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BAJAJ WARDHA COLLEGE OF SCIENCE

BIOGENIC SUSTAINABLE NANOTECHNOLOGY

Trends and Progress

Edited by
Raghvendra Pratap Singh
Alok R. Rai
Ahmed Abdala
Ratiram G. Chaudhary

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Preface

With the emergence of interest in the development of eco-friendly, green-synthesized, cost-effective, and straightforward methods, the nanotechnology has emerged as a foremost research topic that has glimmered extensive curiosity. Nanotechnology signifies a radical path for high-tech expansion of living as well as nonliving material at the nanometer scale (reduced to billion times from a meter).

It comprehends the assembly and application of biological, chemical, and physical systems ranging from single atoms or molecules to submicron dimensions, and further an active and sustainable integration of it into the larger systems.

The driving force of this interest is the possibility to avoid hazardous chemicals and lower energy consumption. In green synthesis methods different materials from biological origins such as microorganism, cells, plants, or their enzymes or extract are employed with the focus on a greener environment minimizing waste generation and implementing sustainable processes. The green synthesis of nanostructured material is an extremely challenging approaches for all the researchers due to the existence of phytochemical agents in the extracts such as various sugars, flavones, saponins, proteins, amino acids, chromone, steroids, phytol, and terpenoids. The phytochemicals present in the plant extracts play a key role in the improvement of reduction rate, size, and stabilization by acting as good reducing surfactants and structure directing and capping agents. Therefore this themed issue is a compilation of articles reviewing the green synthesis of nanomaterials with various biological systems, especially the emphasis is placed on the mechanisms of nanomaterial synthesis, spectroscopic characterization, and their applications in different fields. These synthesized nanomaterials have wide potentiality to change our perceptions and expectations by providing us the competence to resolve comprehensive mankind issues.

Nowadays, nanotechnology is a vital part of our research and reality. A sundry of domains of human resource activities has reached from food production to its conservation, medicine production to its efficient application, biology to biotechnology, computing to communications, transport to space investigation. The chemical mediated nanotechnology inevitably contributes to hazardous ecological contamination. Hence, the editors have planned to upsurge the facts and flaws of nanotechnology and tried to display the radical solution with the compilation of global intellectual ideas in a book.

This book expresses the cumulative efforts of handpicked specific scientist and professors having expertise in nanotechnology, biotechnology, and microbiology and the noble effort of them has been summarized as the biogenic nanotechnology or green nanomaterials and its related areas.

The authored chapters precisely touch upon facets such as microbial factory-based environment-friendly nanosynthesis and their characterization and the use of various biological

items such as plants and its part, bacteria, fungi, etc. to synthesize an efficient and high functional materials compared to traditional chemical synthesis of nanoparticles. Moreover, the historical backgrounds and future prospectives have also been elaborated nicely and finally the economic aids of green nanotechnology to human resource development have been shown.

Editors are highly grateful to all the eminent authors for their outstanding contribution, who are to be recognized not only for their contribution but, most notably, for keeping their promise timely which has facilitated us to finalize this project in the given timeline.

We would also like to thank Cloe Holland-Borosh, Editorial Project Manager at Elsevier, who fortified us to commence editing of the present project; Rachel Pomery, Editorial Project Manager; Emily Joy Grace Thomson, Senior Editorial Project Manager of the book project; and the entire Elsevier team participated into its publication, for their firm work, skillful handling, sustenance, and patience. Finally, we thank the researchers and readers, who perceived this book, read, and found it motivating for nanoresearch. Editors are always welcome for any type of comments regarding its content and presentation manners.

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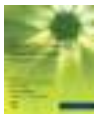

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About the book

Description

Biogenic Sustainable Nanotechnology: Trends and Progress focuses on the green synthesis of nanomaterials with various biological systems, emphasizing the mechanisms of nanomaterial synthesis, spectroscopic characterizations, and applications in a variety of industrial sectors.

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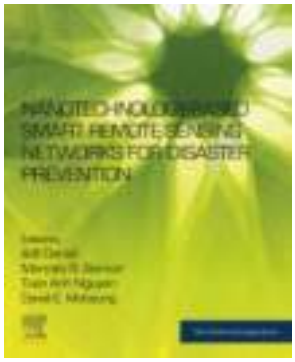

Vijay L. Maheshwari
Ravindra H. Patil *Editors*

Natural Products as Enzyme Inhibitors

An Industrial Perspective

Springer

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Chapter 11
Metal Nanomaterials as Enzyme Inhibitors and Their Applications in Agriculture and Pharmaceutics

Satish V. Patil, Kiran R. Marathe, Hemant P. Borase, and Bhavana V. Mohite

Abstract As enzymes play a vital role in all biological systems, their regulation is also an important mechanism to drive various systems. In modern agriculture practices, the use of enzyme inhibitors to control pests and to regulate soil microbial activity is becoming an essential practice. The synthetic and natural organic chemicals are already documented for their use in pest control and fertilizers as a protectant. But these chemicals have reported some drawbacks like sensitivity to physical factors like temperature, pH, development of resistance and phytotoxicity, etc. The current review focuses on the potential and the use of metal nanomaterials as enzyme inhibitors in important agriculture practices. Various nanomaterials like lead, copper, gold, etc. were reported for their enzyme inhibition potential such as proteases, ureases, nitrate reductase, acetylcholine esterases, etc. Their potential with some conjugates as important agrochemicals such as pest control agents, fertilizers additives is briefly described in this review which will induce researchers for designing future agro formulations.

Keywords Enzyme inhibitors · Pests · Nanomaterials · Fertilizer additives · Agriculture practices

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11.1 Introduction

Enzymes are the biocatalyst of the living system. After nucleic acid, enzymes are considered to be the most vital biomolecules. Around 5000 biochemical reactions are carried out by enzymes (Ahmed et al. 2016). The biological functions of enzymes are diverse ranging from metabolism, nerve impulse transmission, control of biological activities such as replication, translation, organ specialization, blood coagulation, breathing, reproduction, generation of resistance, and diseases to name a few (Katsimpouras and Stephanopoulos 2021; Gomez-Fabra Gala and Vögtle 2021).

Owing to the essential role of enzymes not only in humans but also in microbes and insects, a class of molecules known as "enzyme inhibitors" are researched as a promising drug candidate in diabetes, Alzheimer's, and cancer and as a biocidal agent to kill plant pathogens and pests (Chen et al. 2017). Enzyme inhibitors act on enzymes because of the association of enzymes with life-supporting processes and diseases such as metabolic, neurological, cardiovascular, etc. Inhibition of enzymes can be lethal to pathogens, and therefore, enzyme inhibitors can be used as pesticides (Copland 2005). Enzyme inhibitor binds to an enzyme's active site and partially or completely inhibits its activity. However, they need not cover the complete binding site.

A variety of molecules such as paracetamol, rivastigmine, mupirocin, fosinopril, ritonavir, atorvastatin inhibit target enzymes such as acetylcholinesterase, cyclooxygenase, HIV protease, HMG-CoA reductase and, therefore, are used in the treatment of bacterial and viral infections, pain, cancer, hypertension, hypercholesteremia, etc. (Copeland et al. 2007; Ouertani et al. 2019). However, most of the currently available enzyme inhibitors are organic and have several drawbacks such as instability, degradation, and low catalytic activity (Cha et al. 2015; Ouertani et al. 2019). Moreover, organic enzyme inhibitors work on relatively few major types of inhibitory mechanisms such as competitive, irreversible, or allosteric inhibition with several limitations (Ouertani et al. 2019). There is a need to find enzyme inhibitors having a broad inhibition spectrum.

Global pharma-biotech-agro industries such as Ranbaxy Laboratories, AstraZeneca, Merck, Pfizer, Roche are investing a big amount of money in enzyme inhibitor research (Cision 2017). The global market for enzyme inhibitors was estimated to be more than US$ 95 billion in 2006 and is predicted to grow at an average rate of around 8% per annum (BCC Research 2018). Hence, there is a plenty of space to find versatile and robust enzyme inhibitors with unconventional chemical structures, desirable features, degradation resistance, and diverse inhibitory effects.

11.2 Potential of a Metal Nanomaterial as an Enzyme Inhibitor

According to the national nanotechnology initiative (United States), nanotechnology is science, engineering, and technology conducted at the nanoscale (1–100 nm) and utilizing products obtained from this unique phenomenon in diverse fields from chemistry and physics to medicine and engineering (NNI 2020). Pieces of evidence of the use of nanomaterials by humans trace back to the fourth century AD in the time of Greek and Democritus and can be found in historical objects like the Lycurgus cup and medieval church windows (Bayda et al. 2020). Nanotechnology is holding great economical potential as evidenced by its tremendous industrial applications and incorporation in day-to-day consumer goods (Janković and Plata 2019). Nanoforms of silver, gold, copper, zinc, and other metals are successfully applied in the pharmaceutical and agriculture industry (Khan et al. 2019; Singh et al. 2021).

There are two approaches for the synthesis of metal nanoparticles, viz. top-down and bottom-up. Extensive research was done on chemical, physical, and biological methods of nanoparticles synthesis, but the growing demand for nanomaterials is fulfilled by chemical and physical methods despite growing toxicity issues. On the other hand, biological methods utilizing microbes and plants present a promising approach but are still not feasible for large-scale nano synthesis (Patil et al. 2016; Borase et al. 2014, 2021; Ahmed Mohamed et al. 2020).

Pharmaceutics and agriculture are expected to gain tremendous benefits from nanotechnology interventions (Singh et al. 2021; Contera et al. 2020). Current agricultural practices in most of the world rely on high doses of agrochemicals such as fertilizers, pesticides that adversely affect soil rhizospheric microbiome, causing water pollution and biomagnification, affecting food quality and supply (Singh et al. 2021).

Plant growth is largely affected by various biotic and abiotic factors such as disease-causing pathogens, genetic traits, moisture availability, and soil fertility (Lahiani et al. 2013). Nanoscience is an innovative platform that involves the development of approaches to a range of inexpensive nanotech applications for enhanced seed germination, plant growth, development, and acclimation to environments. In this regard, an extensive number of studies have shown that the application of nanomaterials has positive effects on germination as well as plant growth and development. Likely, the application of multiwalled carbon nanotubes (MWCNTs) positively influences seed germination of different crop species including tomato, corn, soybean, barley, wheat, maize, peanut, and garlic (Khodakovskaya and Biris 2010; Srivastava and Rao 2014). Nanoparticles as enzyme inhibitors are promising due to several properties such as the high surface area to mass ratio, nano size, different shape, chemical functionalization, resistance to degradation in environmental conditions, etc. (Maccormack et al. 2012; Chen et al. 2017). Nanoparticles are expected to act as broad-spectrum enzyme inhibitors due to the above characteristics (Benelli 2018).

11.3 Nanoparticles Inhibiting Vital Enzymes (Some Examples)

Most of the commercially available antibiotics and agrochemicals act on pathogens and insects by targeting important enzymes. However, due to the widespread issue of resistance emergence, nanoparticles offer a promising alternative to the traditional arsenal of enzyme inhibitors (Ahmed et al. 2016; Ali et al. 2018). Nanoparticles have useful properties including shape, size tenability, binding of multiple ligands on the surface, and diverse enzyme inhibitory strategies.

Penicillin inhibits enzyme transpeptidase, which is essential for bacterial cell wall synthesis. Thus, the β-lactam-mediated inhibition of transpeptidation leads to cell lysis. However, after the introduction of penicillin, within a few years, various bacterial strains started showing drug resistance by naturally producing an enzyme, penicillinase (Drawz et al. 2014).

Two-dimensional molybdenum disulfide (2D-MoS_2) nanomaterials were reported to inhibit the β-lactamase enzyme. The negatively charged ligand functionalized MoS_2 materials exhibit electrostatic and other non-covalent interactions between enzyme and inhibitor leading to competitive inhibition of the latter (Ali et al. 2018).

Trypsin (223 amino acid residue protein) is another important target enzyme involved in human diseases and also helps insects to neutralize biocidal agrochemicals (Schnebli and Braun 1986). It was observed that absorption of trypsin on TiO_2 NPs decreased the β sheet content. TiO_2 NP–trypsin interaction altered the secondary structure due to electrostatic force, van der Waals force, and hydrogen bonding (Wang et al. 2011). In another study, gold nanoparticles were also found to inhibit trypsin. Electrostatic and hydrophobic interactions, along with covalent interactions between Cys-58, 42, Lys-60, and gold nanoparticles, might be involved in the binding and inhibition process (Zhang et al. 2014).

Urease (nickel-containing metalloenzymes) is an important factor in peptic ulcers, and commercially available urease inhibitors have multiple side effects such as antibiotic resistance (Naz et al. 2019). Silver nanoparticles functionalized with N-substituted methyl 5-acetamido-β-resorcylate (AgL) with an average size of 20 nm were found to be stable at variable temperatures and pH. AgL showed enhanced urease inhibition as compared to the standard drug (thiourea), N-substituted methyl 5-acetamido-β-resorcylate, and silver nanoparticles. Interestingly, AgL is inactive against other metalloenzymes like xanthine oxidase and carbonic anhydrase II as well as for non-metallic enzymes such as α-chymotrypsin and acetylcholinesterase. Therefore, AgL is very selective to inhibit urease only (Benelli 2018).

Silver nanoparticles fabricated using *Cassia fistula* fruit pulp extract inhibits the fourth instar larvae of *Aedes albopictus* and *Culex pipienspallens* (Coquilett) with a substantial decrease of acetylcholine esterase and α- and ß-carboxylesterase activities. Similar to the above study, silver nanoparticles prepared using salicylic acid and

3,5-dinitrosalicylic acid inhibit *Ae. albopictus*, with the decrease of total proteins, esterase, acetylcholine esterase, and phosphatase enzymes.

