CHAPTER

19

Azotobacter

Satish V. Patil[1,2], Bhavana V. Mohite[3], Chandrashekhar D. Patil[4], Sunil H. Koli[1], Hemant P. Borase[5], Vikas S. Patil[6]

[1]School of Life Sciences, Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, Maharashtra, India; [2]North Maharashtra Microbial Culture Collection Centre (NMCC), Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, Maharashtra, India; [3]Department of Microbiology, Bajaj College of Science, Wardha, Maharashtra, India; [4]PSL Université Paris: EPHE-UPVD-CNRS, USR 3278 CRIOBE, Université de Perpignan, Perpignan Cedex, France; [5]C. G. Bhakta Institute of Biotechnology, Uka Tarsadia University, Surat, Gujarat, India; [6]University Institute of Chemical Technology, Kavayitri Bahinabai Chaudhari North Maharashtra University, Jalgaon, Maharashtra, India

1. Introduction

Every living organism essentially requires a utilizable source of nitrogen to survive and grow. Utilizable sources of nitrogen exemplified as nitrogen gas and urea. However, the nitrate, nitrites, and ammonia are the most preferable sources by living organisms. Majorly nitrogen is present on earth in gaseous form and (more than 1017 metric tons) out of which about 2% is in free form in the atmosphere. Although such a large quantity of nitrogen is present on the planet, unfortunately, it is not utilized directly by most living organisms.

The abundance of nitrogen in the atmosphere can become usable for living organisms when they are converted into its usable form through the process of biological fixation of nitrogen. This practice is taking place via several routes. The prokaryotes are the major members intricate in the biological way of nitrogen fixation. Prokaryotes fix the nitrogen from the atmosphere by reducing molecular nitrogen into ammonia, which further used for assimilation of amino acids. This process was assumed to provide 200 million tons of nitrogen (N_2) per year (Rascio and Rocca, 2008). The biological nitrogen fixation process is categorized into two types, symbiotic and nonsymbiotic fixation. Nonsymbiotic nitrogen fixation process involves a major genus *Azotobacter*, *Azomonas*, *Beijerinckia*, and *Derxia*.

Beneficial Microbes in Agro-Ecology
https://doi.org/10.1016/B978-0-12-823414-3.00019-8

1.1 *Azotobacter* taxonomy

The perception of family Azotobacteraceae is defined in the Bergey's manual eighth edition as Gram-negative, aerobic heterotrophic bacteria capable of nonsymbiotic nitrogen fixation under normal atmospheric partial oxygen of pressure (Becking, 1974). Based on the numerical taxonomy, Thompson and Skerman (1979) concluded that the family Azotobacteraceae is comprised of "the Gram negative bacterial genera that are non-spore former, free nitrogen fixer and not found similarity with genera of other families."

The foremost distinctive genus *Azotobacter chroococcum* was revealed and described by Beijerinck Martinus in 1901. He designated and labeled the species *Azotobacter chroococcum* as the foremost free-living aerobic nitrogen fixer (Beijerinck, 1901a).

Azotobacter vinelandii was described in 1903 by Lipman and a year after it was named as *Azotobacter beijerinckii* (Lipman, 1904), in the honor of Beijerinck. *Azotobacter nigricans* was identified in 1949, by the Russian microbiologist, Nikolai Krasilnikov (Krassilnikov, 1949). Thompson and Skerman (1981) divided it into two subspecies: *Azotobacter nigricans* subsp. achromogenes and *Azotobacter nigricans* subsp. nigricans. In 1981, Thompson and Skerman proposed *Azotobacter armeniacus* (Thompson and Skerman, 1981). The air-tolerant and microaerophilic type *Azotobacter salinestris* was reported by Page and Shivprasad that was dependent on sodium ions (Page and Shivprasad, 1991).

Azotobacter has its place to the Bacteria kingdom; Proteobacteria phylum; Gamma proteobacteria class; Pseudomonadales order; Azotobacteraceae family and the *Azotobacter* genus. The family Azotobacteraceae covered free nitrogen fixer usually present in soil, water, and sediments (Aquilanti et al., 2004).

Azotobacteraceae is the dominant member of Rhizobacterium family which has its major role in dinitrogen fixation. Nitrogen is the important factor for soil fertility. *Azotobacter* is Gram negative nonendospore forming bacteria; some of the *Azotobacter* species cause the dormant structures known as a cyst, which majorly includes genera of *Azomonas*, *Beijerinckia*, *Derxia*, and *Azotobacter*. The *Azotobacter* shows either motile or nonmotile nature and possesses catalase and oxidase positive nature.

In 1901, Beijerinck, who, studying the Chemolithotrophy, was attracted by nitrogen fixer *Azotobacter* and extensively studied the melanin producing coccus, *Azotobacter chroococcum* and the extensive capsule producing nitrogen fixer *Azotobacter agilis*, which was found in Holland soil.

The Beijerinck isolated the *Azotobacter chroococcum*, the first species of *Azotobacter* from the Holland soil (Beijerinck, 1901b). Thereafter in the succeeding period, numerous other types of *Azotobacter* collection have been isolated from rhizosphere and soil which were categorized under the family Azotobacteraceae, e.g., Lipman (1903a,b, 1904), reported *Azotobacter vinelandii*, *Azotobacter beijerinckii*, respectively, while Krassilnikov (1949) and Dobereiner (1966), isolated and characterized unique *Azotobacter* having specific association with wild grassroots *Paspalum notatum*, i.e., *Azotobacter paspali*. Thompson and Skerman in 1981, reported *Azotobacter armeniacus* and in 1991 *Azotobacter salinestris* by William Page and Shivprasad (1991).

Azotobacter is Gram-negative, blunt to oval short rods with 1.5–2 μm or more in diameter, having soil as common habitat, besides that aquatic plant rhizospheric, and phylospheric were also the identified habitat. They are generally aerobic and capable to fix the atmospheric nitrogen in presence of suitable carbon sources.

The Azotobacteraceae family was characterized into two main genera *Azotobacter* and Azomonas based on various characters. The genus *Azotobacter* includes six major species, characterized by their atmospheric nitrogen fixation capacity. In the environment or nitrogen deprived medium *Azotobacter* is aerobic in nature but also able to grow under low oxygen tension. Major six species of the genus are identified and studied extensively are

1. *Azotobacter chroococcum*
2. *Azotobacter vinelandii*
3. *Azotobacter beijerinckii*
4. *Azotobacter nigricans*
5. *Azotobacter armeniacus*
6. *Azotobacter paspali*

Besides all above characterized species, Page and Shivprasad (1991) isolated a cyst forming, nitrogen fixer from saline soil surface in Alberta, Canada. The organism showed specific additional characters, i.e., the presence of Na^+/succinic acid efflux, and it was named as *Azotobacter salinetris*. The organism showed brown-black pigmented colonies on a Burk medium, the brown-black pigmentation is owing to the water-soluble catechol, melanin production. The organism utilizes galactose, mannitol, glucose, fructose, and melibiose as carbon source without producing acid. The organism uses sodium ion as an electron acceptor.

Out of all species, *Azotobacter chroococcum* is very commonly occurring in soil. The genus *Azotobacter* differentiated from Azomonas based on the microcyst formation. *Azotobacter* form microcyst, a dormant spore-like structure, while Azomonas never formed the microcyst. Besides that, *Azotobacter* always has high GC content, i.e., 63−67.5 mol% than the Azomonas have 52−59 mol%.

The typical genus was allocated to the family Azotobacteraceae (Pribram, 1933), but after the 16S rRNA sequencing study, they were shifted to the family Pseudomonadaceae. The phylogenetic study in 2004 discovered that *A. vinelandii* fits the equal clade as *Pseudomonas aeruginosa* (Rediers et al., 2004) bring about with the concept that the genera *Azotobacter*, *Pseudomonas* and *Azomonas* are correlated and might be alternative (Young and Park, 2007).

The taxonomy controversy resulted in usage of immunological attractions present between the several species of Azotobacteraceae family via Immunoelectrophoresis technique. The *Azotobacter chroococcum* strain was immunologically heterogenous than the *Azotobacter vinelandii* and *Azotobacter paspali* strains (Tchan et al., 1983).

Azotobacter has characterized by utilizing a numerical taxonomy into two different phenotypic groups at a similarity level of 79%−85%. One cluster had a colony of white-beige and the other cluster with colonies of yellow-brown (Zulaika et al., 2014).

1.2 Distribution of *Azotobacter*

They exist in dry deserts, hot steppes, rocky terrain, in dry sands, on foothill summits and in valleys. They are also found in the cold and in an aquatic habitat like the Arctic and Antarctic soils (Garg et al., 2001), thus they present in the soil of diverse topographical areas. The *Azotobacter* population in soil is mostly affected by other soil microbiota. Bagyaraj and Patil (1975) reported the dominant presence of *Azotobacter* in the numerous agronomy crops

rhizosphere for instance sorghum, soybean, ragi, sugarcane, rice, green Gram, and cereals. The *Azotobacter* number was most abundant in black soil compared with red soil and observed reduced in number with an increase in depth while the reduction was additional in black soils (Ramaswamy et al., 1977).

1.3 Habitat and reproduction

The *Azotobacter* can flourish in a nonacidic soil, as well as in the cold climate like the Arctic and Antarctic soil. The cyst forming potential is responsible for resistance to severe environments like drought, solar radiations, ultrasound, UV, and gamma radiation. But the extreme heat is not tolerated by *Azotobacter*.

Azotobacter reproduces by simple division (fission), like all bacteria, and respires aerobically with the generation of energy.

When they form cysts, the cysts cannot reproduce, they survive the adverse conditions, and then when the optimal conditions are achieved again, the cysts germinate and form vegetative cells, which then reproduce via simple cell division.

1.4 Molecular characterization

The *Azotobacter* genus is suitable for the Proteobacteria subclass (Becking, 2006; Tchan, 1984) and includes seven species: *A. chroococcum*, *A. vinelandii*, *A. beijerinckii*, *A. nigricans*, *A. armeniacus*, and *A. salinestris*. *A. paspali*, a novel endemic species, was screened from Thailand based on molecular techniques in 2005 (unpublished data). *Azotobacter* is the aerobic-free nitrogen fixer with the heterotrophic mode of nutrition having 63%−67.5% G + C content (Becking, 2006; Setubal et al., 2009), and found presence in the soil, aquatic environment as well as sediments (Tejera et al., 2005; Torres-Rubio et al., 2000). *Azotobacter* contains the highest amount of DNA than the other bacteria, the circular DNA molecule of *Azotobacter* comprises 5,342,073 nucleotide pairs and 4988 genes out of 5043 encode proteins. The Nif genes are the major complex genes. The genome is typical of utmost prokaryotes. The above average amount of DNA is might be due to larger cells of *Azotobacter* than those of other bacteria (Aquilanti et al., 2004).

The *Azotobacter* has 52−67.5 mol% GC content. The DNA content and the number of chromosomes in the cells increase upon aging. *Azotobacter* contains more than 100 copies of a chromosome per cell in old cultures.

The *Azotobacter chroococcum* NCIMB 8003 (ATCC 4412) (Ac-8003) genome, contains 5,192,291 bp making seven circular replicons. The species of *Azotobacter* contains 1.5 g × 10−13 g of DNA, which is approximately 40 times more than that of *E. coli*. It also has presence of six plasmids (Phadnis et al., 1988). The *Azotobacter* sp. DNA shares several similar properties to *Escherichia coli* genome, as gene type and recognition factors. Nif gene studies of *Azotobacter*, nitrogenase holoenzyme of *Azotobacter vinelandii* indicate that the enzyme active site holds the molybdenum iron-sulfide cluster cofactors (FeMoCo), each carrying two pseudocubic iron-sulfido structures. The *Azotobacter* chromosome has 66.27% G + C content. The metabolic pathways and macromolecular designs of this organism appear well-preserved with genes meant for CO-dehydrogenase, formate dehydrogenase, and a soluble NiFe-hydrogenase (Robson et al., 2015).

NifH gene is the source of taxonomic identification of *Azotobacter* for analysis of their nitrogen fixing genetic potential (Zehr et al., 1995). These NifH genes are also valuable as the markers for the finding and recognition of the genetic diversity of the *Azotobacter* residing in the soil (Ueda et al., 1995; Widmer et al., 1999).

