BIOLOGICAL NITROGEN FIXATION (BNF)

Although the atmosphere contains a vast reservoir of molecular nitrogen (N_2) that is about 79% of the air, however, it is not directly available for use by higher plants. Before assimilation can occur, it must be converted to a so-called fixed form, either by oxidation to NO_3-N or by reduction to NH_4-N. As molecular nitrogen is highly inert, due to very stable triple bond (N≡N), these conversions are not easy to bring about. Industrially the chemical fixation of N_2 requires, high temperature and pressure, and natural gas as source of hydrogen (Haber-Bosch process). Biological N_2 fixation (BNF) which is the reduction of atmospheric N_2 to ammonia, by N_2-fixing microbes, is also very energy intensive. The difference being that, for BNF, the energy comes from the oxidation of carbon sources such as glucose.

Global contribution of nitrogen by BNF ranges from 100 to 180 million metric tons per year. Yearly industrial N_2 fixation amounts to 85 million metric tons. BNF has significant contribution in agriculture and offers an alternative to expensive industrial (fertilizer) nitrogen. However, the high-yielding agricultural systems are difficult to sustain solely on BNF.

BNF is mediated exclusively by prokaryotes, including many genera of bacteria, cyanobacteria and actinomycetes. N_2-fixing microbes can exist as free-living or in association with other microbes or plants.

BIOLOGICAL SYSYTEMS OF N_2-FIXATION

1. Symbiotic

Mutually beneficial relationship between certain microorganisms and plants, e.g.:

i) Legume — *Rhizobium* (nodule forming bacteria)

ii) Nonlegume — *Frankia* (Actinomycetes)

iii) Lichens (algae — fungi)

2. Associative symbiotic

In this case the microorganisms may depend on rhizo-deposits as carbon source but do not directly depend on the presence of the higher plant

i) *Grasses — Azotobacter*, Azospirillum (the bacteria live on root exudates etc.)

ii) Azolla — *Anabaena* (association of a fern and blue green algae) biomass incorporated in the soil provides nitrogen to plants. Practicable in rice fields.

3. Nonsymbiotic

Free-living microorganisms are involved and the presence of plant is not necessary, e.g *Azotobacter, Beijerinckia*, cyanobacteria (blue green algae).

THE NITROGENASE ENZYME

After photosynthesis, biological nitrogen fixation (BNF) is the second most important biological process on earth. Central to all kinds of BNF (above described) is the enzyme complex nitrogenase, which is responsible for conversion of N_2 to ammonia.

The nitrogenase enzyme consists of two protein components:

1) the molybdenum-iron (MoFe) protein (mol. wt. 220,000 to 270,000) and
2) the iron (Fe) protein (mol. wt. 55,000 to 66,000).

For nitrogenase to function, both components should be present. In addition nitrogenase enzyme also requires the following for BNF:

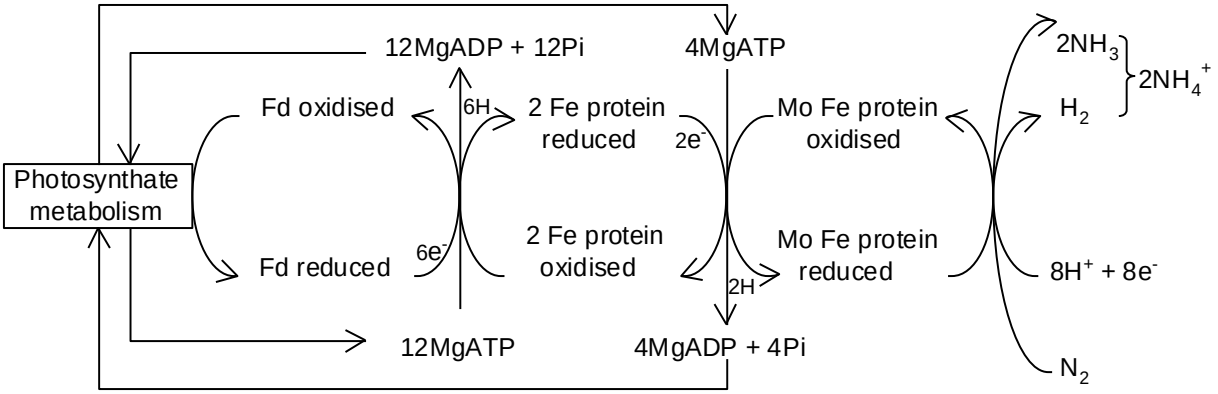
a) source of e^-,
b) ATP,
c) Mg for production of MgATP
d) absence of O_2