Multivalent nanoconstructs are another new advanced technology involving the conjugation of multiple copies of enzyme inhibitors on nanosurface to improve inhibitory potency and selectivity. Nanometer-sized fluorescent hybrid silica (NFHS) particles (size 150 nm) loaded with fluorescent and sulfonamide carbonic anhydrase inhibit carbonic anhydrase activity several-fold because of higher silica nanoparticles adsorption on the border of carbonic anhydrase (Touisni et al. 2015).

Detoxification enzymes (GST, Catalase, SOD) of insects act as the first line of defense against chemical-induced stress and hence are an attractive target for designing insecticidal and pesticide formulations. Several other enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT)) serve an important role in protein and carbohydrate metabolism (Borase et al. 2021). Insects characteristically lack important detoxification enzymes glutathione peroxidase and catalase known to have little affinity with hydrogen peroxide (H_2O_2). Hence, ascorbate peroxidase (APOX) plays a major role in the clearance of (H_2O_2). Beetles species *Blapspolychresta* were treated with 26.27 ± 4.43 nm Nickel (II) oxide nanoparticles (NiO NPs) at sub-lethal concentrations of 0.02 mg/g. NiO NPs were found to cause a significant decrease in the activity of APOX as compared to the untreated group (El-Ashram et al. 2021).

Borase et al. (2019, 2020, 2021) evaluated the effects of the most widely used metal nanoparticles (silver nanoparticles (20 and 40 nm), gold nanoparticles (30 nm), and zinc oxide particles (250 and 500 nm)) on important enzymes of aquatic organism *Moina macrocopa.* Interestingly all nanoparticles under investigation inhibit key enzymes including acetylcholinesterase and digestive enzymes (trypsin, amylase) and β-galactosidase. It was found that the size of nanoparticles is a crucial factor during their interaction with enzymes along with the resistance/sensitivity of the organism. Although *M. macrocopa* is an aquatic organism but finding from the above studies can be a reference for developing enzyme inhibitors for pharma and agriculture sectors.

11.4 Future of Nanoparticles as an Enzyme Inhibitor

Although several studies put forward the superiority of metal nanoparticles as a new class of enzyme inhibitor (BCC Research 2018), more research is needed to study the complete life cycle of nanomaterials including nonspecific interactions with other proteins normally present in biological fluid. Toxicity and accumulation in non-target host and environmental consequences are also unanswered questions.

11.4.1 Nanoparticles as Protease Inhibitors in Pest Management

Nanoparticles have various applications in different fields of agriculture like pest management, vector-pest management, herbicide delivery, and nanosensors for pest detection (Scrinis and Lyons 2007; Rahman et al. 2009). The role of nanoparticles as a protease inhibitor, their mechanism of action in insects, and their possible applications in agriculture are summarized below.

Proteases or peptidases are hydrolytic enzymes that selectively catalyze the cleavage of peptide bonds in proteins. Peptidases participate in various cellular physiological processes and irreversible proteolytic reactions whose control is essential for cell functions. The proteolytic activity of proteases is controlled by regulating secretions, specific degradation, and also by inhibition. Several natural specific and selective protease inhibitors are now known as major regulating proteins to control proteolytic activity in all life forms (Umezawa 1982). These characteristics make protease inhibitors good diagnostic and therapeutic tools for the treatment of various microbial (hepatitis, herpes, AIDS, aspergillosis) and mortal (arthritis, muscular dystrophy, malaria, cancer, obesity), neurodegenerative, and cardiovascular diseases (Karthik et al. 2014). Until now, several protease inhibitors are identified from plants, animals, and microbes for each mechanistic class of proteases, e.g., serine, cysteine, aspartyl, and metalloproteases (Lorito et al. 1994; Joshi et al. 1999; Bijina et al. 2011). Although the role of protease inhibitors have been identified, they are not widely used in agriculture due to ability of insects to produce insensitive proteases or degradation of inhibitors to neutralize their effects. Hence, there is the necessity to look for alternative solutions to overcome protease inhibitor resistance developed by insects. With the emergence of widespread antibiotic, enzyme, and insecticides resistance, a better alternative is the use of different metal nanoparticles. Nanoparticles possess several advantages over conventional protease inhibitors in respect of their stability, reproducibility, and reactivity with other chemicals (Friedman et al. 1993). The different biologically synthesized metal nanoparticles, their size, applications, and enzyme inhibition pattern are summarized in Table 11.1.

The biosynthesized chromium nanoparticles (Cr_2O_3 NPs) from *Hyphaene thebaica* showed antiviral activity against the poliovirus by inhibiting the protein kinase enzymes (Khalil et al. 2018). Similarly, Iqbal et al. (2020) reported the Cr_2O_3 NPs from *Rhamnus virgata* leaves extract showed the inhibition of protein kinase and α-amylase enzymes with biopotential against the fungi and bacterial species. The silver, aluminum oxide, zinc oxide, and titanium dioxide NPs have been successfully used as insecticides to manage different pests, most of these NPs showed detrimental effects on treated insects by inhibiting the gut proteases enzymes (Table 11.1). Of these, silver nanoparticles are the most studied and utilized nanoparticles for biosystem due to their strong inhibitory and bactericidal effects as well as a broad spectrum of antimicrobial activities (Becker et al. 2005). Feng et al. (2000) reported that silver ions interact strongly with the thiol groups of vital enzymes, causing their inactivation. Patil et al. (2016) observed the

Table 11.1 Some examples of metal nanoparticles as an enzyme inhibitor

Sr. No.	Metal nanoparticles	Size (nm)	Shape	Target enzyme	Major finding	Reference
1	Gold nanoparticles (AuNPs)	20–50	Triangular, hexagonal	Protease	Serum protease of *A. aegypt*, *H. armigera, C. maculatus* inhibited due to multiple bonding between enzyme and AuNPs	Patil et al. (2016)
2	Zinc oxide nanoparticles (ZnO NPs)	20	Pyramids, plates, and spheres	β-Galactosidase	Shape-dependent denaturation, electrostatic attraction	Cha et al. (2015)
3	Silica nanoparticles	4, 20 and 100	Spherical	Chicken egg lysozyme	Loss in alpha-helix content. Strong adsorption for large NPs	Vertegel et al. (2004)
4	Copper oxide nanoparticles	<50	Spherical	Nitrate reductase and nitric oxide reductase	Inhibition of electron transport chain	Zhao et al. (2020)
5	Silver nanoparticles (AgNPs)	10	–	Urease, acid phosphatase, arylsulfatase, β-glucosidase	AgNPs negatively affect soil exoenzyme activities, with the urease activity especially sensitive to AgNPs due to non-competitive inhibitor by blocking sulfhydryl groups	Shin et al. (2012)
6	Silver nanoparticles (AgNPs)	20	Spherical	Catalase (CAT) and superoxide dismutase	Disturbing α-helicalsecondary structure of enzymes	Liu et al. (2020)
7	Silver-moxifloxacin nanoparticles	50–60	Spherical	Urease	250 times better compared to moxifloxacin	Nisar et al. (2015)
8	Two-dimensional molybdenumdisulfide (2D-MoS2) nanomaterials	–		β-Lactamase	Electrostatic, non-covalent interactions and steric obstruction. Useful in β-lactamase resistance	Ali et al. (2018)

(continued)

Table 11.1 (continued)

Sr. No.	Metal nanoparticles	Size (nm)	Shape	Target enzyme	Major finding	Reference
9	Lead oxide (PbO) nanoparticles	27		Acetylcholinesterase	Potential use in the treatment of Alzheimer's	Khalil et al. (2018)
10	Gold nanoparticles (AuNPs)	5–70	Triangular	Cytochrome P450	AuNPs likely block the substrate pocket on the CYP surface	Ye et al. (2014)

protease-inhibiting properties of latex fabricated AuNPs, particularly with regard to trypsin when mixed with insect serum. Protease activity was found to be inhibited by 66% in the serum of *A. aegypti* mosquito larvae and pests including *Helicoverpa armigera*, *C. maculatus, C. chinensis,* and *M. hirsutus*. Kantrao et al. (2017) also reported the inhibition of gut protease activity of *H. armigera* due to biosynthesized AgNPs. Raj et al. (2017) reported that increased oral dosages of AgNPs in the larval stage of *Drosophila melanogaster* alter the protein, carbohydrate, and lipid levels by impairing the metabolic activity, which may be due to the inhibition of gut proteases. Similarly, the silver nanoparticles lower the activity of copper-dependent superoxide dismutase and tyrosinase enzymes in *Drosophila melanogaster* which is involved in the antioxidant activity and pigment production, respectively (Posgai et al. 2011; Armstrong et al. 2013; Ávalos et al. 2015). The biosynthesized AgNPs using the fruits of *Cassia fistula* showed a significant decrease in the activity of AChE, a-and b-carboxylesterases, phosphatase enzymes, and total proteins in *Aedes albopictus* and *Culex pipienspallens* larvae and pupa (Fouad et al. 2018). Yasur and Rani (2015) also reported the insecticidal activity of AgNPs against the *Spodoptera litura* F. and *Achaea janata* L. due to the accumulation of nanoparticles in the gut of larvae which decreases larval and pupal weight. Also, the conjugated silica nanoparticles with plant protease inhibitors showed excellent larvicidal activity against the *Helicoverpa armigera* by inhibiting gut proteinase activity (Bapat et al. 2020; Khandelwal et al. 2015). Marathe et al. (2021) also investigated the mode of action of AgNPs and suggested that antifungal activity could be due to inhibition of ergosterol biosynthesis leading to severe damage to the cell membrane. A summary of the effects of different metal nanoparticles on various pests is presented in Table 11.2.

11.4.2 Nano Metals as Urease Inhibitors

Urease is the nickel metalloenzyme produced by various microbes such as bacteria and fungi, which are the major component of soil. They are the key component of urea loss by gaseous ammonia and other minerals of nitrogen. Although very efficient urea inhibitors are available, they have some negative impacts on crop health, i.e., phytotoxicity and get rapidly hydrolyzed by the other soil microbes. They also lose their urease inhibiting potential by various environmental factors. Leaf scorches and necrosis of leaf margin are common examples of phytotoxicity of these synthetic inhibitors. It was also proved that N-(n-butyl) thiophosphoric triamide (NBPT), taken by peas and spinach roots and translocated to leaves, also inhibits endogenous urease of roots and leaves plants or crops (Artola et al. 2011). The glutamate synthetase and amino acid levels were found to be reduced by NBPT (Cruchaga et al. 2011).

These drawbacks led the scientific community to find other nonorganic urease inhibitors. Various metals have already been identified to possess potent urease inhibition potential. Some already identified metals (silver and mercury) forming insoluble sulfides act as potent urease inhibitors by reacting with sulfhydryl groups.

Table 11.2 A summary of effect of metal nanoparticles against different pest

Sr. No.	Nanoparticles	Size (nm)	Application	Mechanism of action	References
1	Cr_2O_3-NPs	25–38	Antiviral, antioxidant	Inhibits the protein kinases in polio virus	Khalil et al. (2018)
2	Cr_2O_3-NPs	28	Antibacterial, antioxidant, antifungal	Inhibition of protein kinase and alpha amylases	Iqbal et al. (2020)
3	AgNPs synthesized with Ficus extracts	20	Insecticidal activity against *H. armigera*	Inhibits larval gut proteases of *H. armigera*	Kantrao et al. (2017)
4	AgNPs	20–100	Insecticidal activity against *Drosophila melanogaster*	Altered the protein, carbohydrate, lipid metabolism	Raj et al. (2017)
5	AgNPs uncoated and polysaccharide-coated	10 and 60	Insecticidal activity against *Drosophila melanogaster*	Depigmentation by inhibiting the tyrosinase enzymes	Posgai et al. (2011)
6	AgNPs	10–50	Insecticidal activity against *Drosophila melanogaster*	Depigmentation, impaired movement, compromised fertility	Armstrong et al. (2013)
7	AgNPs	4.7	Insecticidal activity against *Drosophila melanogaster*	Depigmentation, impaired movement, compromised fertility	Ávalos et al. (2015)
8	AgNPs PVP coated	–	Insecticidal activity against *Spodoptera litura* F. and *Achaea janata* L.	Larval and pupal weight decreased due to the accumulation of nanoparticles in gut	Yasur and Rani (2015)
9	AgNPs synthesized with *Cassia fistula* fruit	148–900	*Aedes albopictus* and *Culex pipienspallens* larvae and pupa	Inhibition of acetylcholinesterase and a- and b-carboxyl esterases activity	Fouad et al. (2018)
10	AgNPs synthesized with *Streptomyces* spp.	20–40	Antifungal activity against *fusarium verticilloides*	Inhibition of ergosterol biosynthesis and membrane damage	Marathe et al. (2021)
11	AuNPs synthesized with plant latex *J. gossypifolia*	20	Insecticidal activity against *H. armigera and A. aegypti*	Inhibition of larval gut protease	Patil et al. (2016)
11	SiNPs conjugated with soyabean trypsin inhibitor (SiNPs-STI)	20–100	Insecticidal activity against *H. armigera*	Inhibition of gut proteinase activity (HGP)	Bapat et al. 2020)

(continued)

Table 11.2 (continued)