The *A. vinelandii* DJ genome sequence has a 5,365,318 bp single circular genome. *A. vinelandii* has a complement of respiratory proteins for oxygen-sensitive processes. It produces the alginate that guards the organism against spare exogenous oxygen. In accordance with the oxygen availability, the alginate conformation may change by several repetitions of alginate modification genes. The investigation of the genome recognized the genes coding for the three-known oxygen-sensitive nitrogenases and more for oxygen-sensitive enzymes, such as formate dehydrogenase and carbon monoxide dehydrogenase. In this way, *A. vinelandii* could work as a mediator for the formation and elucidation of oxygen-susceptible proteins (Setubal et al., 2009).

The varying strain of *Azotobacter* like *A. vinelandii* strain CA (or OP) (ATCC 13,705, accession no. CP005094) was a nongummy, pigment fabricating, native strain (Bush and Wilson, 1959). *A. vinelandii* strain CA6 (accession no. CP005095) is a mutant strain resulted from strain CA by spontaneous alterations. It was reported that tungstate stops growth and nitrogen fixation by strain CA nevertheless it could inhibit CA6 (Bishop et al., 1980). Similarly, strain CA6 also diminished molybdate uptake (Premakumar et al., 1996) but produce a vast amount of hydrogen gas during nitrogen fixation.

1.5 Nutritional requirement

The *Azotobacter* sp. has metabolic aptitudes of atmospheric nitrogen fixation by conversion to ammonia. The three discrete nitrogenase enzymes make these bacteria of interest for studying the nitrogen fixation and its role in agriculture. The *Azotobacter* sp. has the highest metabolic rate (Jensen, 1954).

Azotobacter is a mixotrophic bacteria showing autotrophy or heterotrophic mode of nutrition by making its own food (by sunlight or by chemical reaction [autotropic] or by getting food from other sources, respectively). *Azotobacter* often grows green/brown, slimy, and around 7 mm diameter colonies. The fixation of nitrogen requires source of carbon, which could be fulfilled by carbohydrates and sugars, as per, 1 g of glucose is needed in order to fix 10 g of nitrogen.

1.6 The general characteristic of *Azotobacter*

The *Azotobacter* has specific physiological and morphological characteristics which primarily differentiate it from the other Gram negative and nitrogen fixers (Table 19.1). *Azotobacter* species occur from a range of soil habitat, i.e., slightly acidic to alkaline soil and some species like *Azotobacter paspali* are associated with plant root. But generally, *Azotobacter* species population occurs abundantly in fertile soil; this is due to the *Azotobacter* requirement for high minerals like phosphates.

The *Azotobacter* species are chemoheterotrophic, nitrogen fixers, and motile, other than *Azotobacter beijerinckii* and *Azotobacter nigricans* by peritrichous and polar flagella. They generally produce diffusible and nondiffusible big colonies on the nitrogen-free

TABLE 19.1 Primary morphological characters on Burks medium.

sr. No.	*Azotobacter* species	Gram nature	Cell shape	Cell size (L × W) μm	Pigment production	Motility
1.	*A. vinelandii*	−ve	Round-ended rods	3.0−4.5 × 1.5−2.4	Yellow-green, fluorescent, water-soluble pigment	+ve
2.	*A. beijerinckii*	−ve	Rods or ellipsoidal	3.2−5.3 × 1.7−2.7	Yellowish or cinnamon pigment	−ve
3.	*A. chroococcum*	−ve	Rod-, oval-ovoid-, or coccus	3.0−7.0 × 1.5−2.3	Brown or blackish-brown	+ve
4.	*A. paspali*	−ve	Long filaments	7−12 × 1.3−1.7	Yellow-green, fluorescent or red-violet, water-soluble pigment	+ve
5.	*A. armeniacus*	−ve	Bluntly rounded rods	5.0−5.7 × 1.7−2.0	Diffusible brown-black or red-violet	+ve
6.	*A. nigricans*	−ve	Bluntly rounded rods	4.1−4.9 × 1.5−2.7	Yellow nondiffusible pigment	−ve
7.	*A. salinetris*	−ve	Rods	2−4 × 4.5−5.0	Black brown	+ve

medium containing sugar or alcohol as carbon sources. The colonies are generally smooth, opaque, somewhat convex glistening, though the nature of colony changes accordingly to medium and type of carbon sources used (Thompson and Skerman, 1979), e.g., the colonies with more big size, more transparent and viscous colonies appeared on media containing sucrose and raffinose than the nitrogen-free media with glucose. The species are also characterized by the production of gray-brown, black nondiffusible pigments. The pigment production is also found to be media component dependent, e.g., *Azotobacter chroococcum* produces nondiffusible brown-gray pigment. The pigments production is also found to be the media component dependent. e.g. *Azotobacter chroococcum* produces non diffusible brown-grey pigment on Stainers medium (Pribram, 1933) fortified with the 0.2% gluconate and black diffusible pigment on benzoate fortified medium. But *Azotobacter nigricans* and *Azotobacter armeniacus* not produces brown diffusible pigment in presence of benzoate, whereas *Azotobacter vinelandii* produces brown-black pigment.

Azotobacter is chemoheterotrophic utilizing sugars such as glucose, fructose, ethanol, acetate, carbinol fumarate, pyruvate, and other organic acids as a carbon source. It is also able to use various nitrogen compounds but poorly or unable to use nitrate. *Azotobacter* does not require organic growth factors but requires only minerals, like vanadium and molybdenum, which is an essential component of the nitrogen fixation system.

The combined nitrogen-free medium with suitable carbon source is the preferable condition for the growth of *Azotobacter*. Although the organism is catalase positive and aerobic in nature the reduce or low oxygen tension condition is required for better nitrogen fixation, because dinitrogen fixation is categorized as a reductive process as well as the involvement of the major oxygen labile enzymes, which is get inactivated in presence of oxygen.

The optimum temperature of growth for most of the members of *Azotobacter* is 28−37°C, but another cardinal temperature varies as per the species, e.g., some species of *Azotobacter* require minimum temperature for growth as 14°C, while *A. beijerinckii* and *A. nigricans* have

the minimum temperature requirement is of 9°C and *A. armeniacus* required 28°C as minimum growth temperature. Although optimal temperature meant for most of the *Azotobacter* is 32°C, *A. paspali* and *A. vinelandii* have optimum temperature is 37°C. The optimum temperature also found to vary as per strains, for. e.g., some strains of *A. chroococcum* have optimum temperature is 37°C. The temperature tolerance also found to be varying accordingly strain isolated from subtropical and temperature region. It was repairer that all *Azotobacter* survives at 50°C for to 10 min but not any species able to survive within 10 min at 60°C treatment or incubation. Similarly, *Azotobacter nigricans* and *Azotobacter armeniacus* are unable to grow at 37°C. The growth of *Azotobacter* has observed from pH varies from acidic to alkaline i.e., 4.8–8.5.

1.7 *Azotobacter* phages

As with the other bacteria, the *Azotobacter* species is also reported to be susceptible to bacteriophages. De Jong (1938) given the first idea for presence of bacteriophages for Azotobacter sp. as like with other bacteria. Den Jong observed lysis of Azotobacter cells due to phage infection, although he has not proved it by isolation and characterization of azophage from lysed cultures. But his observation was assumed as the first report on the presence of *Azotobacter* phages. After that, Monsour et al. (1955) isolated Azophages from the soil which have capable of forms plaques on the green pigmented strains of *A. vinelandii*. Hezagi and Jensen (1973), reported that phages showed more lysis of *A. vinelandii* and *A. chroococcum* than *A. beijerinckii*, while other *Azotobacter* were not reported for phage infection.

2. Isolation of the genus

The design of medium for the isolation of *Azotobacter* sp. is based on its basic nature chemoheterotrophy and dinitrogen fixation. Various methods were reported for isolation of *Azotobacter* sp. such as soil paste plate method, and silica gel method. One of the oldest methods, in which soil paste is made and fortified with calcium carbonate, potassium phosphate, and carbon sources like glucose, sucrose, mannitol, and was set in watch glass or gypsum block and kept on a Petri plate containing filter paper and allowed to incubate at 27–30°C for three to up to seven days. After incubation, the slimy colony were raised similarly, and a sieve plate could also be used. The silica gel was fortified with a suitable carbon source on nutrient isolation, and the plates were impregnated or seeded with sieved soil and allowed to incubate at 27–30°C for 48–72 h. The colonies of *Azotobacter* were found to grow around soil particles on silica gel (Becking, 2006).

But currently above methods of isolation are not in use, current isolation methods included use of different nitrogen free solid or agar medium includes Winogradsky (1938) nitrogen-free media, Burk medium (Wilson and Knight, 1952), Ashby's medium (Ashby, 1907), Norris medium (Norris, 1959), and LG medium (Lipman, 1904). These all medium are a general medium for isolation of *Azotobacter* sp. The media composed of all these six methods is somewhat similar vary with only in some carbon sources and percentage of minerals and micro and macronutrient (Table 19.2).

TABLE 19.2 Common nitrogen-free medium for isolation of *Azotobacter*.

Medium				
Burk's N_2-free medium (g/L) (Wilson and Knight, 1952)	**Sergei Winogradsky N_2 free medium(g/L) (Winogradsky, 1938)**	**LG medium (g/L) (Lipman, 1904)**	**Ashby's medium (g/L) (Ashby, 1907).**	**Norris medium(g/L) (Norris, 1959)**
$MgSO_4$ 0.20, K_2HPO_4 0.80, KH_2PO_4 0.20, $CaSO_4$ 0.13, $FeCl_3$ 0.00145, sodium molybdate 0.000253, sucrose 20.00	KH_2PO_4 50.00, $MgSO_4 \cdot 7H_2O$, 25.00, NaCl, 25.00, $FeSO_4 \cdot 7H_2O$ 1.00, $Na_2MOO_4 \cdot 2H_2O$, 1.00, $MnSO_4 \cdot 4H_2O$ 1.00, pH 7.2	Sucrose 5.00, K_2HPO_4, 0.20, KH_2PO_4, 0.60, $MgSO_4 \cdot 7H_2O$ 0.20, $CaCl_2 \cdot 2H_2O$ 0.02, $Na_2MoO_4 \cdot 2H_2O$ 0.002, bromothymol blue (5 g/L in 0.2 N KOH), 5 mL FeEDTA (solution 16.4 g/L), 4 mL vitamin solution, 1 mL. DW: 1000 mL with DW. pH 6.0 to 6.2	Mannitol 20.00, K_2HPO_4 0.200, $MgSO_4$ 0.200, NaCl 0.200, K_2SO_4 0.100, $CaCO_3$ 5.000, agar 15.000 final pH 7.0	Glucose 10.00, K_2HPO_4 1.00, $MgSO_4$ 0.20, $CaCO_3$ 1.00, NaCl 0.20, sodium molybdate 0.005, $FeSO_4$ 0.10, pH 7.0

*The pH was adjusted and autoclaved at 121 °C for 15 min. $Na_2MoO_4 \cdot 2H_2O$ and $FeSO_4 \cdot 7H_2O$ were filtered and sterilized prior to adding into the autoclaved medium

Nevertheless, none of these described media was the perfect medium to isolate specific *Azotobacter* species, although the use of specific enrichment medium help to isolate or enrich specific *Azotobacter* sp., like all above medium, are applicable for getting pure isolated colonies. For further confirmation of *Azotobacter* species, various biochemical and morphological studies are required.

2.1 Preservation of *Azotobacter*

Among the various methods such as cryopreservation, lyophilization, and immobilization, the simple method of preservations of *Azotobacter* is on nitrogen-free agar medium with sucrose and glucose as carbon sources. This method was found significant, which keep the organism viable for 1−10 years; the only essentially required care is that agar should not be dry. The modern method of preservation like lyophilization was found to be nonsignificant for *Azotobacter* as compared to simple preservation method (Antheunisse, 1973; Lapage et al., 1970). Becking (1961) also found the preservation of nitrogen-free agar medium with cotton paraffin seal at room temperature (RT) or at 4°C showed significant viability of *Azotobacter* up to 3−5 years. Besides these, Thompson (1987) reported successful preservation or maintenance of *Azotobacter* up to 10 years by cryopreservation in liquid nitrogen, but there are some reports of damage of membranes and loss of viability during cryopreservation.

In our laboratory at KBCNMU, Jalgaon, it was observed that the cheapest method of *Azotobacter* preservation is with dry sterile soil. The five-day biomass in cyst induced medium (1% Butanol), was aseptically mixed with dry soil and maintained at RT resulted in maintenance of viable *Azotobacter* for five to six years; similar preservation was also advocated by Vela (1974).

2.2 Some practical methods of preservation

2.2.1 Dry soil preservation

Inoculate pure *Azotobacter* culture in sucrose (1%) and 0.5% butanol containing a medium, incubate at 120 rpm at 30°C for 4–5 days. After incubation, recover biomass aseptically by centrifugation at 10,000 rpm for 10 min. Mix the biomass with dried sterile black cotton soil, seal the bottles with cotton, and preserve at RT.