The characteristics of the nitrogenase enzyme are that it:

a) is destroyed by O_2. Oxygen exclusion from its microenvironment is therefore a prerequisite to keep it functional,
b) needs Mg^{2+} ions to be active for the production of MgATP,
c) needs Fe and Mo for the make-up of two proteins of which it comprises of,
d) requires low-redox reductants such as ferredoxins or flavodoxins,
e) converts MgATP to MgADP when functioning,
f) is inhibited by MgADP,
g) reduces dinitrogen,
h) reduces H^+ to H_2 even in the presence of N_2,
i) is inactive below 5^oC and starts to denature at 40^oC

PROCESS OF N_2 REDUCTION

As mentioned earlier, similar to industrial N_2 fixation, BNF also has a high requirement of energy, but in case of BNF the processes of photosynthate metabolism (e.g. electron transport and oxidative phosphorylation) provide it in the form of chemical energy (ATP). As shown in the following scheme that ATP is used at two stages. This input of ATP is so regulated that for every e^- passing, two ATP are hydrolysed with simultaneous production of one H^+.



The iron protein, accepts electrons from a low-redox donor, such as reduced ferredoxin (Fd) or flavodoxin, and is reduced itself. At this stage for six electrons passed, there is an input of 12 MgATP and formation of $6H^+$.

Reduced iron protein passes electrons to the MoFe protein and causes its reduction. At this stage, passage of two electrons mediates the hydrolysis of four MgATP and production of $2H^+$.

The whole process (two stages) requires an input of 16MgATP where by introduction of $8e^-$ results in release of $8H^+$ which is capable of reducing one dinitrogen (N_2) molecule ($N \equiv N$) to two molecules of ammonia or ammonium as shown in the diagram.

The overall BNF reaction can be summarised as:

$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16Pi$$

or

$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_4 + 16MgADP + 16Pi$$

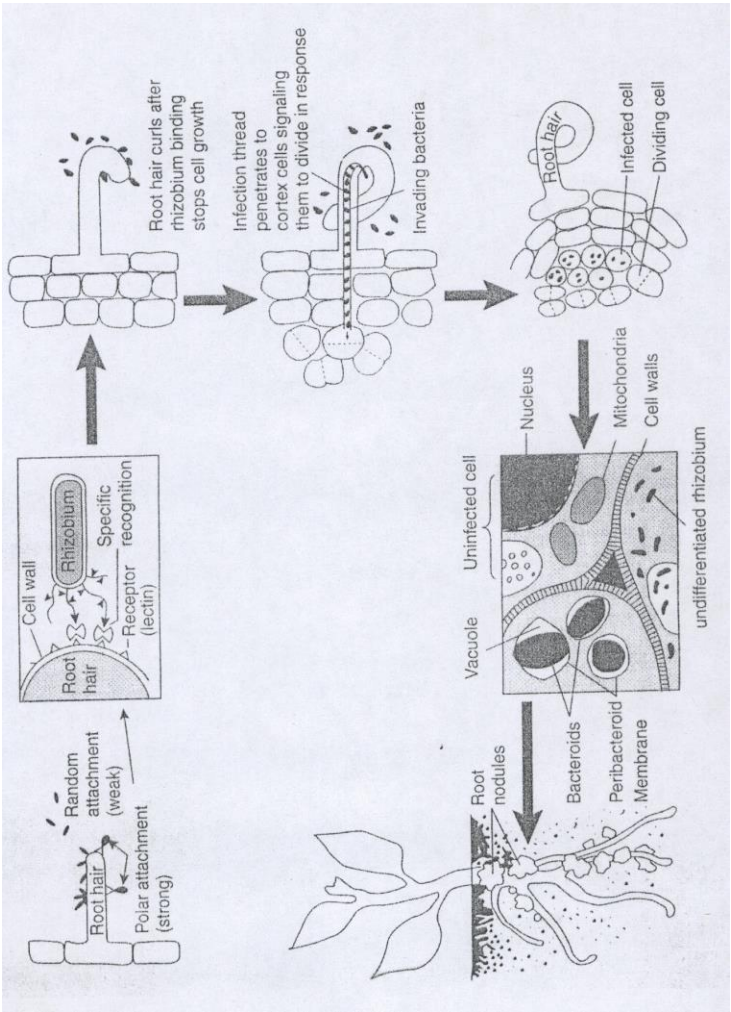
In nature the BNF reaction never operates exactly as outlined in the above scheme (i.e. with 100% efficiency). Some of the H^+ produced get reduced to H_2 even in the presence of N_2. This step incurs a loss of energy. Interestingly some diazotrophs (N_2 fixers) contain an uptake hydrogenase mechanism (not shown in above diagram) that allows them to oxidise some of this H_2 and regenerate a reduced electron carrier or MgATP. This can then be used in N_2 fixation reaction, thereby recapturing some of the energy lost. Diazotrphs having H_2-uptake activity (Hup^+) have significantly higher BNF efficiency than those that do not (Hup^-).