Sr. No.	Nanoparticles	Size (nm)	Application	Mechanism of action	References
12	Silica nanoparticles conjugated with plant protease inhibitors	240	Insecticidal activity against *H. armigera*	Inhibition of gut proteinase activity	Khandelwal et al. (2015)
13	Copper nanoparticles (CuNPs)	10–70	Insecticidal activity against *Tribolium castaneu*	Body deformation throughout the life cycle of insect	El-Saadony et al. (2020)
14	Copper oxide nanoparticles (CuO NPs)	10–70	Insecticidal activity against *Spodoptera littoralis*	Reduced pupation and adult emergence, adult malformation, adult fecundity, and egg hatchability	Shaker et al. (2016)
15	Zinc nanoparticles (ZnO NPs)	25–50	Insecticidal activity against *Spodoptera frugiperda*	Effect on fecundity, fertility and longevity of insect	Pittarate et al. (2021)
16	ZnO nanoparticles (ZnO NPs)	50–60	Insecticidal activity against *Aedes aegypti*	Programmed cell death (apoptosis)	Banumathi et al. (2017) Gunathilaka et al. (2021)
17	Silica nanoparticles (SiO_2 NPs)	50–150	Insecticidal activity against *Bombus terrestris*	Midgut epithelial injury	Mommaerts et al. (2012)
18	Graphene oxide nanoparticles	1–10	Insecticidal activity against *Acheta domesticus*	Increased enzymatic activity of catalase and glutathione peroxidases, as well as heat shock protein (HSP 70) and total antioxidant capacity level	Dziewięcka et al. (2016)
19	TiO_2 nanoparticles	<100	Insecticidal activity against *Bombyx mori*	Upregulation of signaling pathway and downregulation of development and molting period	Li et al. (2014)
20	Alumina nanoparticle	30–60	Insecticidal activity against *Sitophilus oryzae* (L.)	Binding to beetle cuticle resulting in insect dehydration	Stadler et al. (2017)

The general potential of metal ions as urease inhibitors is in the order of $Ag^{2+} > Hg^{2+}$ $Cu^{2+} > Ni^{2+} > CO^{2+} > Fe^{2+} > Mn^{2+}$ (Upadhyay 2012). These data may lead to the use of the above metals/non-metals as urease inhibitors. Although no commercial use of nanomaterial as urease inhibitor for soil application is available as yet, but

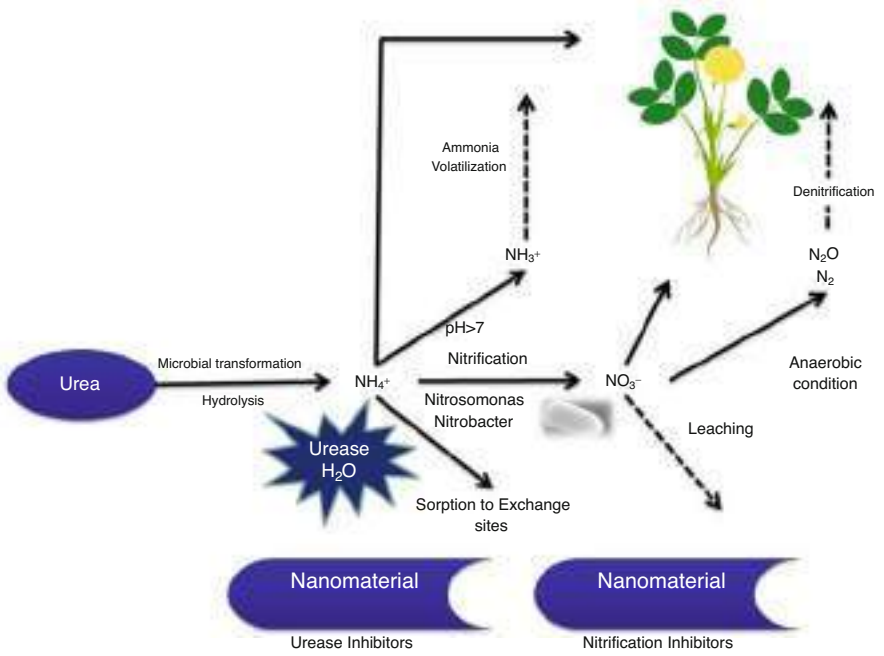

Fig. 11.1 Possible mechanism of urease regulations by metal nanomaterials

various reports of these metal nanomaterials as urease inhibitors make them potential candidates as the future component of nitrogenous fertilizers for controlled release of nitrogen, i.e., ammonia as per the requirement of the crop by controlling urease activity (Fig. 11.1).

Some reports on the use of nanometals as urease inhibitors also indicate their future potential. It was documented that PVP(polyvinyl alcohol)-based silver nanoparticles (AgNPs) having 15 nm size inhibiting the ammonia-oxidizing capacity of soil bacteria after direct application in soil (Huang et al. 2018). Similarly, Vandevoort and Arai (2018) and Simonin et al. (2018) found that copper nanometals, less than 50 nm in size, significantly decreased conversions of gaseous ammonia in nitrogen cycling.

Size-dependent reduction in ammonia oxidation potential of N*itrosomonas eutropea* by zero-valent silver polyvinyl alcohol (PVA) composite was also reported (Yan et al. 2020). Yuan et al. (2013) revealed that Na_2 ATP-doped silver nanoparticle composite retains 58.2% AgNPs after 24 h while PVA-coated AgNPs remain or retain 9.9%; hence, the latter is more efficient in inhibiting NH_3 oxidation. It was also found that this reduction may be due to cell wall damage and disintegration of nuclei. Silver nanomaterial with 20 nm size functionalized with N-substituted methyl acetoamide B-resocyclate was found to be more stable at the wide range of pH, temperature, and salt concentration with significantly increased urease inhibition. It was also proved that Ag-5-Amino-ß-resorcylic acid hydrochloride dihydrate

(AR) had significantly greater urease inhibition than the thiourea. The observed inhibitory activity of the latter was recorded at 11–18 times lower concentration than the former. Similarly, silver nanoconjugates of 5-amino-beta-resorcylic acid hydrochloride dihydrate were found to possess significant in vitro inhibitory potential against important enzymes like urease, xanthin oxidases, cholinesterase, and chymotrypsin which are targeted in various nitrogen fertilizer protection and agricultural pest management (Naz et al. 2014).

Zheng et al. (2017) reported that AgNPs inhibit the nitrification rate which was found to be inversely related to the concentration of AgNPs. Borase et al. (2015) reported significant urease inhibition by plant latex-mediated AgNPs of 21 nm size. Similarly, Jadon et al. (2018) advocated urea coated with 4% neem, 4% pine oleoresins, 35% rock phosphate, and 2% nano zinc particles (ZnO) for reducing the ammonia volatilization by 27%, 41%, 26% and 35% respectively.

11.5 Conclusion

The present literature points to the enzyme inhibitory and entomologic properties of metal nanoparticles. Especially, the silver nanoparticles showed the maximum inhibitory activity against the gut proteases, acetylcholine esterase, and phosphatases. This review suggests the possible use of metal nanoparticles as a biocontrol agent in agriculture, which can withstand alkaline conditions of an insect's gut. Moreover, with the commencement of insecticides and antibiotic resistance, nanoparticle-based enzyme inhibitory strategies could open up a new tool in therapeutics and agriculture as nano-insecticides in pest management and revolutionize insect and phytopathogen control which can benefit humans in the long run.

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Microbial Food-borne Diseases Due to Climate Change

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Climate Change and Microbes

Impacts and Vulnerability

Javid A. Parray | Suhaib A. Bandh | Nowsheen Shameem
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Contents

This book deals with the concept of thermoelasticity which finds its roots and applications in the branch of Physics, Mathematics and Engineering. Thermoelasticity is a fast growing concept and today it is an integral part of engineering and structural designs, rocket science and space engineering. Mathematical aspects of thermoelastic problems in cylindrical and spherical structures have been dealt with and thermal displacements, thermal stress and strains in these structures have been discussed in detail. Functionally Graded Materials, which have gained popularity as high temperature and high thermal stress bearing materials and widely used in space engineering have been discussed. Magneto-thermoelastic stresses and magnetic field vector perturbations for FGM hollow cylinder, FGM hollow sphere have been deduced. Mathematical approach for thermoelastic problems may be of immense use for the researchers, teachers pursuing work on thermoelasticity.

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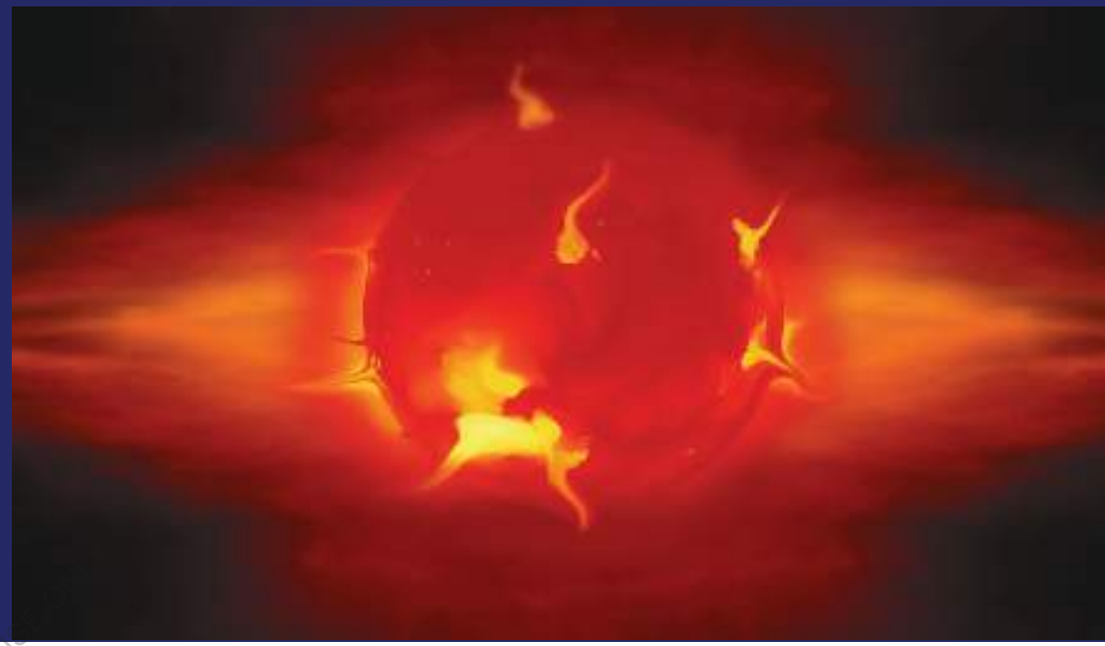

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THERMOELASTICITY-II

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DEDICATED TO MY

PARENTS

PREFACE

The book “THERMOELASTICITY- II” is second book on the topic dealing with thermal stresses, thermal strain and resulting displacements, the first one being THERMOELASTICITY- I. The mutual interactions between Physics, mathematics and Engineering have enriched the mankind in many ways. Thermoelasticity has been in human knowledge since two centuries, but studies related to it are recent developments. The principles of thermoelasticity have affected the way engineers design different structures and machinery. Thermoelastic investigations have now become an inevitable part of all design and construction mechanisms.

The book “THERMOELASTICITY-II “involves three chapters. The mathematical tools essential for this study have been discussed in chapter-I. Chapter-II discusses the thermoelasticity in cylindrical structures while chapter-III deals with thermoelastic behaviour of spherical structures. Thermal strain - stress in these bodies subjected to different heating conditions and boundary conditions are discussed here. Functionally Graded Materials(FGM) which has earned a reputation as the materials that can sustain more temperatures and thermal stresses and hence more useful in rocket science and aerospace engineering have also been discussed in this chapters. Hope that this book will be helpful to the students in understanding the mathematical aspects of thermoelastic problems, enabling them to make further research developments in this field.

SANJAY H BAGADE

CONTENTS

Thermoelasticity is one of the streams in Science where the concepts and ideas in Physics, Mathematics and Engineering are extensively used. The fruitful interactions and applications between these branches have led to many technological advancements. This book deals with the concept of thermoelasticity which finds its roots and application in the above branches. Thermal response of few circular and rectangular geometrical structures subjected to different boundary and heating conditions are discussed here. Thermal stresses and strains under thermal variations and different heating conditions are elaborated. Topics discussed here should be helpful to students and researchers pursuing work on thermoelasticity.

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Thermoelasticity - I

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Thermal Stresses in Circular and Rectangular Structures

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THERMOELASTICITY-I

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DEDICATED TO MY PARENTS

PREFACE

Physics has always played an important role in this fast growing world of Engineering and Technology. The concepts and discoveries from the field of Physics are harnessed by Engineers, and Engineering, in turn provides the incentives to Physics, paving a way for more concepts and discoveries. Such mutual interactions between Physics and Engineering have enriched the mankind in many ways. Thermoelasticity has been in human knowledge since two centuries, but studies related to it are recent developments. The principles of thermoelasticity have affected the way engineers design different structures and machinery. Thermoelastic investigations have now become an inevitable part of all design and construction mechanisms. This book on thermoelasticity is a small effort to acquaint the readers about the topic, making use of the concepts and tools in Physics and Mathematics.

The book "THERMOELASTICITY-I " consists of three chapters. Chapter-I deals with the basic introduction and the necessary mathematical tools used in this study. Chapter-II discusses the thermoelasticity in circular structures while chapter-III deals with thermoelastic behaviour of rectangular structures. Thermal strain -stress in these bodies subjected to different heating conditions and boundary conditions are discussed here. Hope that this book will be helpful to the students in understanding the mathematical aspects of thermoelastic problems, enabling them to make further research developments in this field.