2.2.2 Agar slope preservation

The simplest procedure among the reported process includes streaking on nitrogen-free medium slant with a pure culture of *Azotobacter*, allow to incubate 30°C for 48 h. Then sealed with molted paraffin, allow cooling for proper sealing and preserving the slant at 4°C.

2.2.3 Immobilization with polymers

The polymer sterile solution (1.0%–1.5%) like polymerlike alginate, Gum Acacia, Carrageenan, and Plyox could be mixed with *Azotobacter* cells, dried aseptically and preserved at 15°C with 40 ± 2% relative humidity for maintaining 80% viability up to 60 days (Rojas-Tapias et al., 2013).

3. Identification of the genus

The organism isolated from the nonspecific enrichment culture only gives the idea that the isolated organism is a free nitrogen fixer, but does not give a confirmed idea that an isolated one is a member or species of *Azotobacter*. Primary confirmatory tests for genus *Azotobacter*, done by the principal morphological test, was the cyst formation potential of the isolate. The cyst formation is the only differentiating simple laboratory test which differentiae *Azotobacter* from other nitrogen fixing organism (Table 19.3).

TABLE 3 Major differentiating characteristic i.e. Microcyst formation of *Azotobacter* from another genus

Organism	Microcyst formation
Azotobacter	Positive
Azomonas	Negative
Beijerinckia	Negative
Derxia	Negative
Azospirullum	Negative
Rhizobium	Negative
Klebsiella	Negative
Pseudomonas	Negative
Azotomonas	Negative

3.1 Cyst formation

Azotobacteraceae cells generally show the vegetative growth of a large, plump and rod-to-oval-shaped cell morphology. But during the life cycle, especially in trace conditions, the vegetative cells give rise to bacteria, a spore-like specialized spherical dormant cell known as a cyst. It has very specific morphology appearing as like spherical cell with an outermost rough layer known as exine; inner to that is a homogeneous thin layer known as in tine, which covers the central body containing nuclear material and globules. The cyst formation process is known encystment process.

Jensen (1954) showed that cyst formation is one of the prime criteria for taxonomic identification of *Azotobacter*. Because the other free nitrogen fixers like *Azotominas*, *Azomonas*, *Derxia*, etc. does not show cyst formation potential. Winogradsky in (1938) reported the induction of cyst formation by specific compounds like ethanol and butanol as a carbon source.

3.1.1 Cysts: a unique character of differentiating Azotobacter sp. from other free nitrogen fixers

Batchinskaya (1935) described a very specialized, spherical form of a cell of Azotobacteraceae. These structures are morphologically very different from the normal vegetative cells. These are nothing but the special form of cell "cyst." Those are with contractile and highly vacuolated structures in the cytoplasm. The central body of these cells covered with a thick capsule-like layer, which has been covered by another thin inner layer. Winogradsky (1938) reported the cyst formation could be induced by fortifying some special chemicals like ethanol, butanol in the nitrogen-free medium. Socolofsky and W'yss (1961), Tchan and New (1984) also reported that the use of 0.3% n-butanol induces the cyst formation in *Azotobacter* after 5–7 days incubation. Lin and Sadoff (1968) reported that in *Azotobacter vinelandii*, encystment was induced by Burk's nitrogen-free liquid media added with β-hydroxybutyrate or n-butyl alcohol or crotonate. Nevertheless, butyrate and butyraldehyde do not encourage the encystment. They also observed encystment rate was increased in absence of glucose, β3-hydroxybutyrate, and in the presence of glucose, cells produce abortive encystment with disorganized exine, releasing viscous material. Layne and Johnson (1964) also reported that the induction of cyst by altering sucrose concentration in Burks medium. They reported induction of 80% cyst formation process after reducing 0.05% sucrose in Burks medium.

Various reports proved that as compared to normal cells of *Azotobacter*, these cysts were resistant to various chemical and physical agents like UV rays and stains (Socolofsky and Wyss, 1961.)

3.1.2 Cyst induction and cyst staining

The confirmation of *Azotobacter* species from other free nitrogen fixers, the isolated pure colonies of organism on nitrogen-free medium is streaked on the special cyst-inducing medium i.e., modified Burks medium containing 0.3% n-butanol or 3% ethanol as a carbon source. For preparations, after autoclaving of the Burk medium, the plates were poured and after solidification, 0.3% n-butanol or 3% ethanol was poured on it and allows diffusing it 2–3 h in freeze at 4°C. Then isolated culture of nitrogen fixer should be streaked, and incubated for 5–7 days at 33°C. The comparative vegetative growth was attained on sucrose containing Burk's medium. The sucrose media suppress the cyst formation. After 5–7 days,

the growth on n-butanol and ethanol medium should be morphologically observed by simple staining and like the bacterial spore; the large size spherical, uniform shape structure cyst was observed. Further confirmation is done by comparison with vegetative cells and cyst staining.

3.1.3 *Cyst formation; confirmation by plate assay and staining*

The cyst formation also confirmed by simple plate assay in which the organism streaked on Butanol Burk and control Burk medium and incubated for 5–6 days at 30°C (Daniel et al., 2009). After 5 days of incubation, both the plates were sprayed with cyst identification reagent i.e., a solution containing 0.5% fast blue in 5.0% acetic acid. The colonies on Butanol Burk medium show in red because of reaction of fast blue with alkylresorcinol present in cyst layer while colonies on Burk medium, i.e., vegetative cells, do not show any color change.

The simple staining method like Vela and Wyss method (1965) was reported for cyst staining. This includes the following steps: the cyst suspension from Butanol Burk medium was prepared and used for state. A loopful of culture take on a clean glass slide and add few drops of Vela and Wyss reagent (glacial acetic acid, 8.5 mL; Na_2SO_4, 3.25 g; neutral red, 200 mg; light green SF yellowish, 200 mg; ethanol, 50 mL; distilled water, 100 mL; mixed well), kept for 4–5 min, remove the excess stain by blotting paper and observe wet mount under 40x. The vegetative cell appeared green in color. The cells started encystment appeared light yellowish-green. As encystment proceeds, further, the cytoplasm condenses and appears a deeper green. The matured cyst showed a thick and compacted brownish-red exine and clear unstained area of the intine, which covers the deep green stained distinct central body.

These methods of fast blue staining also very significantly differentiate vegetative cells and cysts of Azotobacter. Fast blue-B staining of cysts is based on the principle that fast blue stains the alkylresorcinol lipids, which essentially present in the layers of cysts. The staining is performed with the Azotobacter cells which grown for 5 days in the cyst inducing/butanol containing Burk medium, and loopful of samples of the culture were placed clean glass slide heat fix it and overlay with the with a solution of 0.5% Fast Blue B in 5% acetic acid for 10 min, remove the excess stain by blotting paper and observe wet mount under 40x or air dry it and observe under oil immersion objective. Cyst will appear with the reddish layers with greenish blue central body of cyst. while the vegetative cells will not retain stains and appear with red blue layers. Carbol Fuchsin (Ziehl-Neelsen) staining is also employed for visualisation of cyst.

The cyst formation is also confirmed by UV radiation test and desiccation test. Because, the *Azotobacter* cysts were found to be comparatively more resistant than the vegetative cells to various deleterious agents and environment i.e., ultraviolet irradiation, desiccation, and sonication. It was reported that cyst required twice UV doses than vegetative cells for 90% inactivation. Similarly, cysts are comparatively high resistance to desiccation than the vegetative cells. Although cyst is shown resemblance to the bacterial endospore structure and also extremely resistant to gamma radiation, sonic treatment, and desiccation, it does not comparable for bacterial heat resistance capacity.

3.2 Confirmatory test for identification of *Azotobacter* sp.

Once the cyst formation ability of organism grown on nitrogen-free media was proved, and then it was assumed that the isolated bacteria growing on any nitrogen-free medium

like Burks, Ashbys, Norris etc. is the member of genus *Azotobacter*. Further, the exact species level identification of the *Azotobacter* can be done by various morphological and biochemical tests. These tests include utilization of specific carbon sources, production of diffusible, nondiffusible pigments, tolerance or sensitivity to specific chemical compounds as the basis of various such tests, the specific species of *Azotobacter* were identified. Such differentiating, morphology and biochemical test for different species is as shown in Table 19.4.

3.2.1 *Azotobacter chroococcum*

A. chroococcum is abundantly occurring in the soil. The major morphological structure is cell appear in coccus form. They also show blunt oval avoids cell with 3.0–7.0 μm length × 1.5–2.3 μm width. Cells remain motile up to 24–48 h only. The major characteristic of *A. chroococcum* is after aging on Ashby's or Burk medium, it produces yellow-brown nonwater-soluble pigment. Beijerinck (1901b) developed an enrichment method for *A. chroococcum* known as nutrient solution method which followed by purification on the solid medium for *A. chroococcum* (Table 19.5).

The solid medium becomes differential because it contains $CaCO_3$ which act buffering agent and maintain pH-7.4–7.5 because this is a favorable condition for growth *A. chroococcum*. Becking (1961) and Jensen (1965) reported that the soil has pH 7.5 and abundantly contains *A. chroococcum*. It was observed that soil with pH range 7.0–7.4 has 89%, 6.5–6.9 contain 57%, and 6.0–6.4 have 42% *Azotobacter* population (Jensen, 1965). Similarly, Jensen and Petersen (1955) advocated that lower pH of nitrogen-free medium is the growth limit factor for *A. chroococcum*.

For the biochemical identification of *A. chroococcum*, caproate and caprylate utilization test is a very important test (Table 19.6). Caproate is nothing but the ethyl hexanoate also known as hydroxyl progesterone caproate. It is ester obtained by condensing hexanoic acid and ethanol. It is also present in fruits, flower gives aromas. Among the all *Azotobacter* species only *A. chroococcum* and *A. vinelandii* were able to utilize this ethyl hexanol as carbon or energy sources. Hence, *Azotobacter* isolate was caproate positive. It indicates that organism may be only *A. chroococcum* or *A. vinelandii*. The confirmation was done by caproate and

TABLE 19.4 Compounds/conditions used in a selective medium.

Sr. No.	Name of organism	Biochemical test
1.	*Azotobacter vinelandii*	Rhamnose, erythritol, butanol, ethylene glycol 10% sodium benzoate, 0.1% phenol
2.	*Azotobacter beijerinckii*	Tartarate, α hydroxyl benzoate, D-galaturonate, pH 4.9–5.5
3.	*Azotobacter chroococcum*	pH 7.0–7.5
4.	*Azotobacter paspali*	0.5% bromothymol blue in sucrose medium/Sample from rhizosphere of *Paspalum notatum*
5.	*Azotobacter armeniacus*	No specific addition of caprylale in Burks medium
6.	*Azotobacter nigricans*	Citrate, n- valerate
7.	*Azotobacter salinetris*	Burk medium fortified with sodium salt 1.0%–2.0%

TABLE 19.5 Enrichment method for *Azotobacter chroococcum*.

Compound	g/L
Glucose	20.0
K_2HPO_4	0.8
$MgSO_4$	0.5
KH_2PO_4	0.2
$FeCl_3 \cdot 6H_2O$	0.005
$CaCl_2 \cdot 2H_2O$	0.005
Agar	15
DW	1000 mL
Or	
$CaCO_3$	20.0
$NaMoO_4 \cdot 2H_2O$	0.05
pH	7.4–7.6

TABLE 19.6 Differentiation characteristic of *Azotobacter vinelandii* and *Azotobacter chroococcum*.

Character	*Azotobacter vinelandii*	*Azotobacter chroococcum*
Rhamnose	Positive	Negative
Caproate	Positive	Positive
Caprylate	Positive	Negative
Malonate	Positive	Detectable
Mesoinositol	Positive	Negative
Cysten	Negative	Detectable
Glutarate	Positive	Negative
Glycolate	Negative	Positive

caprylic acid/utilization tests, which differentiate *Azotobacter chroococcum* from *A. vinelandii* because of *Azotobacter chroococcum* unable to utilize caprylate as carbon sources (Table 19.2). Besides that, a glycolate utilization test was found to be positive by *A. chroococcum* and negative for *A. vinelandii*. Besides these various tests like malonate, myoinositol, and Rhamnose utilization test confirm the presence of *A. chroococcum*.

