**Unit 2 Non-Symbiotic Nitrogen Fixers**

**Free living Azospirillum- free isolation, characteristics, mass inoculums, production and field application.**

Non-symbiotic (NS) N2 fixation includes N2 fixation by free-living soil bacteria (autotrophic and heterotrophic) that are not in a direct symbiosis with plants, and associative N2-fixation (*e.g.* associated with the rhizospheres of grasses and cereals).

In 1925, Beijerinck for the first time described a nitrogen fixing bacterium found in the root of digit grass in Brazil and named *Spirillum lipoferum.* During 1963, its nitrogen fixing ability could be proven by several workers. In 1978, Tarrand and co-workers renamed *Spirillum* as *Azospirillum* (nitrogen fixing *Sprillum*). During 1970s, this bacterium could also be isolated from Indian Soils and rhizosphere regions of many plants.

It is associative symbiont because it effectively colonises the roots and infects cortex also. It is present inside and outside the roots without developing any apparent structure on roots. It has been found to occur in xylem vessels of black-gram and sugarcane. Moreover, it is capable of fixing 20-40 kg nitrogen under microaerophilic conditions.

1. **Isolation of *Azospirillum-***

* ***Azospirillum*** can be isolated from soil and as well as from plant root samples.
* For isolation from plant roots, a suitable plant host is selected and taken out from soil.
* Root system washed with running tap water and roots are cut into 0.5 cm long pieces.
* Roots are sterilized with 0.1% HgCl2 solution from one minute. Therefore, these are serially washed with sterile distilled water.
* One or two pieces of roots are placed on sterile and cool down semi-solid *Azospirillum* medium in screw capped tubes, incubated at 28-30°C for 2 days.

1. **Characteristics of *Azospirillum*-**

* *Azospirillum* is a gram-negative, motile (with a long polar flagellum and occasionally with peritrichous flagella, vibroid bacterium.
* It consists of poly-ß-hydroxybutyrate (PHB) granules.
* It behaves as highly aerobic when grown in ammonium-containing medium and found as micro-aerophilic when grown in nitrogen free medium.
* The carbon sources that provide energy are malic acid, succinate, lactate and pyruvate. Hence, it grows well on media containing these carbon sources. It grows moderately on galactose or acetate-containing medium and poorly on glucose or citrate- containing medium.

1. **Mass inoculums production**

* The starter culture of *Azospirillum* is prepared by transferring its loopful colony to ammonium containing- Okon’s medium in culture flask. On this, it proliferates under aerobic condition when the flask are incubated at 35°C on shaker incubator.
* After 3 days incubation , the cells are harvested from Okon’s medium and filled in fermentor, by maintaining sufficient aerobic conditions, temperarure 35°C for a few days.
* At certain intervals, broth is tested for its purity and cell number. The broth is harvested when cell number reaches to 109 ml.

1. **Field application of *Azospirillum* inoculants**

*Azospirillum* inoculant is applied in the field for various crops. However, it is applied in field as given below-

* **Seed treatment-**slurry of *Azospirillum* inoculants is prepared by mixing with water in a container. Seeds to be sown in field are soaked in slurry (2kg inoculants per hectare overnight). Then seeds are sown in field.
* **Seedling treatment –** slurry of *Azospirillum* inoculants is prepeared by mixing 1Kg of inoculants with 40 litres of water. The roots of transplanted seedlings are dipped in slurry for 15-20 minutes. Then seedlings are transplanted in the field. The remaining slurry is spread in the field.
* **Top dressing-** carrier- based inoculants is mixed with Farmland manure and soil in the ratio of 3:25:25 (w/w). this mixture is top dressed through out field especially young seedlings of rice.

**Positive effects of foliar application such as plant growth, plant biomass and yield are recorded.**