SANJAY H BAGADE

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Practical Handbook on Agricultural Microbiology

Edited by

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Preface

Agricultural Microbiology is a part of the microbiology branch dealing with beneficial or harmful microbes associated with either plants or soil. This manual focuses on beneficial microbes dealing with soil fertility, microbial degradation of organic matter, soil nutrient transformations, and biocontrol agents. Nowadays, techniques involved in the study of beneficial microbes in agricultural microbiology toward enhancing global agricultural productivity are in trend. This manual covers a wide range of basic and advanced techniques associated with research on the isolation of agriculturally important microbes, identification, biological nitrogen fixation, microbe-mediated plant nutrient use efficiency, and biological control of plant diseases and pests. Introduction to each protocol explains the role/importance of chemicals involved, uniqueness, and protocol application. A proper understanding of the protocol helps the researchers to manipulate them as per their need.

This book is composed of seven parts with 52 protocol chapters. Parts I and II represent the importance, isolation, and purification methods of agriculturally important microbes and include mineral-solubilizing microbes. Part III deals with phytohormones quantitative protocols directly or indirectly associated with microbes. Parts IV and V provide deep insights into protocols for screening agriculturally important enzymes and compounds related to biocontrol activity. Part VI represents assessment methods of soil microbial activity by soil respiration. The final Part VII deals with protocols for selecting microbial strains for inoculant production and quality control ultimately representing commercial biofertilizers production criteria. This book will help postgraduate students, research scholars, postdoctoral fellows, and teachers belonging to different disciplines of Plant Microbiology and Pathology. Moreover, this manual may also serve as a textbook for undergraduate courses like Techniques on Plant-Microbe Interaction/Biological Control of Plant Diseases/Nutrient Use Efficiency.

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Chapter 48

Isolation and Screening of ACC Deaminase-Producing Microbes for Drought Stress Management in Crops

Satish V. Patil, Chandrashekhar D. Patil, and Bhavana V. Mohite

Abstract

The compound aminocylopropane-1-carboxylic acid (ACC) is the product of Yang cycle, which plays a vital role in drought response, salt stress, host–pathogen interactions, seed germination, flowering, and fruit ripening. While ACC is produced within the plant, some of it is exuded from the roots where it may alter the rhizosphere microbial composition. Within the plant, ACC may be converted to the phytohormone ethylene by the action of the plant enzyme ACC oxidase (ACO). When the rhizosphere or the plant endosphere contains microbes that produce ACC deaminase, some of the ACC is converted to alpha ketobutyric acid and ammonia. Thus, reducing ethylene production and decreasing the negative effects of ethylene including numerous drought-like symptoms, thereby reduces the deleterious effects of drought stress. ACC deaminase-producing microorganisms may readily be isolated by using minimal medium containing ACC as sole source of nitrogen and a pH indicator. The microbial cleavage of ACC changes the pH of the growth medium and causes an easily detectable color change around the colony. Subsequently, qualitative or quantitative assays for alpha ketoglutaric acid or ammonia may be used to assess the level of ACC deaminase activity.

Key words PGPR, ACC deaminase, Screening, pH dye, Phenol red

1 Introduction

The studies of Yang and his colleagues pioneered the research to unlock the mystery of freshness of fruit, flowers, defoliations, the ripening of fruits, etc. through the Yang cycle [1]. Ethylene has a vital role in host–pathogen interactions, seed germination, flowering, fruit ripening, and the response of plants to both biotic and abiotic stress [2].

Yang's studies proved that the synthesis of S-adenosylmethionine is an intermediate compound which further converted to aminocylopropane-1-carboxylic acid (ACC) and then, by the action of the enzyme ACC oxidase to ethylene (Fig. 1).

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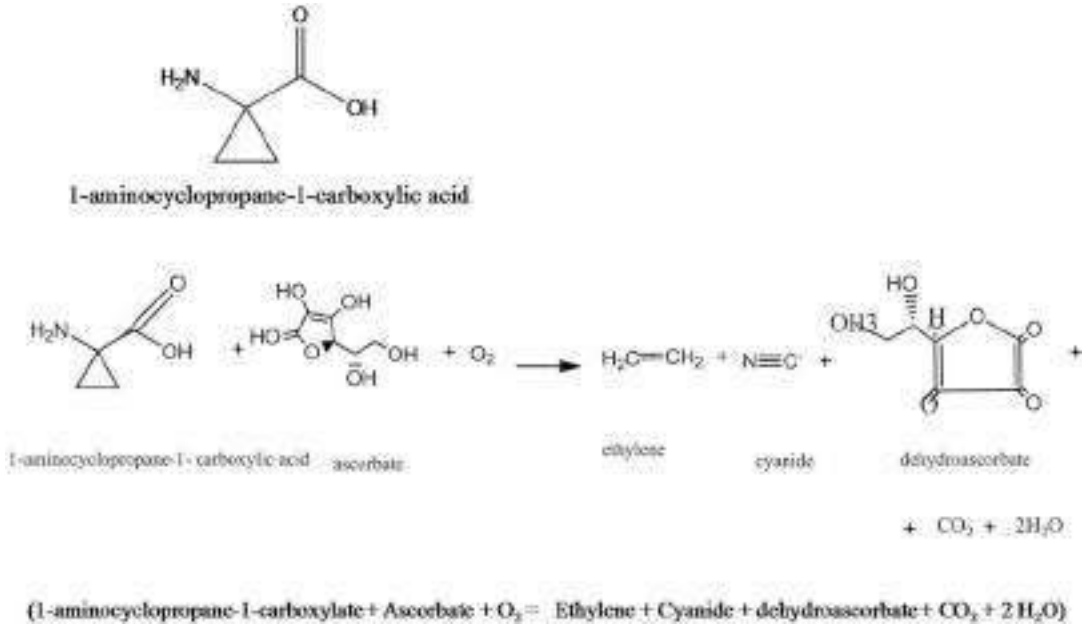

Fig. 1 Conversion of ACC into ethylene

In plants, ethylene level has been found to be rapidly increased following the onset of drought and other stress conditions [2, 3]. This ethylene synthesis is a marker of the stress condition and is sometimes known as stress ethylene.

1.1 Amino-cyclopropane-1-Carboxylic Acid Deaminase and Its Role

Glick et al. [4] described the role and importance of some plant growth promoting *Rhizobacteria* in management of drought stress and various physiological activities of plants. This work showed that the ACC is produced in greater quantities during drought stress; ACC is exudated outside by the root cells. The plant growth inducing bacteria around the roots are recognized for their various beneficial activities. These microbes utilize the plant exudates including ACC by using ACC deaminase which helps to maintain the balance between the inner and outer ACC level. As more of the ACC gets secreted outside the plant roots, it reduces the concentration of ACC that can be used for the synthesis of ethylene inside the plant cells. In this way by utilizing ACC, rhizospheric microbes help to reduce the ethylene production that occurs during drought conditions. This leads to less defoliation, more root elongation, increased nodulations, and increased transpiration activities [5] (Fig. 2).

Various rhizospheric microbes have been reported to have ACC deaminase-producing potential, e.g., *Enterobacter cloacae*, *Pseudomonas putida*, *Pseudomonas alcaligenes*, *Hansenula* spp., *Rhizobium*, *Sinorhizobium* spp., *Pseudomonas chlororaphis*, *Rhizobium leguminosarum*, *Bacillus subtilis*, *Penicillium citrinum*, etc. [6].

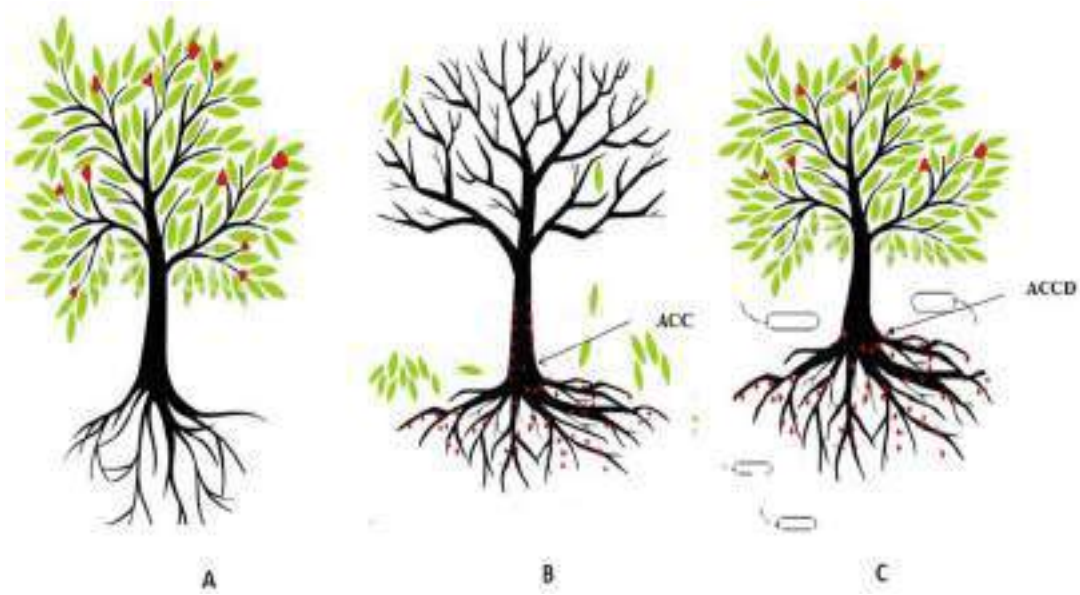

Fig. 2 ACC deaminase-producing microorganisms are attracted towards plants during stress conditions. (**a**) Normal plant, (**b**) Under the stress conditions the level of ACC increases and subsequent formation of ethylene causes defoliation and root growth inhibition. (**c**) The rhizospheric microorganisms utilize ACC in root exudates through the functioning of ACC deaminase and decrease the effects of the stress condition

ACC deaminase is a member of the oxidoreductase enzymes, a multimeric enzyme having monomeric subunit of molecular mass of 35–42 KDa [5]. ACC deaminase catalyzes the ACC to alpha ketobutyric acid and ammonia. It was reported that D-serine and D-cysteine can also act as substrates for ACC deaminase. ACC deaminase has a reported optimum pH and temperature of 8.5 and 30–35 °C, respectively [7–9].

1.2 Principle of Assay

This assay combines the two classical approaches as shown in Fig. 3, "Reproduced from Patil et al. [10] with permission from [Elsevier]" (1) Cultivation of test microorganisms on ACC-containing minimal medium [11] and (2) addition of pH indicator dyes phenol red and bromothymol blue.

The ACC deaminase-producing microbes utilize the synthetic substrate, ACC as a sole nitrogen source in minimal medium. The growing bacteria show a zone of clearance relative to its capacity for substrate utilization. The pH indicator dye shows a zone of color change around the colony due to the production of ammonia, a byproduct of the catalytic reaction of ACC deaminase.

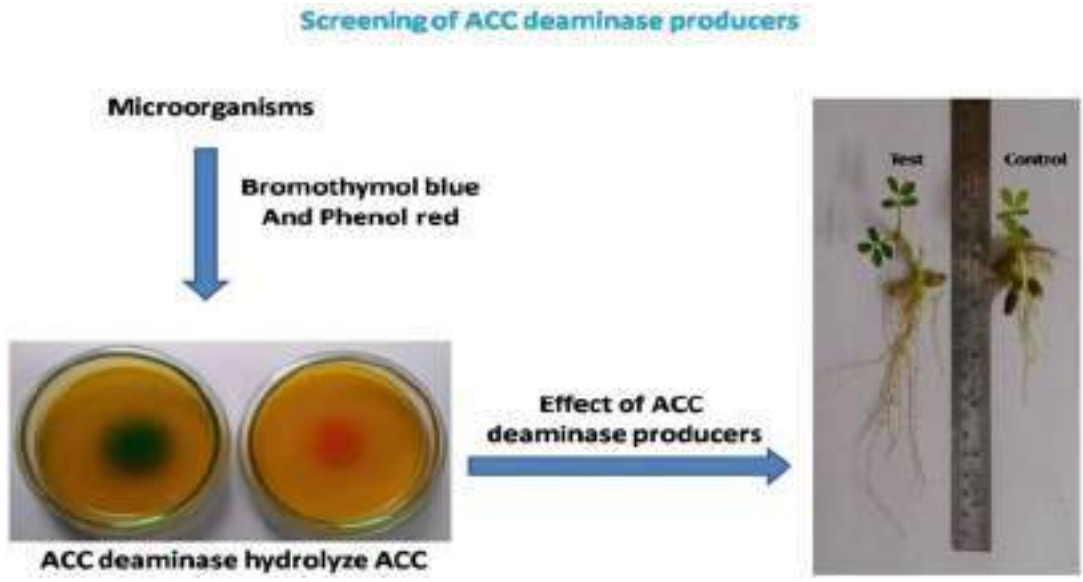

Fig. 3 Example of isolation and application of an ACCD producing microorganism and plant growth assay. ("Reproduced from Patil et al. [10] with permission from [Elsevier]")

2 Materials

2.1 Isolation of Rhizobacteria and Qualitative Estimation of ACC Deaminase Activity

1. Rhizospheric soil of plants, especially from drought areas.
2. Sterile plastic screw cap bottle.
3. Sterile saline tubes for serial dilution of the soil samples.
4. Sterile modified minimal medium.
5. Sterile Nutrient or LB agar.
6. Modified Dworkin and Foster salt medium.
7. Stock solution of trace elements.