3.2.2 *Azotobacter vinelandii*

It was isolated by Lipman (1903a,b) and recognized as *Azotobacter miscellum* by Cohen and Johnstone in (1964). The organism was first isolated from Vineland, New Jersey and so

recognized as *Azotobacter vinelandii* organism showed oval to short rod cell, motile with peritrichous flagella. Cells also show motility up to 24–48 h as with *Azotobacter chroococcum*. The cells show dimension 3.0–4.5 μm long × 1.5–2.4 μm width. The *A. vinelandii* shows the specific character of yellow-green fluorescent and water-soluble pigment production. Becking (2006) reported *A. vinelandii* from alkaline soil with pH 8.0–9.5 and rich in sodium chloride, alkaline sea muds, calcareous soil of Indonesia, and various localities of Boliva and South America (Becking, 1961).

Reuszer (1939) observed that addition of benzoate acid, phenolic compound, and benzoate in soil surprisingly replaces the normal *A. chroococcum* flora to *A. vinelandii*.

Reuszer (1939) mention the organism characteristically produced green pigment, on the basis of this observation, Derx (1951) designed special enriched medium for isolation of *A. vinelandii* (Table 19.7).

The Derx medium preparation with a carbon source, ethanol was used for precise isolation of *A. vinelandii* (Table 19.7). Ethanol (10 mL) should be added after autoclaving and at 40°C added in flask just before pouring plate. Besides that, Jensen (1961) also designs the Rhamnose agar medium for isolation of *A. vinelandii*. The medium is based on the principle that other *Azotobacter* sp. except for *Azotobacter vinelandii* generally unable to utilize Rhamnose as a carbon source (Thompson and Skerman, 1979). Although there are very few other strains of *Azotobacter* sp. variants either reported for Rhamnose utilization or nonutilization (Thompson and Skerman, 1979; Claus and Hempel, 1970).

Besides all of these biochemical characterizations, there are various chemical compounds selectively used by *Azotobacter vinelandii*, and hence used for its characterizations such as resorcin, ethylene glycol, and glutarate utilization test. Hence the addition of 0.1% or 0.2% of the above compound in nitrogen-free medium selectively allows *A. vinelandii* isolation from soil and other sources.

Thompson and Skerman (1979) also observed the *Azotobacter vinelandii* exceptionally uses the caproate, caprylate, and mesoinositol as a carbon source. Besides that, they also observed 0.1% phenol in nitrogen-free medium. The medium become a selective medium for isolation of *Azotobacter vinelandii.*

Further, Thompson and Skerman identified selective carbon sources like caproate (C6), caprylate (C8), short chain fatty acids, and among which mesoinositol utilization test become

TABLE 19.7 Derx medium for selective screening of *Azotobacter vinelandii*.

Composition	g/L
Mannitol	5.0
Or ethanol	10 mL
K_2HPO_4	5.0
Sodium benzoate	10.0
pH	7.5–8.0
DW	1000

an important test to differentiate the *Azotobacter vinelandii* from *Azotobacter chroococcum.* Because among all species of *Azotobacter*, *Azotobacter chroococcum* and *Azotobacter vinelandii* only utilize caproate, but for further differentiation caprylate or caprylic acid is one most important biochemical test.

Hexanoate is straight-chain saturated fatty acid anion and which is the conjugate base of hexanoic acid or caproic acid. It has also found as a human metabolite and a plant metabolite (Fig. 19.1).

Caprylic acid is the saturated fatty acid made up of octanoic eight-carbon. It is apparently present in the mammal milk, in slight amount as a component of coconut oil, palm kernel oil and even formed during yeast fermentation. It gives unpleasant smell. The caprylic acid or caproate only utilized by *Azotobacter vinelandii* and not by *Azotobacter chroococcum*.

Similarly, the mesoinositol is also utilized by only *Azotobacter vinelandii* not used by *Azotobacter chroococcum*.

3.2.3 *Azotobacter beijerinckii*

Azotobacter beijerinckii shows morphological similarities with *Azotobacter chroococcum*. Lipman (1904) isolated *Azotobacter beijerinckii* and named subspecies acid tolerance. *Azotobacter beijerinckii* although not viewed as discrete species and assumed as per a pigment deficient strain of *Azotobacter chroococcum* in the Bergey's manual edition restored species level (Buchanan and Gibbons, 1974). Then, on the basis of nonmotile nature of *Azotobacter beijerinckii*, it was clearly differentiated from *Azotobacter chroococcum*. After aging, it produces yellowish and cinnamon pigment while *Azotobacter chroococcum* produces blackish-brown pigment. *Azotobacter beijerinckii* is only species among *Azotobacter*, which is nonmotile and can use malonate, propionate benzoate, and D-Galacturonate as a carbon source. These are the important biochemical tests to identify the *Azotobacter beijerinckii*, from morphologically resemble species i.e., *A. chroococcum.* Similarly, the strains are exceptionally recognized as amylase positive, hence utilizes starch as a carbon source. Jensen and Petersen (1955) designed a selective medium for *A. beijerinckii which* have some composition as like nitrogen-free medium but instead of $CaCO_3$ essentially use $CaCl_2$ media component and pH of medium maintain slightly acidic i.e., 4.9−5.5. Jensen and Peterson medium for *A. beijerinckii* is based on their previous finding that all *A. beijerinckii* strains grow and fix nitrogen at pH 5.1, although they also reported that at alkaline pH more atmospheric

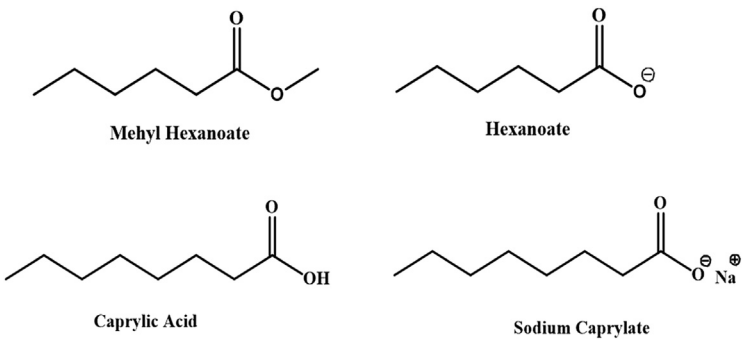

FIGURE 19.1 Structure of unusual carbon sources for *Azotobacter* differentiation.

TABLE 19.8 Differentiation characteristic: *Azotobacter beijerinckii* and *Azotobacter nigricans*.

Test	*Azotobacter beijerinckii*	*Azotobacter nigricans*
Motility	Negative	Negative
Black, red, violet pigments	Negative	Positive
Malonate	Positive	Detectable
Propionate	Positive	Negative
Benzoate	Positive	Negative
Glycerol	Detectable	Negative
D-galacturonate	Positive	Negative
Glucuronate	Positive	Negative
Glutarate	Negative	Negative
Citrate	Positive	Negative

nitrogen was fixed by *A. beijerinckii* (Jensen and Petersen, 1955). All strain of *A. beijerinckii* exceptionally produces urease enzyme; hence utilize urea as a nitrogen source (Table 19.8).

3.2.4 *Azotobacter nigricans*

A. nigricans was originally detected by Krasilnikov from USSR in 1949. The strain shows similarity with A. beijerinckii e.g., both is the only nonmotile species of *Azotobacter*. Cells are blunt, rounded rods with a dimension of 4.1−4.9 μm length × 1.5−2.7 μm width. The strain is characterized by various biochemical aspects like unable to utilize malonate, ethanol, pentanol, benzoate etc. To differentiate from *Azotobacter beijerinckii* benzoate, propionate, malonate, and galacturonic utilization test are the important differentiating biochemical test because *A. beijerinckii* shows these entire tests positive and *A. nigricans* gives it totally negative. The confirmation is also possible by testing the glutarate utilization (Table 19.8).

3.2.5 *Azotobacter paspali*

This species originally isolated by Winogradsky described a method of isolation of *Azotobacter* sp. i.e., silica gel plate containing a mineral solution and the sole carbon source as calcium citrate. It was a character used for identification, as younger filamentous long rods cells with yellowish-green fluorescent or red-violet water-soluble pigment colonies. In 1966 Döbereiner studied it thoroughly by growing it on N_2 free Lipman (1903a,b) and modified medium with sucrose as a sole carbon source and bromothymol blue as an indicator (Table 19.9).

Organic acid production is one of the unique characteristics of *A. paspali*, the sucrose minerals medium contains a pH indicator bromothymol blue, which differentiate colony by yellow color on a blue background after 3−4 days (Table 19.10). Similarly, the large filamentous structure is another characteristic of *A. paspali* which shows 1.12 μm length and 1.3−1.7 μm in width. A. paspali is the only *Azotobacter* species which had the specific rhizospheric association. Döbereiner (1970) found that *A. paspali* have specifically for a wild grass *Paspalum notatum* and few other *Paspalum* sp. i.e., *P. virgatum*, *P. plicatulum* etc.

TABLE 19.9 Döbereiner sucrose mineral medium.

Component	g/L
Sucrose	20.0
K_2HPO_4	0.05
KH_2PO_4	0.15
$MgSO_4 \cdot 7H_2O$	0.20
$CaCl_2$	0.02
$CaCo_3$	1.0
$N_2MoO_4 \cdot 2H_2O$	0.02
$FeCl_3$ (10% solution)	1 drop
Agar	20
DW	1000 mL
pH	7.0
Bromothymol (0.5% ethanol)	10 mL

TABLE 19.10 Identification test for *Azotobacter armeniacus* and *Azotobacter paspali*.

Test/characters	*Azotobacter armeniacus*	*Azotobacter paspali*
Large filamentous cells in young cultures	Negative	Positive
Peroxidase	Detectable	Negative
H_2S production from thiosulphate	Negative	Positive
Cysteine	Detectable	Negative
Propionate	Detectable	Negative
Glycolate	Positive	Negative
Malonate	Positive	Negative
Growth at 14°C	Negative	Positive
Peroxidase	Positive	Negative
Nitrate to nitrite	Negative	Negative

3.2.6 *Azotobacter armeniacus*

This *Azotobacter* species was first isolated from US Armenia in 1964, fully described by Thompson and Skerman (1981). The organism was majorly differentiated by its motile cells, production of brown-black and reddish violet pigment. They also have the ability of esterase

production and citrate utilization as a carbon source. Also utilizes n-valerate and caprylate. A. armeniacus also deferentially characterized by peroxidase production, glycolate and malonate utilization from other *Azotobacter* like *Azotobacter paspali.*

The identification of important *Azotobacter* sp. could be outlined based on simple morphological and biochemical characteristics as shown in Fig. 19.2.

4. Beneficial role of the *Azotobacter* in agroecology

Azotobacter has played an important role in sustainable agriculture as plant growth promoting properties as well as biocontrol agent production against phytopathogen (Fig. 19.3).

4.1 Mechanism of crop productivity benefit

Azotobacter is the genus of interest for reviewing nitrogen fixation and effect on plant owing to its fast advancement and efficiency of dinitrogen fixation. The bacteria fix the

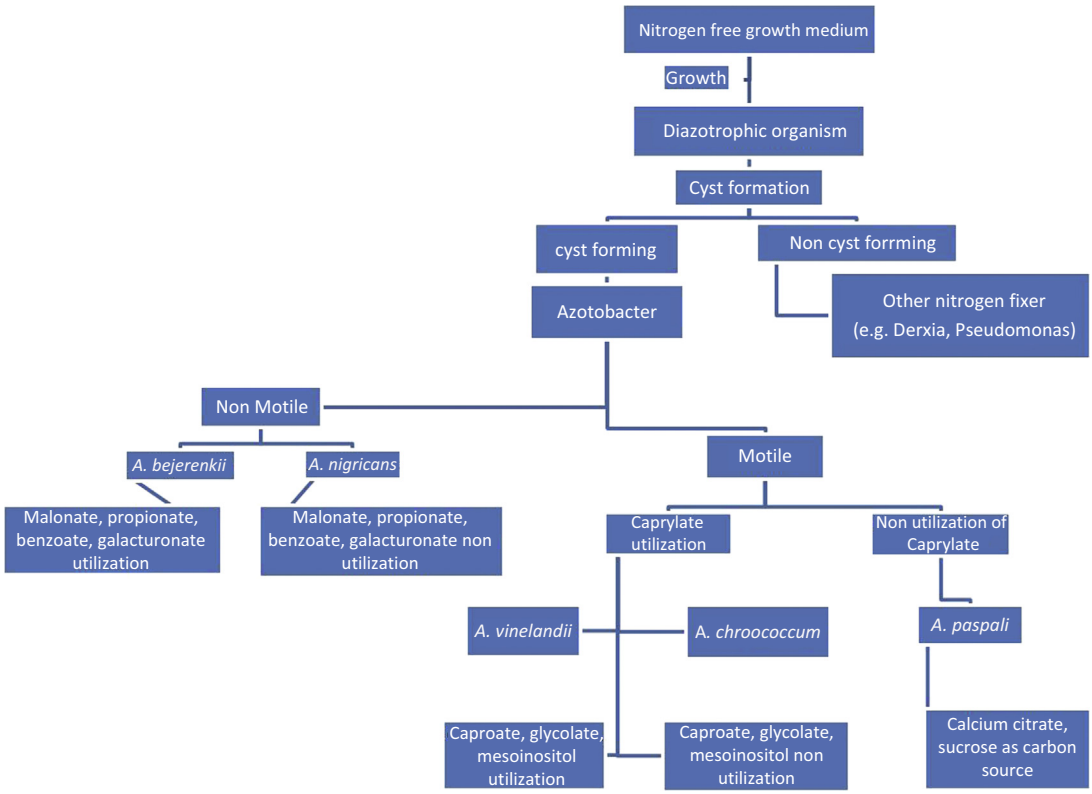

FIGURE 19.2 Key for identification of *Azotobacter* sp. based on important morphological and biochemical characteristics.