INFECTION AND NODULE FORMATION

Most nodules form through the infection of root hairs, although peanut nodules do not and *sesbania rostrata* forms nodules on its stem. The nitrogen fixing bacteria associated with stem nodulation are called *Azorhizobium*. The mchanisms behind these infection processes are not well studied as are the root nodules that form via root hairs.

The first step in nodule formation is for the rhizobia in soil to recognize that a suitable host is present. The plant releases specific compounds-flavones-that attract, stimulate, or signal rhizobia. These flavones are specific for specific rhizobia strains.

The next step is rhizobia attachment to the root hairs (Fig.) The initial binding to the root hairs is random and reversible. Subsequent binding is ploar and irreversible. Rhizobia then multiply in the rhizosphere. During this period, the root hair curls and forms a structure called the shepherd's crook. As the shepherd's crook forms, the rhizobia begin the process of invading the plant root cell, which is apparent because an infection thread forms. The infection thread is a hollow, cellulose-lined tube in which rhizobia multiply. Usually only one type or strain of rhizobia is in an infection thread.



Less than 5% of the infected root hairs go to form nodules. For nodules to form, the plant must play a role. Even before the rhizobia reach the cortex, the cortical cells divide. Rhizobia are released into the cortical cells and surrounded by a plant-produced membrane called the peribacteroid membrane. Within the peribacteroid membrane,

rhizobia change shape to form cells called bacteroids-pleomorphic-shaped rhizobia. Up to 10,

000 bacteroids are found per root cell and all are compartmentalized within peribacteriod membranes.

The nodule morphology

is characteristic of the plant host. Clover nodules are club shaped. Alfalfa and pigeon pea nodules are branched. Soybean nodules are spherical. Nodule number and size vary. Some nodules can approach the size of a baseball. Most are smaller-less than 0.5 cm in diameter of length. Grain legumes (pulses) have fewer nodules than do forage legumes, but the nodules are larger. As a general rule, the more nodules a plant has, the smaller they are and the less N_2 they fix. Effective nodules (N_2-fixing nodules) are larger than non effective (non-N_2-fixing) nodules. The simple way to tell which is effective nodule is to cut the nodule open. A red color indicates the presence of leghemoglobin and also indicates an effective nodule (some nodules form dark pigments, so occasionally this simple method doesn't work). New nodules form throughout the growing season and old nodules slough off.