2.2 ACC Deaminase Enzyme Assay

1. α-ketobutyrate.
2. 0.1% 2, 4-dinitrophenyl hydrazine solution.
3. 2 N sodium hydroxide.
4. 0.1 M Tris (hydroxymethyl amino methane) buffer (pH 7.5).
5. 0.56 N hydrochloric acid (HCl).
6. Trichloroacetic acid.
7. Nesslers Reagent.
8. Ammonium sulfate.
9. Potassium bromide.

3 Methods

3.1 Stepwise Procedure for the Isolation and Screening of ACC Deaminase-Producing Microbes from Soil

1. Prepare 100 ml of medium by mixing sterile modified minimal medium and the sterilized agar-agar solution, mix well by rotating flask and pour solution in plates.
2. Allow the plates to solidify.
3. Make serial dilutions of the test soil sample.
4. Streak the diluted soil suspension of dilutions 10^4–10^7 on solidified ACC plates, and incubate for 48 h at 37 °C.
5. Observe the color change around the colonies (Fig. 3) [10].
6. Subculture the pure colonies on nutrient or LB agar.
7. Make spot of diluted colony at the center of ACC plates and then incubate the plates for 48 h.
8. Measure the zone of color change as a function of time.
9. Compare the isolated colonies with the zone of color changes and note the colony characters.

3.2 Collection of Soil

For the isolation of ACC deaminase producers, rhizosphere soil of plants, especially from drought areas, should be collected in sterile plastic bottles. In addition, other rhizosphere soils from different locations may also be useful. Collect 1 g of rhizosphere soil in a sterile plastic screw cap bottle, close the bottle, label it, and store at 4 °C or freeze until further processing.

3.3 Isolation of Rhizobacteria and Qualitative Estimation of ACC Deaminase Activity

Dilute the collected soil sample up to 10^{-9} dilution in sterile 10 mL tubes, mix well and incubate for 10 min at room temperature. Then prepare modified Dworkin and Foster salt medium containing in g/l: Glucose, 2.0; KH_2PO_4, 4.0; Citric acid, 2.0; Gluconic acid, 2.0; $MgSO_4 \cdot H_2O$ 0.2; Na_2HPO_4, 6.0; and 3 mM amino cyclo propane, 594 ml; Distilled water to 1 l; mix this solution well.

Prepare stock solution of trace elements as follows: add $FeSO_4 \cdot 7H_2O$, 10 mg in 10 ml distilled water; 100 mg H_3BO_3 in 10 ml DW; 110 mg of $MnSO_4 \cdot H_2O$ in 10 ml DW; 1240 mg $ZnSO_4 \cdot 7H_2O$ in 10 ml DW; 780 mg $CuSO_4 \cdot 5H_2O$ in 10 ml DW; and 100 mg MoO_3 in 10 ml DW. Add 1 ml of each trace solution into above prepared Modified DF medium; maintain final pH of the medium 6.8–7.2. Then add 1 ml of bromophenol blue or phenol red into the medium mix, plug with cotton, and wrap with paper. To a separate flask, add 30 g of agar powder in 400 ml DW, mix well, plug the flask with cotton, wrap with paper and sterilize both preparations. After sterilization, allow the medium to cool to 40 °C and mix in agar solution into the modified DF medium and prepare plates.

3.4 ACC Deaminase Enzyme Assay

Preparation of standard curve of α-ketobutyrate**:** For quantitative assay, prepare stock of alpha-ketobutyrate (0.1–1.0 μM) and add 0.3 ml of 0.1% 2, 4-dinitrophenyl hydrazine solution to it and incubate mixture at 30 °C, for 30 min. Thereafter add 2 ml of 2 N sodium hydroxide to the above mixture and measure the absorbance at 540 nm, and prepare a standard graph. For test organism capacity, estimate protein content of crude enzyme/fermented broth of isolate by the Bradford method and express activity by measuring the nano mole/1 μM of alpha keto butyrate liberated per mg of cellular protein per hour.

3.4.1 ACC Deaminase Assay

For estimation of α-ketobutyrate, mix the enzyme extract (0.2 ml) and ACC (50 mM) and incubate in 0.2 ml of 0.1 M Tris (hydroxymethyl amino methane) buffer (pH 7.5) at 30 °C for 30 min. Terminate the reaction by adding 1.8 ml of 0.56 N hydrochloric acid (HCl). Add 0.3 ml of 0.1% 2,4-dinitrophenyl hydrazine solution and keep the mixture at 30 °C for 30 min. Thereafter, add 2 ml of 2 N sodium hydroxide in the above mixture and measure the absorbance at 540 nm. One unit of enzyme activity is defined as the "amount of enzyme liberating 1 μM of α-ketobutyrate/min." Perform the experiment at least in triplicate. (Reaction mixture can also be used for confirmation of activity by FTIR (*see* **Notes 1** and **2**).

3.5 Confirmatory Test for ACC Deaminase Production

In addition to α-ketobutyrate measurement, ACC deaminase production can be confirmed qualitatively and quantitatively by analyzing the release of ammonia from ACC. The procedure includes: Take a 100 μl mixture of positive bacteria and 400 μl 0.3 M ACC in Tris buffer solution and incubate at 37 °C for exactly 45 min. After the incubation add 250 μl trichloroacetic acid 24.5% (w/w) and centrifuge the mixture at 50,000 rpm. Collect the supernatant and add 250 μl of the supernatant to the Nesslers solution (2000 μl water plus 250 μl Nesslers reagent). Measure the optical density at 450 nm and compare the absorbance with an ammonium sulfate-Nesslers-standard curve based on daily calibration at concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, and 2.5 mM. The units of enzyme activity are defined as micromoles of ammonia released per minute.

3.6 Quantitative Estimation

By using standard graph of α-ketobutyrate, extrapolate the reading of test sample (ACC deaminase-producing bacteria) and determine amount of α-ketobutyrate released where one unit of enzyme activity measured is the amount of enzyme liberating 1 μg of α-ketobutyrate/min.

4 Notes

1. For confirmation of the α-ketobutyrate liberation after a deamination reaction by microbial ACC deaminase activity, the reaction mixture or broth may be used for potassium bromide

(KBr) cell pellet preparation and analysis with the help of Fourier-transform infrared spectroscopy (FTIR).

2. In FTIR spectra analysis results, observe peaks at 1689 and 3343 cm^{-1}, which confirm the presence of a ketonic group and amino functional group, respectively recognized as α-ketobutyrate [12].

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Preface

Agricultural Microbiology is a part of the microbiology branch dealing with beneficial or harmful microbes associated with either plants or soil. This manual focuses on beneficial microbes dealing with soil fertility, microbial degradation of organic matter, soil nutrient transformations, and biocontrol agents. Nowadays, techniques involved in the study of beneficial microbes in agricultural microbiology toward enhancing global agricultural productivity are in trend. This manual covers a wide range of basic and advanced techniques associated with research on the isolation of agriculturally important microbes, identification, biological nitrogen fixation, microbe-mediated plant nutrient use efficiency, and biological control of plant diseases and pests. Introduction to each protocol explains the role/importance of chemicals involved, uniqueness, and protocol application. A proper understanding of the protocol helps the researchers to manipulate them as per their need.

This book is composed of seven parts with 52 protocol chapters. Parts I and II represent the importance, isolation, and purification methods of agriculturally important microbes and include mineral-solubilizing microbes. Part III deals with phytohormones quantitative protocols directly or indirectly associated with microbes. Parts IV and V provide deep insights into protocols for screening agriculturally important enzymes and compounds related to biocontrol activity. Part VI represents assessment methods of soil microbial activity by soil respiration. The final Part VII deals with protocols for selecting microbial strains for inoculant production and quality control ultimately representing commercial biofertilizers production criteria. This book will help postgraduate students, research scholars, postdoctoral fellows, and teachers belonging to different disciplines of Plant Microbiology and Pathology. Moreover, this manual may also serve as a textbook for undergraduate courses like Techniques on Plant-Microbe Interaction/Biological Control of Plant Diseases/Nutrient Use Efficiency.

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Chapter 45

Isolation and Screening of Phytase Producing Microorganisms: An Essential Bioinput for Soil Fertility

Bhavana V. Mohite, Kiran Marathe, Narendra Salunkhe, and Satish V. Patil

Abstract

Phytase is one of the identified enzymes which plays a vital role in phosphorous (P) cycle by mobilization of immobilized P from plant material. Although there is various phosphate solubilizing biofertilizer agents reported, they are majorly targeted for mobilizing rock phosphates in soil. The Phosphate solubilizing biofertilizers are required for mobilizing the immobilized form of P from plant source i.e., from phytate. The protocol is based on the principle that specific phytate agar medium contains calcium/sodium phytate, which is utilized as P source by phytase producing microorganism indicated by zone of clearance around the colony. The further quantification is executed by measuring the free phosphate by spectrophotometric method.

Key words Phytase, Phosphate, Phosphorous, Phytate, Macronutrient

1 Introduction

Phosphorous (P) is one of the major macronutrients for plant growth. It contributes approximately 0.2% of plant's dry biomass. It is important component in nucleic acid, ATP, phospholipids, etc. which contribute for plant growth and metabolic activity. P determines many vital activities like development of flower, seed development, germination and maturity, and consequently majorly affects the crop maturity and yield [1]. Besides these, various vital activities like N-fixation in legumes, energy metabolism, synthesis of membranes, photosynthesis, respiration, enzyme regulation, crop quality, and resistance against various biotic and abiotic stress are also subject to P nutrition. Soil has always less utilizable form of P for plant; it warrants the application of P fertilizers in soil. The reaction with metals, absorption and precipitation of inorganic P in soil are major physical phenomenon which cause added fertilizers unavailable to plants. The high accumulation of unavailable phosphate also leads to eutrophication and infertility of soil. In plants, P

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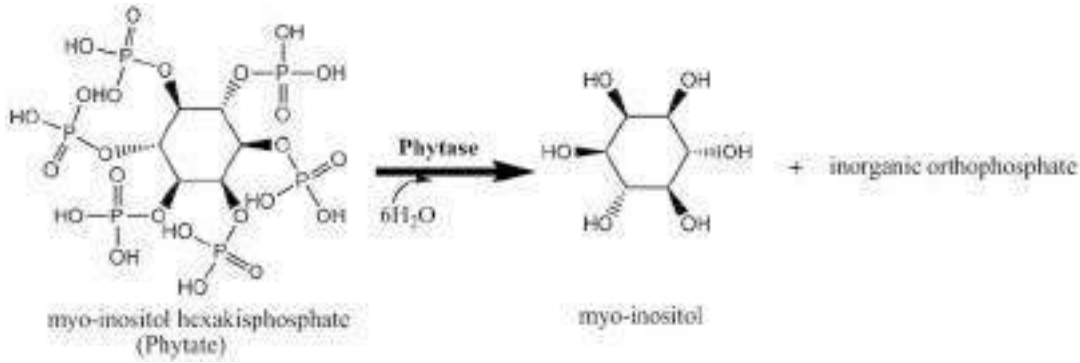

Fig. 1 Mechanism of phytase action for phytate solubilization. (Reproduced from Mohite et al. [3] with permission from Springer Nature)

is in organic form of phytate which forms salts with monovalent and divalent cations like Fe^{2+}, Mn^{2+}, K^{+}, Mg^{2+}, and Ca^{2+}, making it unavailable for assimilation [2]. Phytases are group of enzymes which naturally make this P available for recycling in nature (Fig. 1). Phytases are classified according to pH activity, catalytic mechanism, source and initiation of site of dephosphorylation of substrate. As per IUPAC-IUBMB, phytase grouped in major three classes, i.e., 3-phytases (EC 3.1.3.8), 6-phytases (EC 3.1.3.26), and 5-phytases (EC 3.1.3.72), which further divided as acid and alkaline phytases on basis of optimum pH [4] (Fig. 2). As per recent reports, microbial phytases are considered as new bioinputs for soil as various fungi and bacteria utilize phytate as substrate and make available in assimilable form to crops or plants [5, 6].

1.1 Principle of the Assay

The microorganism having phytase producing capacity will utilize phytate as phosphorus source and indicate by calcium/sodium phosphate dissolution. The phytate dissolution by organic acid will be differentiated by flooding cobalt chloride, ammonium molybdate, and ammonium vanadate solution, these agents reprecipitate the acid solubilized phytases, and the zone due to organic acid dissolution will be disappeared (*see* **Note 1**).

2 Materials

All the culture media used are from HiMedia, Mumbai including the sodium selenite. Pure grade distilled water was used for dilution.