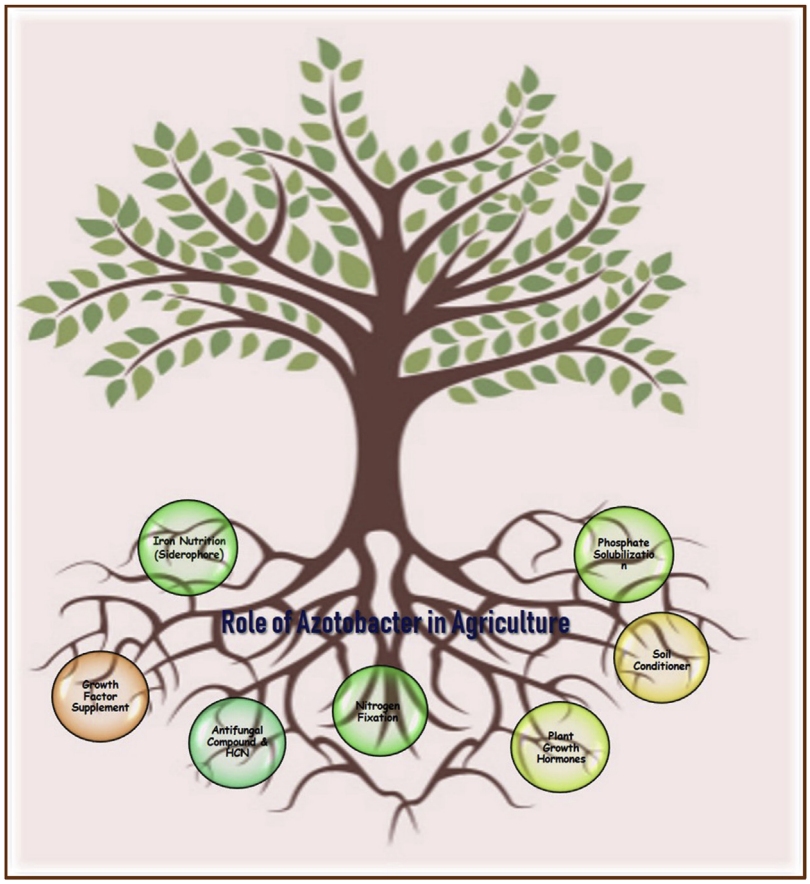

FIGURE 19.3 Role of *Azotobacter* in sustainable agriculture.

atmospheric nitrogen into ammonia and remaining used for the synthesis of protein, which is further return to the soil after the death of bacteria by mineralization and this will be available for the plant from soil (EI-Lattief, 2016).

The possible mechanism involves dinitrogen fixation by the transformation of atmospheric nitrogen (N_2) gas into ammonia (NH_3). The conversion to a utilizable form of nitrogen, i.e., ammonia is required for biosynthetic pathway and molecular nitrogen cycle (Murcia et al., 1997; Diaz-Barrera and Soto, 2010). The fixation of atmospheric dinitrogen by these heterotrophic aerated shaking cultures is achieved by protection of oxygen labile nitrogenase enzyme by consumption of O_2 (Robson and Postgate, 1980). This is possible with the potential of production of highly active cytochrome oxidases (Jurtshuk et al. 1978, 1981), then superoxide dismutase and catalase (Jurtshuk et al., 1984).

The nitrogen fixation; has several effects on plant growth, as provide the collective molecular nitrogen to the vegetation; the phytohormones formation affecting plant productivity and reduction of nitrate which surges accumulation of nitrogen in the plants. As stated

with the estimate, fertilizer equivalent of 20 kg N/ha for *Azotobacter* is needed (Tandon, 1991) which increase the yield of crop plants about 10%−12% (Jaga and Singh, 2010). The combined incubation of *Azotobacter* with other microorganisms like *Azospirillum*, *Pseudomonas* increased grain yield (Yousefi and Barzegar, 2014; Singh et al., 2015). The inhibitory consequence of excess ammonium ions on nitrogenase synthesis and action as well as ammonium accumulation could be prevented by nitrogen fixation by bacteria and carry fixed nitrogen to plant.

The control of dinitrogen fixation gene expression is able to control ammonia synthesis in response to 2-oxoglutarate via NifA with the balance of nitrogen and carbon (Mus et al., 2017).

Azotobacter vinelandii is the model organism for the study of nitrogen fixation under raised atmospheric oxygen condition. The protection of nitrogenase enzyme in the presence of air is possible due to respiratory and conformational resistance of enzyme and position of the enzyme in a cell. The nitrogenase activity is associated with soil moisture. The increase in soil moisture is associated with the exponential rise in the rate of acetylene reduction (Dighe et al., 2010). The growth augmentation is due to mutual communication of the strains along with growth promoting substance synthesis. This shows that the efficiency outcome is subject to the phases of plant growth (Vikhe, 2014).

4.2 Role of *Azotobacter* in crop productivity

Azotobacter is the dynamic, free-living heterotrophic nitrogen fixer majorly found in the alkaline or neutral soil habitat. *Azotobacter* works as plant growth promoting rhizobacteria (PGPR) apart from the fixation of molecular nitrogen.

The effect of *Azotobacter* on plant productivity include, increase in seed germination capacity, promote germination (20%−30%) through the making of the plant growth encouraging substance, which causes a decrease in chemical phosphorus and nitrogen by 25% that resulted in stimulation of crop growth. The straight promoting effect PGPR on plant growth comprise of formation and release of subordinate metabolite like growth regulator as well as smoothing uptake of nutrients from the root environment (Glick, 1995; Polyanskaya et al., 2002).

Azotobacter produces various vegetal growth promoting elements, for instance, cytokinin, auxins, and Gibberellic acid, which are the principal constituents for governing plant growth by improvement in nitrogen content and plant mass. These hormonal constituents from the root surface or rhizosphere also affect the growth of the familiarly related developed plants (EI-Lattief, 2016). Hormones enhance phosphate solubilization (Ramon et al., 1972) influence nutrient uptake by increasing phosphatase activity (Hoflich et al., 1994), increases water and mineral uptake (Bashan and Levanony, 1991), production of amino acids and vitamins, the bioactive constituents. The phytohormone synthesis is also associated with the production of extracellular compounds such as riboflavin, Vit B12, biotin, thiamine, pyridoxine, cyanocobalamin, folic acid and pantothenic acid.

The *Azotobacter* number is usually less within the uncultured soils and rhizosphere of the crop plants. The organic matter from the soil and root exudates nourishes the *Azotobacter* and help further to fix atmospheric N (Maryenko, 1964). *Azotobacter* biofertilizer with C: N ratio (20:1) is indicating the stability of biofertilizer.

The *Azotobacter chroococcum* is having a worthy influence on soil fertility and plant nutrition by promoting significant uptake of N and P (Wani et al., 2016).The synthesis and secretion of bioactive compounds like gibberellic acid, vitamin B, biotin, pantothenic acid, nicotinic

acid resulted in boosting the growth of plant root (Rao, 1986). The secretion of ammonia by *Azotobacter* in the rhizosphere is also important in modification of crop nutrient uptake (Narula and Gupta, 1986). The multi-copper protein family bacteria produced polyphenol oxidases (PPOs) and phenol oxidases (POs). The family Azotobacteraceae is also assumed to produce POs (Herter et al., 2011).

4.2.1 *Vitamins produced by* Azotobacter

Vitamins play a crucial role in the physiological functioning of lives formed by numerous groups of bacteria (Revillas et al., 2000). The vitamin production by *Azotobacter* species is observed in a favorable environment. The B-group vitamins such as pantothenic acid, biotin, niacin, riboflavin are produced by *A. chroococcum* strain H23 (CECT 4435) and *Azotobacter vinelandii* strain ATCC 12837.

4.2.2 *Amino acids produced by* Azotobacter

At the diazotrophic conditions in glucose-supplemented culture media, *Azotobacter* species produced different amino acids such as tryptophan, lysine, glutamic acid, and methionine (Gonzalez-Lopez et al., 1983). *A. vinelandii* and *A. chroococcum* are recognized for the production of aspartic acid, serine, glutamic acid, glycine, histidine, threonine, arginine, alanine, proline, cysteine, valine, lysine, isoleucine, phenylalanine, tyrosine, methionine, and leucine (Revillas et al., 2005).

4.2.3 *Phosphate solubilization*

Phosphobacteria is the noteworthy microorganisms for the transformation of phosphorous. The hydrolysis of organic and inorganic phosphorus from insoluble compounds is occurred by phosphate solubilizing bacteria. Thus, P-solubilization efficiency of the microorganisms intended for phosphate nourishment of plant is a very significant character.

4.2.4 *Plant growth hormones (IAA, GA)*

Several *Azotobacter* species produce Indole acetic acid (IAA; 2.09−33.28 μg/mL) (Spaepen et al., 2007). IAA producing PGPR strains upsurge root length resulting in larger root surface zone, which allows plants to access additional nutrients from the soil. The IAA is accountable for the division and differentiation of plant cells and tissues as well as stimulation of root elongation (Ahmad et al., 2008).

Azotobacter chroococcum is the signature model for its role in plant nourishment and its influence on soil richness by synthesis of plant development hormone. *A. vinelandii* cells can biosynthesize at least three molecules the intracellular polyester poly-β-hydroxybutyrate (PHB), the extracellular polysaccharide alginate, and catechol compounds (siderophores).

4.2.5 *Biopolymers as a soil conditioner*

The biopolymers in the rhizosphere have different natural functions such as self-adhesion of cells into biofilms, surface adhesion, the creation of defensive barriers, water retention about roots, and nutrient accumulation. The biopolymer has its role as a soil modifier to build up slope stability, lessen transport of objects in overflow water, cut the passage of heavy metals, and generation of dust (Larson et al., 2012).

Biopolymers strengthen the soil by ecofriendly way and required in low concentration. The polysaccharide biopolymer has hydroxyl groups on its surfaces that encourage hydrogen bond formation with water molecules to make hydrophilic nature that allowing the formation of viscous hydrogels or hydrocolloids.

In drought condition, the biopolymer can create hydrogen bonding toward clay particle or subsidiary ionic bonding with clay particle in existence of earth metals. The direct and indirect bonding primes the creation of a steady biopolymer-clay matrix, which resulted in a substantial rise in soil cohesion. The mixing of biopolymers with coarse and clay particles is therefore anticipated to offer best firming possessions, owing to the mixture of amplified motorized resistance among rough particles, and a cementation outcome among biopolymer-clay matrices (Chang et al. 2016).

Azotobacter species (*A. beijerinckii*, *A. chroococcum*, *A. vinelandii*) produce extracellular (alginate) and intracellular (Poly-β-hydroxybutyrate) polymer (Haleem Khan et al., 2015). Pseudomonas and *Azotobacter* species produced alginates (Remminghorst and Rehm, 2006). Alginate is a straight-chain polymer composed of a varying number of (1−4)-β-D-mannuronic acid and a-L-guluronic acid, its C-5-epimer has an extensive variety of applications such as a stabilizer, thickener, emulsifier and gelling agent in food, and in addition to textile and pharmaceutical industries. The polyhydroxyalkanoate (PHAs) is intracellular polyesters polymer.