FACTORS AFFECTING BIOLOGICAL NITROGEN FIXATION

a) Source of energy

The energy requirement of the BNF reaction has to be met by the diazotroph. That is, per one N_2 fixed, a minimum of 16 ATP or under natural conditions probably 20-30 ATP are required. This high ATP requirement means that abundant supply of energy-yielding substrates must be readily available for vigorous N_2 fixation.

b) Effects of combined nitrogen

Since N_2 fixation is so expensive to a cell, it is not surprising that the nitrogenase activity is repressed by the supply of combined nitrogen (i.e. ammonium, nitrate, and organic nitrogen). In this way the organisms avoid the high expense of synthesizing and operating an enzyme system that is not needed under conditions of nitrogen sufficiency.

c) Effects of oxygen on nitrogenase activity

An interesting characteristic of nitrogenase, its extreme sensitivity to molecular oxygen (O_2) can be a problem for the diazotroph. In many bacteria the nitrogenase is permanently damaged due to exposure to oxygen while others have adapted some unique strategies for protecting the enzyme from oxygen (detail of these is given in the next section).

d) Effect of environmental and other factors

Nitrogenase is active over a fairly narrow temperature range. At the lower limit of 5-10^{o}C, nitrogenase activity is low, whereas at the upper limits, 37-40^{o}C, nitrogenase activity falls rapidly because of the sensitivity of the enzyme to heat.

Various other factors can affect the growth and survival of diazotroph and thus directly or indirectly influence N_2 fixation. Among these are adequate supplies of phosphorus (N_2 fixation requires high levels of phosphorus), other nutrients, especially trace elements, e.g. of iron, molybedenum, vanadium.

Anaerobic condition of the soil, water-logging and ethylene accumulation has adverse effect on nodulation and N_2-fixation.

HOW MICROBES SOLVE THE OXYGEN PROBLEM FOR NITROGENASE

a) Avoidance

Anaerobes and facultative anaerobes fix N_2 only in the absence of oxygen.

b) Microaerophily

Most aerobic diazotrphic bacteria fix N_2 maximally at low partial pressure of oxygen, thereby lessening the exposure of nitrogenase to oxygen.

c) Respiratory protection

Respiration functions in all aerobes to divert oxygen away from nitrogenase to some extent. In certain Azotobacter species the high respiration rate serves to almost deplete oxygen from the microenvironment of the enzyme. In case of Rhizobium although the leghaemoglobin maintains an efficient supply of O_2 to the nodule but low O_2 concentration is maintained in close proximity of nitrogenase (bacterial surface).

d) Production of specialised cells

Some nitrogen fixing organisms produce thick walled cells (heterocysts) or vesicles with nitrogenase compartmented in, that keeps the external O_2 excluded.

e) Production of slime

Production of extracellular polysaccharides serves as a diffusion barrier to the entry of O_2 into the cell housing the nitrogenase enzyme.

f) Conformational protection

Some Azotobacter species produce a protein that binds to the nitrogenase and by changing its conformation (shape) protects it from oxygen. This arrangement saves the enzyme from O_2 damage at times when respiration is inadequate to lower the oxygen level.

g) Temporal or spatial separation of N_2-fixation and oxygen evolving processes

Temporal separation (or time separation), for example in some cyanobacteria N_2-fixation occurs in dark hours when O_2 evolution is not occurring and respiration serves to keep O_2 away. The example of spatial separation are the formation of aggregate cells, out of those some have O_2 free environment, suitable for nitrogenase.

Fate of biologically fixed nitrogen (NH_3 or NH_4)

The nitrogen fixed by free living diazotrphs (N_2 fixing microbes) is initially incorporated into their biomass. That is available to plants only after the death and decomposition (mineralisation) of the biomass.

In case of symbiotic N_2-fixation, the fixed N (NH_4^+ ion) is translocated out of the bacteroid to the host plant where it is utilised after further metabolism.

In the cystol of bacateriod-containing cells, NH_4 is converted into glutamine, glutamic acid, and asparagine and in many species nitrogen-rich compounds called ureides are formed. The two principal uriedes in legumes are allantoin and allantioc acid. Asparagine and ureides move from a bacteriod containing cells into pericycle cells adjacent to the vascular bundles. The pericycle cells then secrete nitrogen compounds into the conducting xylem cells. Those move these compounds to the xylem of root and shoot to which the vascular bundles of the nodule are connected.