1. Calcium or sodium phytate.
2. 0.2 M sodium acetate buffer (pH 4.0).
3. Trichloroacetic acid.

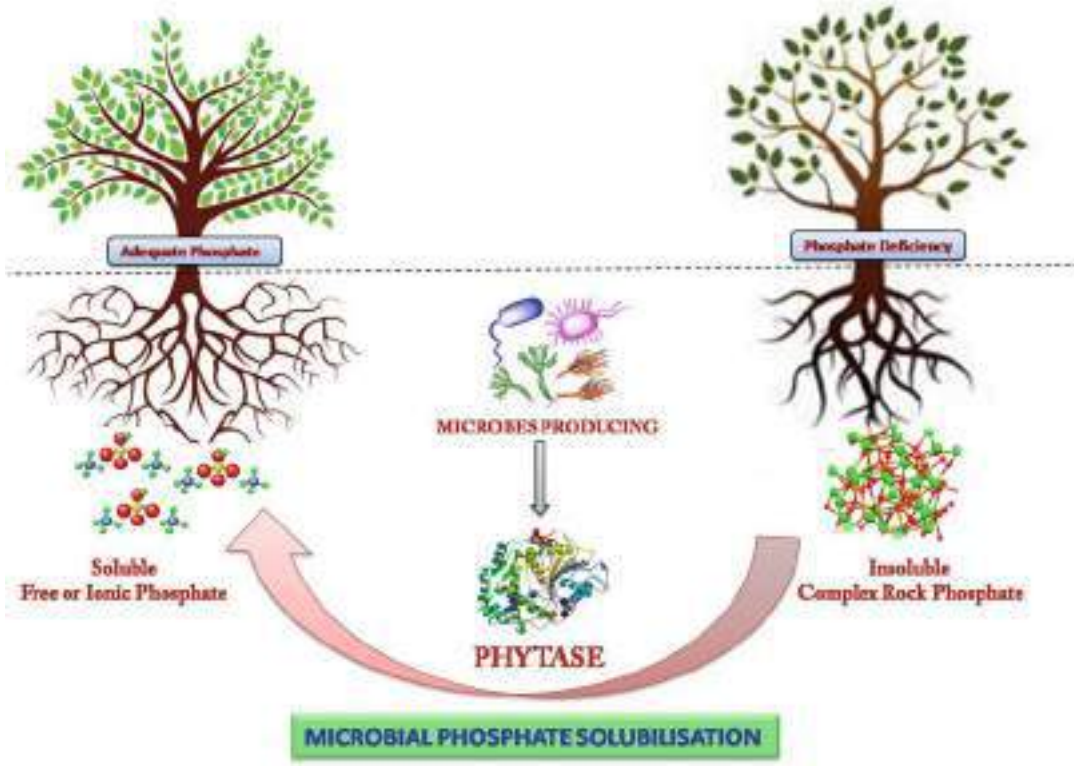

Fig. 2 Microbial phosphate solubilization. (Reproduced from Mohite et al. [3] with permission from Springer Nature)

4. 100 mM glycine/HCl buffer (pH 2.5).
5. Rhizospheric soil sample from the agricultural crops such as wheat or maize and root nodules of the bean crops.
6. **Phytase Isolation agar** (g/l): Glucose 10.0, Calcium Phytate 1.0, Ammonium nitrate 0.1, KCL 0.5, NH_4NO_3 0.5, $MnSO_4 \cdot 7H_2O$ 0.01, and $FeSO_4 \cdot 7H_2O$ 0.01. Dissolve in slightly hot water, add 3.0 g agar agar powder, and sterilize at 21 °C for 15 min.
7. 10 mM sodium phytate and 100 mM glycine/HCl buffer (pH 2.5).
8. Confirmation reagents: **Reagent A**—2% (w/v) aqueous cobalt chloride solution and **Reagent B**—10 ml (25% (w/v) ammonium molybdate + 10 ml 0.42% (w/v) ammonium vanadate solution.
9. Ammonium molybdate solution (AMS): 1.5 g ammonium molybdate in 5.5% sulfuric acid.
10. 2.7% Ferrous sulfate w/v.

11. Reagent for phosphate estimation: 4 ml of AMS + 1 ml of ferrous sulfate solution.

3 Methods

1. Take 1.0 g of collected soil sample, add in 9.0 ml sterile saline, mix well by shaking and then further dilute up to ten folds, i.e., 10^{-9}.
2. Prepare phytase agar by using pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize them at 121 °C for 20 min under 15 psi pressure.
3. Let the media cool at 40 °C, pour it into the sterile petri plates and incubate it at room temperature for 2–3 h, for solidifying. Also prepare control plates of each culture media but without calcium phytate.
4. Take loopful of soil suspension 10^{-5} to 10^{-6} dilution tube and streak on phytate agar plates.
5. Incubate the plates at room temperature up to approximately 48 h, and observe that the plates for growth appear with hollow zone around colonies.
6. Compare the phytate containing plates with the control plates, colonies showing high zone of dissolution, subculture it for further study.
7. The confirmation of phytase production, i.e., phytate solubilization due to phytase but not by organic acid or other metabolic product, could be confirmed by counterstaining treatment [7]. In brief, do spot inoculation of potent phytase producer on sterile phytate agar, and allow growing at 48 h as per organism optimum incubation period. After incubating flood plate with reagent A (2% cobalt chloride solution), incubate for 5 min. Remove extra solution from the plate and overlay the petri plates with Reagent B (ammonium molybdate solution and ammonium vanadate). Incubate plates for 5 min. Observe for the zone of clearance (*see* **Note 2**).
8. The zone of dissolution after counterstain indicates the real phytate dissolution by phytase.
9. **Quantitative assay**: In quantitative phytase assay, the active culture grow in 0.1% sodium phytate containing medium for 24 h, and centrifuge supernatant as 10,000 lb for 10 min [8]. The crude enzyme in supernatant is collected. Add 1 ml of supernatant broth in 600μl of sodium phytate solution, incubate at 37 °C for 30 min. Terminate the reaction by adding 700μl of 5% TCA solution. After that take 1 ml of this reaction mixture, add 0.5 ml AMS reagent. Thereafter phytase activity is

determined as micromole inorganic phosphate release per min at 700 nm by using standard graph of $K_2H\ PO_4$ (*see* **Note 3**).

4 Notes

1. The zone after counterstaining avoids false results of phytate dissolution and pseudo phytase activity. The colony shows dissolution on phytase agar but not after counter staining confirm that phytate utilization is not either by metabolic product of media utilization or by organic acid production.
2. Prepare counterstain **Reagent A**, i.e., (cobalt chloride solution) and **Reagent B** (ammonium molybdate solution and ammonium vanadate mixture) in distilled water.
3. One unit phytase activity (U/mL) was defined as the amount of enzyme required to liberate 1μmol of inorganic phosphate per minute.

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Chapter 31

Isolation of Selenium Biotransforming Microbes as New Age Bioinputs

Pradnya B. Nikam, Narendra Salunkhe, Vishal Marathe, Bhavana V. Mohite, Satish V. Patil, and Vikas S. Patil

Abstract

Selenium (Se) has a very narrow gap between its toxicity and benefits to the different forms of life which makes it as an essential micronutrient for living creatures including its importance in plant growth. It is highly soluble having toxic form in the environment such as the oxyanions; selenate and selenite needed to be transformed to the less toxic elemental selenium which has wide number of benefits to the ecosystem, if provided in appropriate required amount. Till now most of the microorganisms have been studied for biotransformation of selenite by using them as electron acceptor in the respiratory mechanism and ultimately reduced to elemental selenium in the form of nanoparticles. Despite of this, many of the microbes are unexplored and the Se-nanoparticles still less studied for their applications. This chapter explains a simple method for isolating the selenium biotransforming microorganisms from nearby soil sample into the red elemental Se in its nano form.

Key words Selenium, Selenite, Thioredoxin reductase, Selenobacter, Antioxidant

1 Introduction

Selenium (Se) has been proven as an essential micronutrient for almost all living organisms, which is also found important for plants in various aspects. Its presence in the environment ranges from inorganic oxyanions to organic selenium containing amino acids. In human beings, various enzymes such as glutathione peroxidase, thioredoxin reductase, iodothyronine deiodinase, and formate dehydrogenase have selenium as their important component and thus function as an antioxidant, preventing tumors, metabolism, other therapeutic uses and even in reproduction [1, 2]. In the environment, Se exists as highly soluble oxyanions such as Selenate (SeO_4^{-2}) and Selenite (SeO_3^{-2}) which are toxic for humans, hence

Satish V. Patil and Vikas S. Patil contributed equally to this work.

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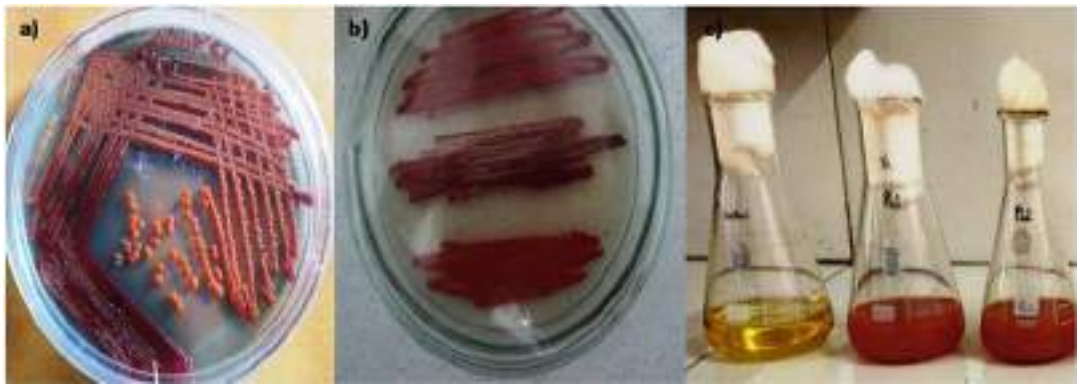

Fig. 1 (**a** and **b**) Isolated colonies of selenium transforming bacteria showing brick red color colonies; (**c**) Transformation of selenium in enrichment medium showing color change from yellow (control) to brick red (test isolates, PI1 and PI2) (Source: Satish V Patil's Laborotory KBC NMU, Jalgaon)

transformed into less toxic and less soluble elemental form (Se^0) by different microbial species through Se cycle. Many of the plants are seen to accumulate these Se oxyanions from the soil and form selenocysteine or may carry formation of selenomethionine, further converting it into volatile dimethylselenide (DMSe). Though Se is not essential for plant growth, it helps the crops to withstand the drought conditions, protects against oxidative stress and sometimes from the pests too [3].

As the distribution of selenium is uneven in the environment, it becomes important to provide it as less toxic and less soluble Se^0, mostly in colloidal form for the living creatures. This can be achieved by isolating the microbes from different areas especially the agricultural rhizospheric soil or also from the endophytic microbes within the roots, as plants have the potential of accumulating the Se at any available concentrations from the soil. The agricultural rhizospheric soil and nodule samples are preferred considering the compatibility of synthesized Se-nanoparticles for further applications in agricultural practices and also most of them will be non-pathogenic [3–5]. When the sample is enriched in the growth medium supplied with selenite or selenate as a Se source, within 24–48 h the visual brick red color starts appearing along with the growth which signifies the transformation of Se by the respective organism (Fig. 1). This transformation relies on the growth phase and also the initial Se concentration in the medium, as reported earlier. Several reports declare this transformation as synthesis of the Se^0 nanomaterial either extra- or intracellular by the microbes [6–8].

2 Materials

All the culture media used are from HiMedia (Mumbai, India) including the sodium selenite. Pure grade distilled water was used for dilution.

1. Rhizospheric soil sample from the agricultural crops such as wheat or maize and root nodules of the bean crops.
2. Sterile nutrient agar media for bacterial isolation.
3. Sterile Sabouraud dextrose agar for fungal isolation.
4. Sterile yeast extract mannitol agar (YEMA) to culture root nodule bacteria (*Rhizobia*).
5. Sterile saline tubes (10 tubes) for serial dilution of the soil samples.
6. Sodium selenite (Na_2SeO_3) (MW—172.94 g/mol).

3 Methods

1. Take 1.0 g of collected soil sample, add in 9.0 ml sterile saline, mix well by shaking and then further dilute up to tenfolds, i.e., 10^{-9} (*See* **Note 2**).
2. Prepare nutrient agar media, Sabouraud dextrose agar, and yeast extract mannitol agar by using pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize them at 121 °C for 20 min under 15 psi pressure in the autoclave.
3. Let the media cool at 40 °C, and then add sodium selenite in nutrient agar media as well as in Sabouraud dextrose agar (5 mM per volume of medium). Rotate flask clockwise and anticlockwise for uniform distribution of selenite (*See* **Note 1**).
4. Pour it into the sterile petri plates and allow solidifying at room temperature for 2–3 h. Also prepare control media plates of each culture media without sodium selenite.
5. Take loopful of soil suspension from 10^{-5} to 10^{-6} dilution tube and streak on selenite containing nutrient agar media and Sabouraud dextrose agar plates.
6. Incubate the plates at room temperature up to approximately 48 h; observe the plates for growth changed to red color.
7. Compare the sodium selenite containing plates with the control plates to examine the exact biotransformation of Se and formation of red colored colonies.
8. These isolates can further be inoculated in liquid enrichment broth medium supplemented with sodium selenite at 5 mM

concentration for selenite biotransformation and conversion to the elemental Se-nanoparticles which can be optimized and characterized further.

9. The synthesized nanoparticles are separated by centrifugation at 10,000 rpm for 5–8 min, and collecting the pellet and purifying with double distilled water and ethanol by washing three times to remove the untreated sodium selenite or other impurities. The nanosuspensions then pass through the dialysis membrane (6000–8000 KD cutoffs). The purified particles then lyophilized and stored at low temperatures which will be used for characterization by various spectroscopic methods.
10. Se-nanoparticle is characterized by UV–Vis absorption spectrophotometer by recording the spectra (200–500 nm).
11. The lyophilized powder is used for further particle characterization such as particle size, shape, and crystallinity by using scanning electron microscopy, transmission electron microscopy, dynamic light scattering (DLS), and X-ray diffraction.

4 Notes

1. Avoid adding sodium selenite into the media preparation before autoclaving as this may result into precipitation of the salt and forming red color due to heat and pressure, prior to the addition of microbial culture sample.
2. For the rhizospheric endophytes and the rhizobia, surface sterilize the material with 70% ethanol or 0.01% mercuric chloride washing. Then crush the sample in sterile saline, further dilute and streak the specific dilution on sterile YEMA or endophyte isolation agar medium.