The bacterium *Azotobacter vinelandii* produce the alginate and PHAs as important polymers with an excess of carbon source and limiting phosphorous and oxygen. Alginate is produced as extracellular polysaccharide in *A. vinelandii* and *P. aeruginosa*, while PHAs is involved in cyst differentiation in *A. vinelandii* (Sadoff, 1975). The formation of the cyst is the result of the intracellular accumulation of PHA/PHB inside the cytoplasm outlined by lipoprotein double wall in the environment of excess carbon and limited nitrogen, phosphorus, or oxygen condition. Once the carbon source is exhausted, cysts are oxidized to work as energy source quickly with the involvement of PHB depolymerases enzyme (da Silva and Garcia-Cruz, 2010).

A. vinelandii also produces the intracellular polyester PHB (polymer of the polyhydroxyalkanoates family), which is a biodegradable and biocompatible thermoplastic and benefited as a supplementary for majority plastics such as polyethylene and polypropylene. The bacteria can accumulate PHB polyester intracellularly as both a carbon and energy reserve material. PHB is made up of around 150 diverse hydroxyl alkanoic acids (Schroth and Hancock, 1982).

The other important function that is achieved with PHB in *A. vinelandii* is the protection of nitrogenase enzyme by ensuring the bacterial respiration even in absence of exogenous carbon and energy source by avoiding decrease of oxygen and maintaining respiratory function (Page et al., 1992; de Almeida et al., 2004).

In our Lab at KBC North Maharashtra University, Jalgaon, *Azotobacter* biopolymer was exploited for various benefits like improved germination, water holding capacity, soil porosity, organic content as soil conditioner along with its role as bioflocculent, for toxic heavy metal and dye removal (Patil et al., 2010, 2011; Mohite and Patil, 2014; Mohite et al., 2017) (Fig. 19.4).

Azotobacter is renowned for the production of diverse forms of subsidiary metabolites, for instance, plant growth hormones (IAA, nicotine, and gibberellins), amino acids (Thiamine),

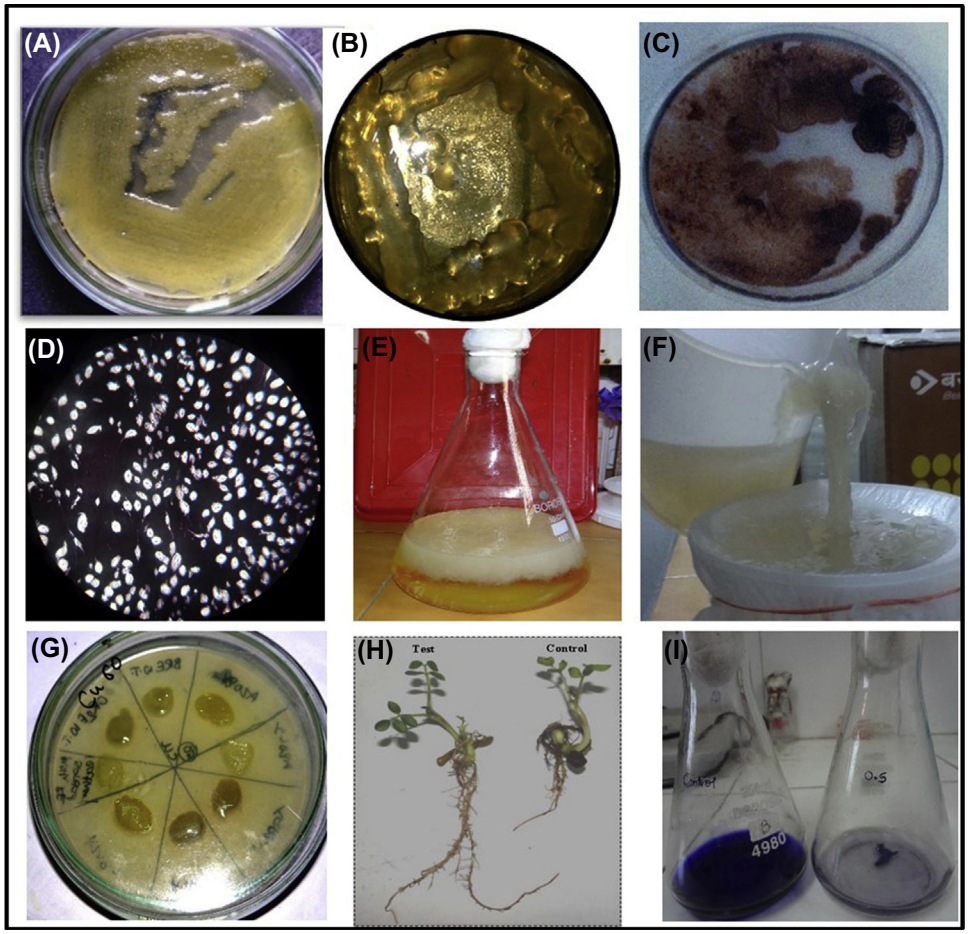

FIGURE 19.4 *Azotobacter* biopolymer and its explored applications (At KBC NMU laboratory), (A), (B), and (C) Different *Azotobacter* sp. on nitrogen-free medium, (D) *Azotobacter* with its biopolymer sheath by negative staining, (E) and (F) *Azotobacter* biopolymer production and recovery; (G), (H), and (I) *Azotobacter* biopolymer applications for heavy metal accumulation, plant growth promotion, and dye removal, respectively.

vitamins (Riboflavin), siderophores, antifungal substance (Myresiotis et al. 2015). The growth encouraging effects like effect on the shoot and root length, germination of seeds is due to the growth encouraging elements like IAA, gibberellic acid (GA), and nicotinic acid (Ahmad et al., 2005).

These subordinate metabolites inspire the growth promoting effect by expelling auxins, vitamins, amino acids, provision of iron to plants by siderophores and poly hydroxyl butyrate (PHB) for large-scale production of alginic acid. The phytohormone production capacity is dispersed among plant-related bacteria, about 80% of plant rhizosphere bacteria can make plant growth encouraging elements.

4.3 *Azotobacter* as biocontrol agent

4.3.1 *Antifungal compounds*

The antibiotic production is among the most focused biocontrol mechanisms for fighting against phytopathogens. *Azotobacter* can offer protection from drought and generates antifungal compounds that are responsible for inhibition of the growth of soil-borne fungi such as *Alternaria*, *Curvularia*, *Aspergillus*, *Helminthosporium*, and *Fusarium* (Mali and Bodhankar, 2009). The *Azotobacter* species produce diverse types of antibiotics like 2,3-dihydroxybenzoic acid, azotochelin, aminochelin, azotobactin and protochelin (Kraepiel et al., 2009).

Azotobacter species worked as biocontrol agents against various plant pathogens (Mali and Bodhankar, 2009; Agrawal and Singh, 2002). The species of Arthrobacter and *Azotobacter* inhibited *F. verticillioides* root colonization which has suppressed fumonisin B-1 creation by *A. armeniacus. F. oxysporum* causes infection to several crops such as chilli, and pigeon pea. The *A. vinelandii* showed a maximum zone of inhibition (40 mm) against *F. oxysporum* confirming the antifungal activity (Cavaglieri et al., 2005; Bhosale et al., 2013).

The growth of various pathogenic fungi in the rhizosphere is inhibited by the antibiotic secreted by *Azotobacter chroococcum* (Subba Roa, 2001). *Azotobacter* inhibited the growth of *Rhizoctonia solani* by producing an antifungal antibiotic which inhibits it (Vikhe, 2014). *Azotobacter* sp. could produce antifungal compounds opposing the pathogens alike *Trichoderma* sp., *Alternaria* sp., *Fusarium* sp. (Bjelić et al., 2015).

4.3.2 *HCN production*

In addition to the production of antibiotics, some of the rhizobacteria accomplished production of HCN, which is a subordinate metabolite of volatile nature that inhibits the microbes and affect the crop growth and development. It is a powerful inhibitor of various metal containing enzymes, particularly copper encompassing cytochrome C oxidases. HCN synthetase enzyme is responsible for formation HCN from glycine. The enzyme is present in association with the plasma membrane of rhizobacteria.

4.3.3 *Siderophore production by* Azotobacter

Siderophores are low molecular weight complexes formed by fungi and bacteria as iron (Fe) chelating agents. Under the iron deficient condition, at neutral to alkaline pH, different bacteria produce the siderophores (Sharma and Johri, 2003). *Azotobacter* excretes siderophores under deprivation of iron as *A. vinelandii* (Page and Von Tigerstrom, 1988), which can combine to iron and form sturdy complexes which are later transferred into the cell by extreme precise transporters (Page et al., 2003). The reported siderophore for *A. vinelandii* is the azotochelin (bis(catechol)), aminochelin (monocatechols), the 2,3-dihydroxybenzoic acid and protochelin (tris(catechol)) and the yellow-green fluorescent pyoverdine-like azotobactin (Kraepiel et al., 2009). *A. vinelandii* generate minimum five diverse siderophores having an antibacterial effect such as 2, 3- dihydroxybenzoic acid, azotochelin (bis-catechol), protochelin (tris-catechol), aminochelin (monocatechols), and the yellow-green fluorescent pyoverdine-like azotobactin.

The pathogenic microorganism proliferation could be prevented by siderophore produced by *Azotobacter* by confiscating Fe^{3+} in the vicinity of the root.

Siderophore producing *Azotobacter* can prevent the proliferation of pathogenic microorganisms by requisitioning Fe^{3+} in the locality of the root. Although the plant can use the iron with the help of bacterial siderophores, the whole concentration is perhaps too little to pay significant iron uptake by the plant.

In modern agriculture practices, *Azotobacter* could support to reduce the chemical fertilizers practice. The urea adaptive nature of *Azotobacter* facilitates the plant growth improvement by combined inoculation of chemical and bacterial fertilizer (Shrivastava et al., 2015).

5. Outlook

Azotobacter is identified for its potential in diverse fields. India is well known for its geographical and biological diversity, and there are tremendous chances to get the more potent and versatile *Azotobacter* strains, which will act as a potential candidate for agriculture, fermentation, and other industrial applications. Hence, the present chapter focuses on simple morphological and biochemical keys and techniques for screening of *Azotobacter* which is the need for the economic laboratory studies in developing country like India.

Acknowledgment

Authors are thankful to Late Prof. Jan-Hendrick Becking (the pioneer Dutch biologist) and **Prof. Bernard Glick** (University of Waterloo, Canada) for the inspiration and encouragement.
The corresponding author, SVP is kindly acknowledging the Department of Biotechnology, New.
Delhi for the Indo-US Foldscope Major Research Project grant (Grant No. BT/IN/Indo-US/Foldscope/39/2015).
And Co-author, BVM acknowledge the Science and Engineering Research Board (SERB) under the Start Up Research Grant, Young Scientist (YSS/2015/001722) for providing financial support.

References

Agarwal, S.K., Singh, S.S., Verma, S., Kumar, S., 2000. Antifungal activity of anthraquinone derivatives from Rheum emodi. J. Ethnopharmacol. 72 (1–2), 43–46.

Agrawal, N., Singh, H.P., 2002. Antibiotic resistance and inhibitory effect of *Azotobacter* on soil borne plant pathogens. Indian J. Microbiol. 42 (3), 245–246.

Ahmad, F., Ahmad, I., Khan, M.S., 2005. Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. Turkish J. Biol. 29 (1), 29–34.

Ahmad, F., Ahmad, I., Khan, M.S., 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol. Res. 163 (2), 173–181.

Antheunisse, J., 1973. Viability of lyophilized microorganisms after storage. Antonie Van Leeuwenhoek 39 (1), 243–248.

Aquilanti, L., Favilli, F., Clementi, F., 2004. Comparison of different strategies for isolation and preliminary identification of *Azotobacter* from soil samples. Soil Biol. Biochem. 36 (9), 1475–1483.

Ashby, S.F., 1907. Some observations on the assimilation of atmospheric nitrogen by a free living soil organism.—*Azotobacter chroococcum* of Beijerinck. J. Agric. Sci. 2 (1), 35–51.

Bagyaraj, D.J., Patil, R.B., 1975. *Azotobacter* research in Karnataka. Curr. Res. 4, 181–184.

Bashan, Y., Levanony, H., 1991. Alterations in membrane potential and in proton efflux in plant roots induced by *Azospirillum brasilense*. Plant Soil 137 (1), 99–103.

Batchinskaya, A.A., 1935. Structure and development of *Azotobacter*. Trudy vsesoyuznogo nauchno isledovotel. Skogo Instituta Selkskokhozyaistvennoi Mikrobiologya 6 (1), 3.

