fixation. Natl Rev Microbiol, 2: 621 -631.
- Galloway, J.N., Dentener, F.J., Capone, D.G., Boyer, E.W., Howarth , R. W, & Seitzinger, S.P. 2004. Nitrogen cycles: past, present and future. Biochemistry, 70: 153-226.
- Hardy, R.W.F., Holsten, R. D., Jackson, E. K. & Burns R . C. 1968. The acetylene ethylene assay for n2 - fixation: Laboratory and field evaluation Plan Physiol, Vol.43**:**1185-1207.
- Inomura, K., Bragg J., Riemann L. & Follows, M.J. 2018. A quantitative model of nitrogen fixation in the presence of ammonium. PLoS ONE, 13(11): e0208282.
- Jensen H. L. 1954. The *Azotobacteriaceae*. *Bacteriology Review*.18:195-214.
- Lanne, C., Krone, W., Konnings, W., Haaker, H. & Veeger, C. 1980. Short-term effect of ammonium chloride on nitrogen fixation by *Azotobacter vinelandi* and by bacteroids of *Rhizobium leguminaserum.* Eur J Biochem, 103: 39-46.
- Lee, S.B. & Wilson, P.W. 1943. Hydrogenase and nitrogen fixation by *Azotobacter*. J Biol Chem, 151: 377-388.
- Nosrati, R., Owlia, P., Saderi, H., Olamaee, M., Rasooli, I. and Akhavian Tehrani, A. 2012. Correlation between nitrogen fixation rate and alginate productivity of an indegeneous *Azotobacter venelandii* from Iran. Iranian Journal of Microbiology, 4(3): 153-159.
- Post, E., Kleiner, D. & Oelze, J. 1983. Whole cell respiration and nitrogenase activities in *Azotobacter vinelandii* growing in oxygen controlled continuous culture. Arch Microbiol, 134: 68-72.
- Reed, S.C., Cleveland, C.C. & Townsend, A. R. 2011. Functional ecology of free-living nitrogen fixation: a contemporary perspective. Annual Review of ecology, evolution and systematics, 42:489 –512.
- Rittmann, B. E. & Mc Carty. P.L. 2001. Stoichiometry and biological energetics. In: Environmental Biotechnology: Principal and Applications. McGrew-Hill; New York 2001. Pp 126-164.

- Saha, B., Saha, S., Das. A. Bhattacharya, P., Basak, N. & Sinha A. 2017. Biiological nitrogen fixation for sustainable agriculture. In: Agriculturally important microbes for sustainable agriculture, eds V. S. Meena, *et al. (Singapore Springer) pp- 81-128.*
- Vitousek, P.M., Menge, D. N. L., Reed, S. C. & Cleveland, C. C. 2013. Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Phil Trans R Soc B 368: 20130119.
- Zoonad, A. 1926. The relation of combined nitrogen to the physiological activity of *Azotobacter*. Brit J Exptl Biol. 4: 105-113.