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Chapter 30

Isolation and Screening of Silicate Solubilizing Microbes: Modern Bioinputs for Crops

Chandrashekhar D. Patil, Bhavana V. Mohite, Rahul K. Suryawanshi, and Satish V. Patil

Abstract

Pertaining to the importance of silica as fertilizer for various crops, silica solubilizers become new component of biofertilizers for sustainable crop and fertility management. Importance of silica metal has been identified for tolerance of biotic and abiotic factors, growth enhancement, etc. Soil is rich source of silica however; it is majorly available in un-utilizable form of silicates. Therefore, silicate solubilizing microbes are essential part of micronutrient management program. The present protocol describes method of isolation and screening of silicate solubilizers from various sources. The application of pH indicator dyes in medium will also help to find out microbe-mediated mechanism of silicate solubilization. Medium with differential sources of immobilized or polymeric silicates will allow isolation of potent and versatile silicate solubilizers useful as modern bioinputs for crops.

Key words Silicate, Rice, Drought, Organic acid, Ammonia

1 Introduction

The silicon (Si) is one of the abundant component of soil. Si was previously considered as nonessential element for crop development, but recent findings proved significance of Si to induce tolerance of biotic and abiotic factors and growth enhancement by carbon photoassimilation in crops like rice, maize, wheat, etc. [1–3] (Fig. 1) and play vital role in development of pathogen resistance in silicon accumulating crops like rice. Various diseases of rice crop, rice blast, powdery mildew, brown spot, stem rot and sheath brown rot on rice were found to be successfully managed by new bioinputs such as silicon fertilizer application [5–7]. Additionally, Si is also known to facilitate the availability of other micro- and macronutrients such as phosphorus, potassium, zinc, and copper [4] (Fig. 1).

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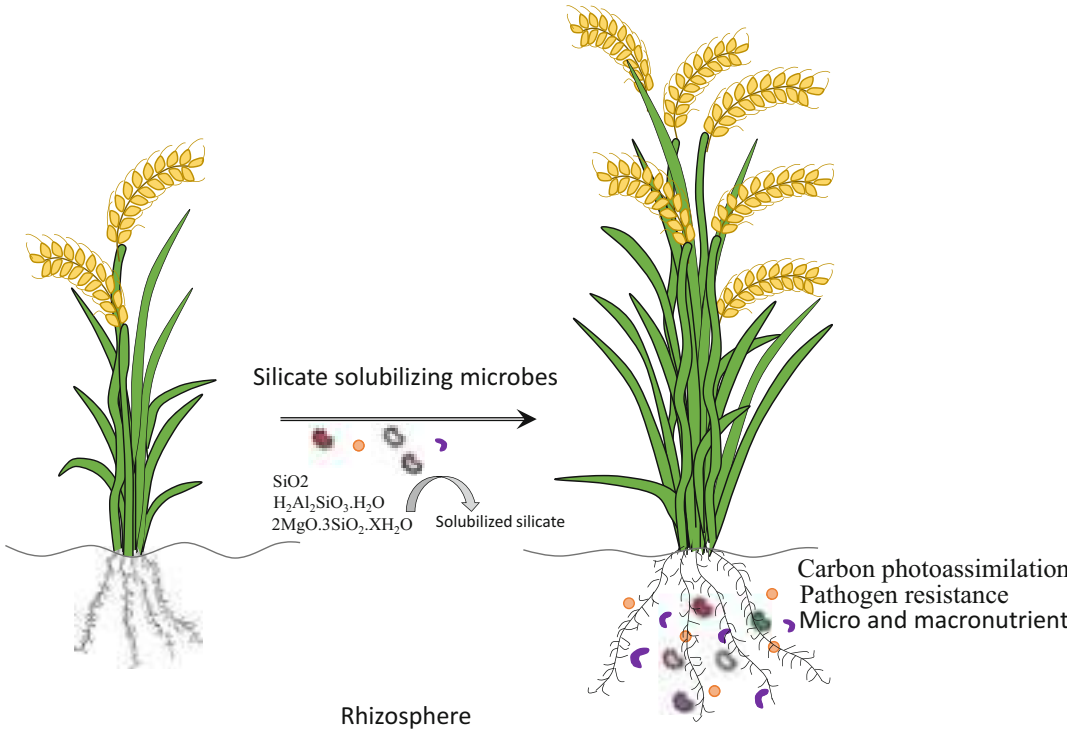

Fig. 1 Graphical representation of silicate solubilizing microorganisms for improving silicate bioavailability

Silicon is abundantly available in soil but in immobilized or mineral form which is unavailable for direct assimilation by plant. The need of plant is fulfilled by extraneous addition of silicon fertilizers, but at majority times silicon fulfillment is done by adding industrial slag, which causes other heavy metal contamination in farm soil. Besides these, silicon fertilizers in market such as bentonite, mica feldspars, and diatomaceous earth are present but they contain very little bioavailable Si and so require application of higher dose. Currently, potassium silicate is applied as popular silicon fertilizer but it is too expensive, and may cause phytotoxicity, eutrophication, etc. The application of Si solubilizing biofertilizers proved most ecofriendly, cheap, and significant to fulfill the crop Si need.

Silicon solubilization is first reported by Aleksandrov et al. [8] during their work on microbial phosphoric acid liberation by silicate bacteria. Various microbes were reported for silicon depolymerizing/solubilizing potential, i.e., Rhizobium, Burkholderia, *Pseudomonas* sp. [9]. Some studies proved that fungi actively solubilize the silicate mineral from rock mineral, e.g., *Fusarium oxysporum* [10, 11].

The naturally occurring silicon in the form of soil minerals is majorly converted by the process of withering into the monosilicic acid which is assimilated by plant roots. The withering of silicon

Fig. 2 (**a**) Isolation of silicate solubilizers on silicate agar, (**b**) silicate solubilization by bacteria, (**c**) silicate solubilization by fungi, (**d**) confirmation silicate solubilization mode of action by pH indicator silicate (Phenol red). (Isolates From Dr. SV Patil, Laboratory School of Life Sciences, KBC, NMU, Jalgaon)

minerals take place by various ways, i.e., by organic and inorganic acids, actions of divalent cations, by nucleophilic attacks of alkali compounds, etc. Majority of these products are produced by microbial action.

Recent studies revealed that the foliar applications of silicate solutions stimulate growth of wheat cereals, soybean, rapeseed, sugar beet, potato, meadows, berries and vegetables, as well as orchard and ornamental plants in diverse abiotic stress like drought by stimulating chlorophyll production, maintaining water content and cellular membrane integrity [12].

1.1 Principle of Assay

Plant rhizosphere is a rich source of silicate solubilizers. Screening of silicate solubilizers uses a classical approach of cultivating test microorganisms isolated from plant rhizosphere on complex silicate mixture containing minimal medium and isolating microbes capable of making zone of clearance (Fig. 2). pH indicator dye can be used to detect acid or alkali based microbial mechanisms of silicate solubilization. The solubilized zinc and its presence in plant can further be estimated through colorimetric, coupled plasma mass spectrometry or absorption/emission spectrometry.

2 Materials

1. Rhizospheric soil sample from the agricultural crops such as rice, wheat or maize and root nodules of the bean crops (*see* **Note 2**).
2. Sterile saline tubes (10 tubes) for serial dilution of the soil samples.
3. **Following media will be needed for isolations of different silicate solubilizers**
   (a) **Sterile Silicate Agar for Bacteria:** (g/L) Magnesium sulfate 0.1, Calcium carbonate 0.1, magnesium trisilicate [$Mg_2O_8Si_3$] 1.0 or Potassium alumino silicate 1.5,

Glucose 1.0, Ferric chloride 0.005, Calcium phosphate 1.0, ammonium sulfate 0.2, Agar 30 g, Final pH (at 25 °C) 7.2 ± 0.2 + 5 ml of 10 mg% Phenol red or 20 mg% bromothymol blue per liter (*see* **Note 1**).

(b) **Sterile Silicate Agar for Fungi:** (g/L) Glucose 0.5, Yeast extract 0.5, Ammonium sulfate 0.4, magnesium trisilicate [$Mg_2O_8Si_3$], Potassium alumino silicate 1.5, Calcium phosphate 1.0, Agar 30.0, Final pH (at 25 °C) 6.5 ± 0.2 + 5 ml of 10 mg% Phenol red or 20 mg% bromothymol blue per liter (*see* **Note 1**).

(c) **Sterile Silicate Agar for Actinomycetes:** (g/L) Magnesium sulfate 0.1, calcium carbonate 0.1, potassium alumino silicate 1.5, Starch 2 g ferric chloride, asparagine 0.01, Casein 0.02 g, calcium phosphate 1.0 g, ammonium sulfate 0.2, Agar 30 g, final pH (at 25 °C) 7.2 ± 0.2 + 5 ml of 10 mg% Phenol red or 20 mg% bromothymol blue per liter (*see* **Note 1**). (d) Aleksandrov medium used for potassium solubilizers is also applicable for isolation of silicate solubilizers.

3 Methods

1. Take 1 g of collected soil sample, add in 9 ml of sterile saline, mix well by shaking and then serially dilute up to tenfold, i.e., 10^{-9}.
2. Prepare silicate agar for bacteria, fungi, and actinomycetes by using pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize them at 121 °C for 20 min under 15 psi pressure.
3. Let the media to cool at 40 °C, and mix well the settled silicate and carbonate.
4. Pour it into the sterile petri plates and allow solidifying at room temperature for 2–3 h. Also prepare control media plates of each culture media without silicate.
5. Take loopful of soil suspension from 10^{-5} to 10^{-6} dilution tube and streak on selenite containing silicate agar plates.
6. Incubate the plates at room temperature approximately up to 48 h; observe the plates for growth and zone of clearance around colony (Fig. 2).
7. Compare the silicate containing plates with the control plates to examine the solubilization of Si and thereby formation of zone around the colonies.
8. These isolates can be further purified in liquid enrichment medium supplemented with magnesium silicate at low concentration of silicate. Isolate specific media required for the

conversion of silicate into depolymerized form of soluble silicon/nanoparticles can be further optimized and the silicon particles can be characterized for solubilization properties (Fig. 2).

9. For confirmation of solubilizing mechanism, use pH indicator in the medium. Add 3–5 ml of 10 mg% phenol red or 10 mg% bromophenol blue and mix well (*see* **Note 3**).
10. The color change will indicate whether solubilization might be due to acid or alkali.
11. **Estimation of Solubilized silica**: Centrifuge the broth medium of silica solubilizers, take 5 ml of supernatant, mix with 15 ml 2.5% boric acid and 5 ml 54% ammonium molybdate, incubate for 5 min and add 1 ml of 0.5% ascorbic acid solution, mix the solution by vortex and measure absorbance at 650 nm. The concentration of silicate could be determined by the standard graph of SiO_2 by spectrophotometric method.
12. **Bioassay and analysis of Si content in test plant**: Plant ten rice *oryza sativa/Triticum aestivum*, sugar cane, maize, wheat seedling in the 5 × 3 cm pots, filled with sterilized black cotton soil and one pot can be inoculated with silicate solubilizers inoculum. The uninoculated pots should be considered as control which should be allowed to grow for 3 weeks, then uproot the plants, wash with distilled water and dry it at room temperature and measure weight of control and test plant. Dry the plant at 60 °C for 3 h and use for silicate analysis in plant (*see* **Notes 4** and **5**).
13. **Analysis of Si content of the plant:** Ground the 0.5–1.0 g of dried biomass of control and test plant. As per method described by Kaya et al [13] take 0.5 g of the lyophilized crushed powder and soak in 0.5 M HCl for 1 h, then wash by distilled water and dry in oven. Add the dried powder in the mixture of nitric acid, sulfuric acid, and perchloric acid (10:1:4 v/v/v) keep it for 1 h, then this digested sample can be analyzed by coupled plasma mass spectrometry (*see* **Notes 4** and **5**).

4 Notes

1. For isolation of silicate solubilizers, various polymeric silica sources can be used in medium such as quartz, bentonite, magnesium trisilicate), aluminum silicate, calcium fuller's earth, kaolin potassium alumino silicate), potassium, aluminum and calcium silicates, phyllosilicates, etc.
2. For the rhizospheric endophytes and the rhizobia, surface sterilize the material with 70% ethanol or 0.01% mercuric chloride. Then crush the sample in sterile saline, further dilute and

streak the specific dilution on sterile silicate solubilization agar medium.

3. Add pH indicators like bromocresol green, phenol red, bromophenol blue, etc. in medium to know mechanism of solubilization of silicates. Prepare pH indicator solution in 70% ethanol to avoid contaminations. pH indicators can be added after sterilization.
4. Many times these depolymerized or solubilized Si particles are in nanoform as hence as nano silicates, which have various significant potential for various industrial and agriculture applications. The nanoforms are characterized by various spectroscopic methods. Si-nanoparticle is characterized by UV–Vis absorption spectrophotometer by recording the spectra (200–500 nm).
5. The lyophilized powder is used for further particle characterization such as particle size, shape, and crystallinity by using scanning electron microscopy, transmission electron microscopy, dynamic light scattering (DLS), and X-ray diffraction.