Becking, J.H., 2006. The family *Azotobacter*aceae. In: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes. Springer, New York, pp. 759–783.
Becking, J.H., 1961. Studies on the nitrogen-fixing bacteria of the genus *Beijerinckia*: II. Mineral nutrition and resistance to high levels of certain elements in relation to soil type. Plant Soil 297–322.
Becking, S., 1974. Family Azotobacteraceae. In: Bergey's Manual of Systematic Bacteriology, vol. 1. Williams and Wilkins, London, pp. 219–230, 1984.
Beijerinck, M.W., 1901a. Uber oligonitrophile mikroben. Zbl. Bakt. Abf. II 7, 561.
Beijerinck, M.W., 1901. On oligonitrophilous bacteria. Proc. Koninklijke Nederl. Akademie van Wetenschappen 3, 586–595.
Buchanan, R.E., 1925. General Systematic Bacteriology, first ed. Villiem and Wilkins, Co, p. 194.
Bhosale, H.J., Kadam, T.A., Bobade, A.R., 2013. Identification and production of *Azotobacter vinelandii* and its antifungal activity against *Fusarium osporum*. J. Environ. Biol. 34, 177–182.
Bishop, P.E., Jarlenski, D.M., Hetherington, D.R., 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. U.S.A. 77 (12), 7342–7346.
Bjelić, D.Đ., Marinković, J.B., Tintor, B.B., Tančić, S.L., Nastasić, A.M., Mrkovački, N.B., 2015. Screening of *Azotobacter* isolates for PGP properties and antifungal activity. Zbornik Matice srpske za prirodne nauke (129), 65–72.
Buchanan, R.E., Gibbons, N.E. (Eds.), 1974. Bergey's Manual of Determinative Bacteriology, eighth ed. Williams & Wilkins Co., Baltimore, Md, p. 21202.
Bush, J.A., Wilson, P.W., 1959. A non-gummy chromogenic strain of *Azotobacter vinelandii*. Nature 184 (4683), 381.
Cavaglieri, L.R., Andrés, L., Ibáñez, M., Etcheverry, M.G., 2005. Rhizobacteria and their potential to control *Fusarium verticillioides*: effect of maize bacterisation and inoculum density. Antonie Van Leeuwenhoek 87 (3), 179–187.
Chang, I., Im, J., Cho, G.C., 2016. Introduction of microbial biopolymers in soil treatment for future environmentally-friendly and sustainable geotechnical engineering. Sustainability 8 (3), 251.
Cohen, G.H., Johnstone, D.B., 1964. Extracellular polysaccharides of *Azotobacter vinelandii*. J. Bacteriol. 88 (2), 329–338.
Claus, D., Hempel, W., 1970. Specific substrates for isolation and differentiation of *Azotobacter vinelandii*. Arch. Mikrobiol. 73 (1), 90–96.
Daniel, S., Odon, V., Yanet, R., Soledad, M., Miguel, C., Guadalupe, E., 2009. Isolation and characterization of *Azotobacter vinelandii* mutants impaired in alkylresorcinol synthesis: alkylresorcinols are not essential for cyst desiccation resistance. J. Bacteriol. 191 (9), 142–148.
da Silva, A.N., Garcia-Cruz, C.H., 2010. Biopolymers by azotobacter vinelandii. In: Biopolymers. In Tech., pp. 413–438
de Almeida, A., Ruiz, J.A., López, N.I., Pettinari, M.J., 2004. Bioplásticos: una alternativa ecológica. Química Viva 3 (3).
De Jong, L.D.D., 1938. Das Verhalten von *Azotobacter chroococcum* unter abnormen Lebensbedingungen. Arch. Mikrobiol. 9 (1–5), 223–252.
Derx, H.G., 1951. L'accumulation spécifique de l'*Azotobacter*agile Beijerinck et de *l' Azotobacter vinelandii* Lipman. Proc. Koninklijke Nederl. Akademie Wetenschappen - Ser. B Palaeontol. Geol. Phys. Chem. Anthropol. 54, 624–634.
Diaz-Barrera, A., Soto, E., 2010. Biotechnological uses of *Azotobacter vinelandii*: current state, limits and prospects. Afr. J. Biotechnol. 9 (33).
Dighe, N.S., Shukla, D., Kalkotwar, R.S., Laware, R.B., Bhawa, S.B., Gaikwad, R.W., 2010. Nitrogenase enzyme: a review. Der Pharm. Sin. 1 (2), 77–84.
Döbereiner, J., 1970. Further research on *Azotobacter paspali* and its variety specific occurrence in the rhizosphere of *Paspalum notatum* Flugge. Zentralblatt fürBakteriologie, Parasitenkunde, Infektionskrankheitenund Hygiene, Abt. 2 (124), 224–230.
Döbereiner, J., 1966. *Azotobacter paspali* sp. n., uma bactéria fixadora de nitrogênio na rizosfera de Paspalum. Pesqui. Agropecu. Bras. 1 (1), 357–365.
EI-Lattief, E.A., 2016. Use of *Azospirillum* and *Azotobacter* bacteria as biofertilizers in cereal crops: a review. Int. J. Res. Eng. Appl. Sci. 6 (7), 36–44.
Garg, S.K., Bhatnagar, A., Kalla, A., Narula, N., 2001. In vitro nitrogen fixation, phosphate solubilization, survival and nutrient release by *Azotobacter* strains in an aquatic system. Bioresour. Technol. 80 (2), 101–109.
Glick, B.R., 1995. The enhancement of plant growth by free-living bacteria. Can. J. Microbiol. 41 (2), 109–117.
Gonzalez-Lopez, J., Salmeron, V., Moreno, J., Ramos-Cormenzana, A., 1983. Amino acids and vitamins produced by *Azotobacter vinelandii* ATCC 12837 in chemically-defined media and dialysed soil media. Soil Biol. Biochem. 15 (6), 711–713.

Haleem Khan, A.A., Naseem, B., Vardhini, V., 2015. Synthesis of extracellular and intracellular polymers in isolates of *Azotobacter* sp. Int. J. Renew. Energy Technol. 4 (12), 231–233.

Hegazi, N.A., Jensen, V., 1973. Studies of *Azotobacter* bacteriophages in Egyptian soils. Soil Biol. Biochem. 5 (2), 231–243.

Herter, S., Schmidt, M., Thompson, M.L., Mikolasch, A., Schauer, F., 2011. A new phenol oxidase produced during melanogenesis and encystment stage in the nitrogen-fixing soil bacterium *Azotobacter chroococcum*. Appl. Microbiol. Biotechnol. 90 (3), 1037–1049.

Höflich, G., Wiehe, W., Kühn, G., 1994. Plant growth stimulation by inoculation with symbiotic and associative rhizosphere microorganisms. Experientia 50 (10), 897–905.

Jaga, P.K., Singh, V., 2010. Effect of biofertilizer, nitrogen and sulphur on sorghum-mustard cropping system. In: Proceedings of National Seminar on Soil Security for Sustainable Agriculture Held at College of Agriculture, Nagpur *(MS on February 27-28, 2010)* "Xxii Savetovanje O Biotehnologiji" Zbornik Radova, Knjiga, vol. 1, p. 2017.

Jensen, H.L., 1954. The *Azotobacter*iace. Bacteriol. Rev. 18, 195–214.

Jensen, H.L., 1965. Non-symbiotic nitrogen fixation. 436–480. In: Bartholomew, W.V., Clark, F.E. (Eds.), Soil Nitrogen, Monograph 10. American Society of Agronomy, Madison.

Jensen, V., 1961. Rhamnose for detection and isolation of *Azotobacter vinelandii* Lipman. Nature 190 (4778), 832–833.

Jensen, V., Petersen, E.J., 1954. Studies on the Occurrence of *Azotobacter* in Danish Forest Soils. 95–110. Royal Veterinary and Agricultural College Yearbook 1954. Kandrup & Wunsch, Copenhagen.

Jensen, V., Petersen, E.J., 1955. Taxonomic studies on *Azotobacter Chroococcum* Beij. And A*zotobacter Beijerinckii*. In: Royal Veterinary and Agricultural College, Yearbook 1955. Kandrup and Wunsch, Copenhagen, pp. 107–126.

Jurtshuk Jr., P., Mueller, T.J., Wong, T.Y., 1981. Isolation and purification of the cytochrome oxidase of *Azotobacter vinelandii*. Biochim. Biophys. Acta Bioenerg. 637 (2), 374–382.

Jurtshuk, P., Liu, J.K., Moore, E.R., 1984. Comparative cytochrome oxidase and superoxide dismutase analyses on strains of *Azotobacter vinelandii* and other related free-living nitrogen-fixing bacteria. Appl. Environ. Microbiol. 47 (5), 1185–1187.

Jurtshuk, P., Mueller, T.J., McQuitty, D.N., Riley, W.H., 1978. The cytochrome oxidase reaction in *Azotobacter vinelandii* and other bacteria. In: Functions of Alternative Terminal Oxidases, pp. 99–121.

Kraepiel, A.M.L., Bellenger, J.P., Wichard, T., Morel, F.M.M., 2009. Multiple roles of siderophores in free-living nitrogen-fixing bacteria. Biometals 22 (4), 573.

Krassilnikov, N.A., 1949. Opredelitelv Bakterii I Actinomicetov. Akademii Nauk SSSR Moscow, p. 328.

Lapage, S.P., Shelton, J.E., Mitchell, T.G., Mackenzie, A.R., 1970. Chapter II culture collections and the preservation of bacteria. In: Methods in Microbiology, vol. 3. Academic Press, pp. 135–228.

Larson, S.L., Newman, J.K., Griggs, C.S., Beverly, M., Nestler, C.C., 2012. Biopolymers as an alternative to petroleum-based polymers for soil modification; ESTCP ER-0920: treatability studies (No. ERDC-TR-12-8). Eng. Res. Dev. Cent. Vicksburg Ms Environ. Lab 1–34.

Layne, J.S., Johnson, E.J., 1964. Natural factors involved in the induction of cyst formation in *Azotobacter*. J. Bacteriol. 87 (3), 684–689.

Levine, N.D., 1975, 21202. xxvi+ 1246 pp. $45.00. J. Protozool. In: Buchanan, R.E., Gibbons, N.E. (Eds.), 1974. Bergey's Manual of Determinative Bacteriology, vol. 22. Williams & Wilkins Co., Baltimore, Md, p. 7 (1).

Lin, L.P., Sadoff, H.L., 1968. Encystment and polymer production by *Azotobacter vinelandii* in the presence of β-hydroxybutyrate. J. Bacteriol. 95 (6), 2336–2343.

Lipman, J.G., 1903a. Experiments on the transformation and fixation of nitrogen by bacteria. Rep. NJ St. Agric. Exp. Stn 217–285.

Lipman, J.G., 1903b. Nitrogen-fixing Bacteria (Ph.D. thesis). Cornell University, Ithaca, New York. June 1903. New Jersey State Agricultural Experiment Station, 16th Annual Report. State Printers. New Jersey.

Lipman, J.G., 1904. Soil bacteriological studies. Further contributions to the physiology and morphology of the members of the *Azotobacter* group. Rep. New Jersey State Agric. Exp. Stn. 25, 237–289.

Lipman, J.G., 1905. Further contributions to the physiologie and morphologie of members of the *Azotobacter* group. Ann. Rep. New Yersey State Agricult. Exper. Stat 25, 237. Seventeenth Annual Report over 1904. State Printers. New Jersey.

Mali, G.V., Bodhankar, M.G., 2009. Antifungal and phytohormone production potential of *Azotobacter chroococcum* isolates from Groundnut (*Arachis hypogea* L.) rhizosphere. Asian J. Exp. Sci. 23 (1), 293–297.

Maryenko, V.G., 1964. Dokt TSHA 99 (2), 399–406, 357-360.

Mohite, B.V., Patil, S.V., 2014. A novel biomaterial: bacterial cellulose and its new era applications. Biotechnol. Appl. Biochem. 61 (2), 101–110.
Mohite, B.V., Koli, S.H., Narkhede, C.P., Patil, S.N., Patil, S.V., 2017. Prospective of microbial exopolysaccharide for heavy metal exclusion. Appl. Biochem. Biotechnol. 183 (2), 582–600.
Monsour, V., Wyss, O., Kellogg Jr., D.S., 1955. A bacteriophage for *Azotobacter*. J. Bacteriol. 70 (4), 486.
Murcia, R., Rodelas, B., Salmeron, V., Martínez-Toledo, M.V., González-López, J., 1997. Effect of the herbicide simazine on vitamin production by *Azotobacter chroococcum* and *Azotobacter vinelandii*. Appl. Soil Ecol. 6 (2), 187–193.
Mus, F., Tseng, A., Dixon, R., Peters, J.W., 2017. Diazotrophic growth allows *Azotobacter vinelandii* to overcome the deleterious effects of a glnE deletion. Appl. Environ. Microbiol. 83 (13), e00808–e00817.
Myresiotis, C.K., Vryzas, Z., Papadopoulou-Mourkidou, E., 2015. Effect of specific plant-growth-promoting rhizobacteria (PGPR) on growth and uptake of neonicotinoid insecticide thiamethoxam in corn (*Zea mays* L.) seedlings. Pest Manag. Sci. 71 (9), 1258–1266.
Narula, N., Gupta, K.G., 1986. Ammonia excretion by *Azotobacter chroococcum* in liquid culture and soil in the presence of manganese and clay minerals. Plant Soil 93 (2), 205–209.
Norris, J.R., 1959. The isolation and identification of *Azotobacter*. Lab. Pract. 8, 239–243.
Nosrati, R., Owlia, P., Saderi, H., Rasooli, I., Malboobi, M.A., 2014. Phosphate solubilization characteristics of efficient nitrogen fixing soil *Azotobacter* strains. Iran. J. Microbiol. 6 (4), 285.
Page, W.J., Shivprasad, S., 1991. *Azotobacter salinestris* sp. nov., a sodium-dependent, microaerophilic, and aeroadaptive nitrogen-fixing bacterium. Int. J. Syst. Evol. Microbiol. 41 (3), 369–376.
Page, W.J., Von Tigerstrom, M., 1988. Aminochelin, a catecholamine siderophore produced by *Azotobacter vinelandii*. Microbiology 134 (2), 453–460.
Page, W.J., Kwon, E., Cornish, A.S., Tindale, A.E., 2003. The csbX gene of *Azotobacter vinelandii* encodes an MFS efflux pump required for catecholate siderophore export. FEMS Microbiol. Lett. 228 (2), 211–216.
Page, W.J., Manchak, J., Rudy, B., 1992. Formation of poly (hydroxybutyrate-co-hydroxyvalerate) by *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 58 (9), 2866–2873.
Patil, S.V., Salunke, B.K., Patil, C.D., Salunkhe, R.B., 2011. Studies on amendment of different biopolymers in sandy loam and their effect on germination, seedling growth of *Gossypium herbaceum* L. Appl. Biochem. Biotechnol. 163 (6), 780–791.
Patil, S.V., Salunkhe, R.B., Patil, C.D., Patil, D.M., Salunke, B.K., 2010. Bioflocculant exopolysaccharide production by *Azotobacter indicus* using flower extract of *Madhuca latifolia* L. Appl. Biochem. Biotechnol. 162 (4), 1095–1108.
Phadnis, S.H., Dimri, G.P., Das, H.K., 1988. Segregation characteristics of multiple chromosomes of *Azotobacter vinelandii*. J. Genet. 67 (1), 37–42.
Polyanskaya, L.M., Vedina, O.T., Lysak, L.V., Zvyagintsev, D.G., 2002. The growth-promoting effect of *Beijerinckia mobilis* and *Clostridium* sp. cultures on some agricultural crops. Microbiology 71 (1), 109–115.
Pribram, E., 1933. Klassification der Schizomyceten. F. Deuticke, Leipzig, pp. 1–143.
Premakumar, R., Jacobitz, S., Ricke, S.C., Bishop, P.E., 1996. Phenotypic characterization of a tungsten-tolerant mutant of *Azotobacter vinelandii*. J. Bacteriol. 178 (3), 691–696.
Ramaswamy, P.P., Mathan, K.K., Nair, K.S., 1977. *Azotobacter* population in red and black soils of Tamil Nadu (India). Mysore J. Agric. Sci. 11, 364–366.
Ramon, A., Barea, J.M., Callao, V., 1972. Phosphate dissolving and nitrogen fixing microorganism and its possible influence on soil fertility. Agrochimica 16, 345–350.
Rao, D.L.N., 1986. Nitrogen Fixation in Free Living and Associative Symbiotic Bacteria. In: Soil Microorganisms and Plant Growth. Oxford and IBH Pub., New Delhi, pp. 116–140.
Rascio, N., La Rocca, N., 2008. Jorgensen Editors-In-Chief: Sven Erik, Fath Brian. Biological Nitrogen Fixation, pp. 412–419.
Rediers, H., Vanderleyden, J., De Mot, R., 2004. *Azotobacter vinelandii*: a *Pseudomonas* in disguise? Microbiol.-SGM 150, 1117–1119.
Remminghorst, U., Rehm, B.H., 2006. Bacterial alginates: from biosynthesis to applications. Biotechnol. Lett. 28 (21), 1701–1712.
Reuszer, H.W., 1939. The effect of benzoic acid compounds upon the abundance of microorganisms, including *Azotobacter* organisms, in a soil. Proc. Third Comm. Int. Soc. Soil Sci. A 151–160.

Revillas, J.J., Rodelas, B., Pozo, C., Martinez-Toledo, M.V., López, J.G., 2005. Production of amino acids by *Azotobacter vinelandii* and *Azotobacter chroococcum* with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. Amino Acids 28 (4), 421–425.

Revillas, J.J., Rodelas, B., Pozo, C., Martínez-Toledo, M.V., González-López, J., 2000. Production of B-group vitamins by two *Azotobacter* strains with phenolic compounds as sole carbon source under diazotrophic and adiazotrophic conditions. J. Appl. Microbiol. 89 (3), 486–493.

Robson, R.L., Postgate, J.R., 1980. Oxygen and hydrogen in biological nitrogen fixation. Annu. Rev. Microbiol. 34 (1), 183–207.

Robson, R.L., Jones, R., Robson, R.M., Schwartz, A., Richardson, T.H., 2015. *Azotobacter* genomes: the genome of *Azotobacter chroococcum* NCIMB 8003 (ATCC 4412). PloS One 10 (6), e0127997.

Rojas-Tapias, D., Ortiz-Vera, M., Rivera, D., Kloepper, J., Bonilla, R., 2013. Evaluation of three methods for preservation of *Azotobacter chroococcum* and *Azotobacter vinelandii*. Univ. Sci. 18 (2), 129–139.

Sadoff, H.L., 1975. Encystment and germination in *Azotobacter vinelandii*. Bacteriol. Rev. 39 (4), 516.

Schroth, M.N., Hancock, J.G., 1982. Disease-suppressive soil and root-colonizing bacteria. Science 216 (4553), 1376–1381.

Setubal, J.C., dos Santos, P., Goldman, B.S., Ertesvåg, H., Espin, G., Rubio, L.M., Valla, S., Almeida, N.F., Balasubramanian, D., Cromes, L., Curatti, L., 2009. Genome sequence of *Azotobacter vinelandii*, an obligate aerobe specialized to support diverse anaerobic metabolic processes. J. Bacteriol. 191 (14), 4534–4545.

Sharma, A., Johri, B.N., 2003. Growth promoting influence of siderophore-producing *Pseudomonas* strains GRP3A and PRS9 in maize (*Zea mays* L.) under iron limiting conditions. Microbiol. Res. 158 (3), 243.

Shrivastava, R., Shrivastava, A.K., Dewangan, N., 2015. Combined application of *Azotobacter* and Urea to improve growth of rice (*Oryza sativum*). IOSR J. Environ. Sci. Toxicol. Food Technol. 1 (3), 67–72.

Singh, R., Babu, S., Avasthe, R.K., YadavG, S., Chettri, T.K., Phempunadi, C.D., Chatterjee, T., 2015. Bacterial inoculation effect on soil biological properties, growth, grain yield, total phenolic and flavonoids contents of common buckwheat (*Fagopyrum esculentum* Moench) under hilly ecosystems of North East India. Afr. J. Microbiol. Res. 9 (15), 1110–1117.

Socolofsky, M.D., Wyss, O., 1961. Cysts of *Azotobacter*. J. Bacteriol. 81 (6), 946.

Spaepen, S., Vanderleyden, J., Remans, R., 2007. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol. Rev. 31 (4), 425–448.

Subba Roa, N.S., 2001. An appraisal of biofertilizers in India. In: Kannaiyan, S. (Ed.), *The Biotechnology of Biofertilizers* (Doctoral Dissertation. Narosa Pub. House, New, p. 375.

Tandon, H.L.S., 1991. Role of sulphur in plant nutrition. Fertil. News 36 (69), 79.

Tchan, Y.T., New, P.B., 1984. *Azotobacteraceae,* 219-225. In: Krieg, N., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. I. Williams & Wilkins Co., Baltimore.

Tchan, Y.T., 1984. Genus 1. *Azotobacter Beijerinck* 1907, 567. In: Bergey's manual of systematic bacteriology, 1, pp. 220–229.

Tchan, Y.T., Wyszomirska-Dreher, Z., New, P.B., Zhou, J.C., 1983. Taxonomy of the *Azotobacteraceae* determined by using Immunoelectrophoresis. Int. J. Syst. Evol. Microbiol. 33 (2), 147–156.

Tejera, N., Lluch, C., Martinez-Toledo, M.V., Gonzalez-Lopez, J., 2005. Isolation and characterization of *Azotobacter* and *Azospirillum* strains from the sugarcane rhizosphere. Plant Soil 270 (1), 223–232.

Thompson, J.P., Skerman, V.B.D., 1979. Azotobacteraceae: The Taxonomy and Ecology of the Aerobic Nitrogen-Fixing Bacteria. Academic Press Inc, (London) Ltd.

Thompson, J.P., Skerman, V.B.D., 1981. *Azorhizophilus*, new genus. *Azorhizophilus Paspali*, pp. 215–218.

Thompson, J.P., 1987. Cryopreservation of *Azotobacteraceae* in liquid nitrogen. MIRCEN J. Appl. Microbiol. Biotechnol. 3 (3), 323–336.

Torres-Rubio, M.G., Valencia-Plata, S.A., Bernal-Castillo, J., Martínez-Nieto, P., 2000. Isolation of *Enterobacteria, Azotobacter* sp. and *Pseudomonas* sp., producers of indole-3-acetic acid and siderophores, from Colombian rice rhizosphere. Revista latinoamericana de microbiología 42 (4), 171–176.

Ueda, T., Suga, Y., Yahiro, N., Matsuguchi, T., 1995. Remarkable N_2-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of nifH gene sequences. J. Bacteriol. 177 (5), 1414–1417.

Vela, G.R., Wyss, O., 1965. Radiation resistance of soil *Azotobacter*. J. Bacteriol. 89 (5), 1280–1285.

Vela, G.R., 1974. Survival of *Azotobacter* in dry soil. Appl. Microbiol. 28 (1), 77–79.

Vikhe, P.S., 2014. *Azotobacter* species as a natural plant hormone synthesizer. Res. J. on Recent Sci. 3, 59–63 (IVC).

Wani, S.A., Chand, S., Wani, M.A., Ramzan, M., Hakeem, K.R., 2016. *Azotobacter chroococcum*—a potential biofertilizer in agriculture: an overview. In: Soil Science: Agricultural and Environmental Prospectives. Springer, Cham, pp. 333–348.

Widmer, F., Shaffer, B.T., Porteous, L.A., Seidler, R.J., 1999. Analysis of nifH gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range. Appl. Environ. Microbiol. 65 (2), 374–380.

Wilson, P.W., Knight, S.C., 1952. Experiments in Bacterial Physiology, vol. 49. Burguess, Minneapolis, USA.

Winogradsky, S., 1938. Etudes sur la microbiologie du sol et des eaux. Annales de l'Institut Pasteur 60, 351–400 (Platenos. V, VI, and VII).

Wyss, O., Neumann, M.G., Socolofsky, M.D., 1961. Development and germination of the *Azotobacter* cyst. J. Cell Biol. 10 (4), 555–565.

Young, J.M., Park, D.C., 2007. Probable synonymy of the nitrogen-fixing genus *Azotobacter* and the genus Pseudomonas. Int. J. Syst. Evol. Microbiol. 57 (12), 2894–2901.

Yousefi, A.A., Barzegar, A.R., 2014. Effect of *Azotobacter* and *Pseudomonas* bacteria inoculation on wheat yield under field condition. Intl. J. Agric. Crop Sci. 7 (9), 616.

Zehr, J.P., Mellon, M., Braun, S., Litaker, W., Steppe, T., Paerl, H.W., 1995. Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. Appl. Environ. Microbiol. 61 (7), 2527–2532.

Zulaika, E., Shovitri, M., Kuswitasary, N.D., 2014. Numerical taxonomy for detecting the *Azotobacter*ial diversity. In: The 8th Korean-Asean Joint Symposium on Biomass Utilization and Renewable Energy.