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Preface

Agricultural Microbiology is a part of the microbiology branch dealing with beneficial or harmful microbes associated with either plants or soil. This manual focuses on beneficial microbes dealing with soil fertility, microbial degradation of organic matter, soil nutrient transformations, and biocontrol agents. Nowadays, techniques involved in the study of beneficial microbes in agricultural microbiology toward enhancing global agricultural productivity are in trend. This manual covers a wide range of basic and advanced techniques associated with research on the isolation of agriculturally important microbes, identification, biological nitrogen fixation, microbe-mediated plant nutrient use efficiency, and biological control of plant diseases and pests. Introduction to each protocol explains the role/importance of chemicals involved, uniqueness, and protocol application. A proper understanding of the protocol helps the researchers to manipulate them as per their need.

This book is composed of seven parts with 52 protocol chapters. Parts I and II represent the importance, isolation, and purification methods of agriculturally important microbes and include mineral-solubilizing microbes. Part III deals with phytohormones quantitative protocols directly or indirectly associated with microbes. Parts IV and V provide deep insights into protocols for screening agriculturally important enzymes and compounds related to biocontrol activity. Part VI represents assessment methods of soil microbial activity by soil respiration. The final Part VII deals with protocols for selecting microbial strains for inoculant production and quality control ultimately representing commercial biofertilizers production criteria. This book will help postgraduate students, research scholars, postdoctoral fellows, and teachers belonging to different disciplines of Plant Microbiology and Pathology. Moreover, this manual may also serve as a textbook for undergraduate courses like Techniques on Plant-Microbe Interaction/Biological Control of Plant Diseases/Nutrient Use Efficiency.

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Chapter 21

Isolation of Bacterivorous Protozoan, *Acanthamoeba* Spp., as New-Age Agro Bio-Input

Chandrashekhar D. Patil, Bhavana V. Mohite, and Satish V. Patil

Abstract

Protozoan is widely distributed in the environment. *Acanthamoeba* genus are free-living protozoan of nonpathogenic and pathogenic nature. Its intrinsic ability to feed on soil bacteria contributes greatly to the soil nutrient turnover and thereby plant growth. This protocol provides a firsthand guide to isolate *Acanthamoeba* from soil samples and their preliminary identification through staining procedure. The isolates could be further tested for plant beneficial roles in laboratory settings.

Key words Protozoan, *Acanthamoeba*, Agriculture, Cyst, Soil fertility

1 Introduction

Protozoa are single-celled eukaryotic microorganisms. They are heterotrophic and mostly predate on the soil bacteria. They are widely present in water and soil habitat. Through their feeding behavior, they play an important role in recycling of soil organic nutrients. Members of *Acanthamoeba* genus are free-living Protozoa and most predominant bacterivores species in the environment. The classification scheme of *acanthamoeba* genus is as follows:

Kingdom: *Protista*.

Subkingdom: *Protozoa*.

Phylum: *Sarcomastigophora*.

Subphylum: *Sarcodina*.

Superclass: *Rhizopoda*.

Class: *Lobosea*.

Subclass: *Gymnamoebia*.

Chandrashekhar D. Patil and Satish V. Patil contributed equally to this work.

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Order: *Amoebida*.

Family: *Acanthamoebidae*.

Genus: *Acanthamoeba*.

Free-living *Amoebae* of *Acanthamoeba* genus include non-pathogenic and pathogenic strains that are currently classified in 18 different genotypes, T1–T18 [1]. The life cycle of *Acanthamoeba* consists of two stages, metabolically active, trophozoite, and metabolically inactive cyst. Both trophozoite and cysts are characterized by a nucleus surrounded by a dense central nucleolus [2]. The interchange between two life stages depends on the external stimuli such as nutrient depletion or availability. A gram of soil generally contains 10^3–10^7 amoeba with varying size <10 μm. The differences between cyst size and shape were the early markers for the differentiation between *Acanthamoeba* spp.

However, distinction between closely similar species was a major challenge due to the irregularity and variation in shapes. Latest molecular tools are highly recommended to profile the soil isolates and characterize their genotypes [3]. Due to the most abundant nature of *Acanthamoeba* in the environment, it is a suitable model organism for soil protozoan function studies [4].

Top organic soil layer (10–15 cm depth) harbors the high number of protozoa and microbial communities. *Acanthamoeba* voraciously feeds on soil bacteria at a rate from 0.2 to 1465 bacterial h^{-1} [5, 6]. Therefore, presence of protozoan, microorganisms, and soil nutrition quality are closely associated with conditions for the successful nutrient turnover via formation of "microbial loop in soil" [7]. Plant root exudates contain the nutrient that triggers the bacterial growth through assimilation of carbon and nitrogen in the rhizosphere. The abundance of bacteria in rhizosphere favors the progression of bacterivorous protozoan and ultimately regulation of bacterial population in soil. Protozoa preying on bacteria thus make nitrogen available for plant growth [4].

Considering the potential of bacterivorous protozoan in organic farming and as a next generation of agriculture bio-inputs [8], this chapter outlines the practical steps to isolate the *acanthamoeba* spp. as a model organism and their preliminary identification.

2 Material and Methods

2.1 Requirements

1. Soil sample.
2. Minimal nutrient medium (peptone: 1.25 g/l, yeast extract: 1.25 g/l, dextrose: 3.0 g/l, agar: 20.0 g/l).
3. Petri plates.
4. *E. coli* culture.

5. Autoclave.
6. Heating chamber.
7. Microscope.
8. Staining reagents.

2.2 Culture Medium Preparation

Acanthamoeba spp. can be easily cultivated on non-nutrient agar or agar media containing minimal concentrations of nutrients. Sterilize the medium by autoclaving at 15 lb. pressure for 20 min, allow the medium to cool at 45 °C, then pour the medium into petri plates, and allow them to solidify for 1 h at room temperature or 30 °C for 2 h. After solidification, aseptically overlay 1 ml of 24 h old culture of *E. coli* heated at 60 °C for 10 min, then incubate the plates at room temperature for 1 h.

2.3 Collection and Plating of Soil Sample

In sterile plastic bags, collect 1 g of rhizospheric soil from the irrigated soil/farms and crop plants as per need. Close the bag and label the sample appropriately (e.g., location, crop, date, etc.). The sample should be store at 4 °C until further experimentation (*see* **Note 1**).

2.4 Primary Identifications

The cultivated bacterivorous protozoan can be identified by observing loopful of culture with loopful of water under 40×, note down the structure (Fig. 1). The *Acanthamoeba* cyst and trophozoite stage can be identified by using simple staining methods like methylene blue, cotton blue, and Giemsa staining.

2.5 Detail Procedure

2.5.1 Part I

1. Dilute the collected rhizospheric soil with sterile saline by routine serial dilution techniques up to 10^9 dilution and mix well.
2. Aseptically remove 0.5 ml from diluted sample up to 10^6 and plate it on sterile culture medium, and spread the sample by rotating plates clockwise and anticlockwise to spread sample uniformly.
3. Incubate plates at 30 °C for 24–72 h.
4. At every 24 h, take loopful of culture and dilute with drop of sterile water.
5. Put this drop carefully at the center of cavity slide with wax at four corners, and invert the slide to make hanging drop preparation and observe under microscope at 40×.
6. Note down the shape and structure of cultivated protozoan (*see* **Note 2**).
7. Further observation for identification can also be done by using methylene blue, cotton blue, iodine, and Giemsa staining (*see* **Notes 3**–7).

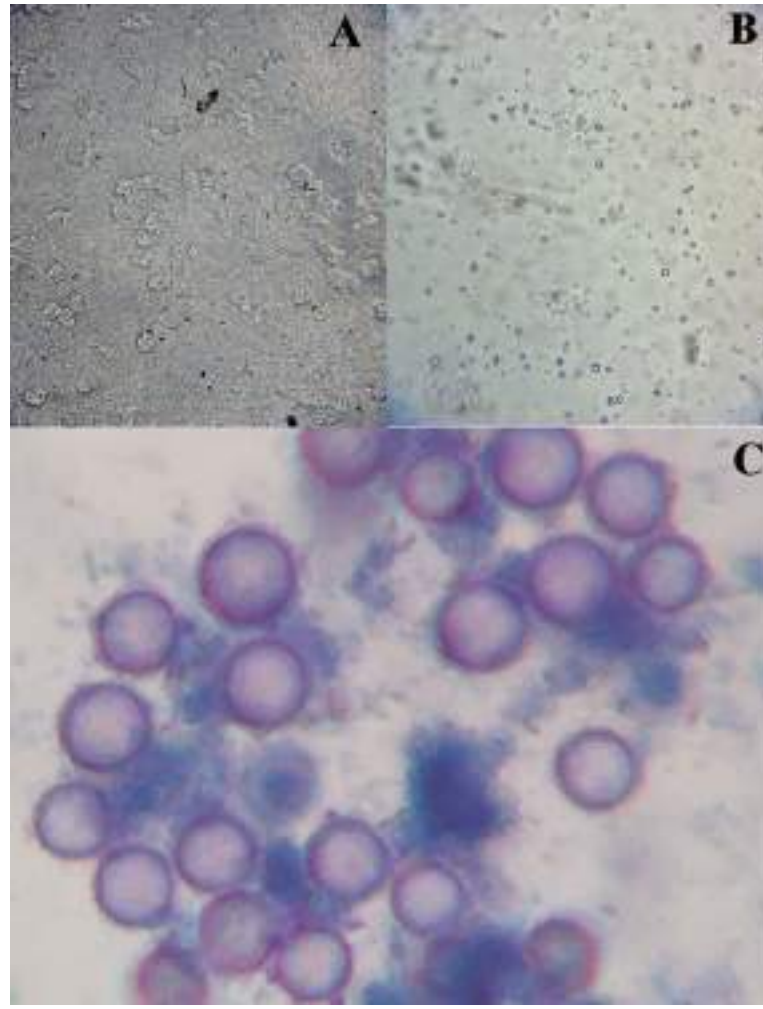

Fig. 1 Microscopic observation of different *Acanthamoeba* life forms. (**a**) *Acanthamoeba* cyst (10×). (**b**) Free-living trophozoites of *Acanthamoeba*. (**c**) Stained *Acanthamoeba* cyst (methylene blue staining, 40×)

2.5.2 Part II: Staining

1. Take a loopful culture of protozoan and spread on a glass slide and gently heat on the flame.
2. Flood the fixed smears of protozoan cyst with freshly filtered carbol fuchsin solution.
3. Then wash the smear in tap water and flood the smear with malachite green as counterstain for 10 s.
4. Then rinse the smear again in tap water and air-dry.

2.5.3 Part III

Axenic Culture of *Acanthamoeba*

The pure culture of *Acanthamoeba* can be done by following steps:

1. Harvest the cysts from the peptone–yeast extract–dextrose (PYD) plate cultures by adding 5 ml sterile saline in 2-week-old *Acanthamoeba* seeded plates (*see* **Note 8**).
2. Collect the suspension and then centrifuge at 7000 rpm (centrifugal g force 16052). For 10 min, then collect the pellet.
3. Incubate the pellet with 3% HCl overnight for elimination of the bacteria.
4. Then wash the cysts 2–3 with physiological saline (7000 rpm for 10 min) to remove remaining HCl.
5. Then transfer it to liquid cultures sterile PYD medium.
6. The culture should be maintained axenic by transferring every second week to fresh sterile PYD medium.

3 Notes

1. Since *Acanthamoeba* is both pathogenic and nonpathogenic in nature, appropriate safety measure should be taken to handle the samples. Sample collection from natural sites with minimum human exposure should be preferred for the isolation of nonpathogenic *Acanthamoeba* spp.
2. Cysts can be counted using a hemocytometer.
3. Basic fuchsin stain in aqueous solution with phenol and ethanol will stain *Acanthamoeba* cysts in red/magenta color against a greenish-blue background.
4. Eosin, iodine, and methylene blue stains are useful for wet mount staining. Eosin stain can be prepared by adding 1 g eosin in 100 ml distilled water, and 0.5 ml acetic acid.
5. Prepare methylene blue stain by dissolving 1 g of methylene blue powder in 100 ml physiological saline.
6. For wet mount staining, spread two drops (25–30 μl) of *Acanthamoeba* suspension on a glass slide; add a drop of the stain, cover with glass coverslip, and observe under microscope at 40× power.
7. The all above staining methods allow to observe cysts of *Acanthamoeba* known to form double-walled cysts, the outer wall ectocyst which is differentiated from the variably stained surrounding background. The inner wall appears as stellated, polygonal, round, or oval forms.
8. Sodium dodecyl sulfate (SDS, 0.5% final concentration) can be used to confirm the transformation of trophozoites into cysts. Trophozoites are SDS-sensitive and any remaining are lysed immediately upon addition of SDS, while cysts remain intact (Dudley, 2005).

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We acknowledge Prof. Michael Bonkowski, University of Cologne, Germany for pioneering the work in ecosystem engineers in shaping rhizosphere microbial diversity and functioning and their feedback on plant productivity. We are also thankful to our research collaborator Prof. Marilise B Rott, Universidade Federal do Rio Grande do Sul, Brazil for inspiring us on protozoan study.

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Preface

Agricultural Microbiology is a part of the microbiology branch dealing with beneficial or harmful microbes associated with either plants or soil. This manual focuses on beneficial microbes dealing with soil fertility, microbial degradation of organic matter, soil nutrient transformations, and biocontrol agents. Nowadays, techniques involved in the study of beneficial microbes in agricultural microbiology toward enhancing global agricultural productivity are in trend. This manual covers a wide range of basic and advanced techniques associated with research on the isolation of agriculturally important microbes, identification, biological nitrogen fixation, microbe-mediated plant nutrient use efficiency, and biological control of plant diseases and pests. Introduction to each protocol explains the role/importance of chemicals involved, uniqueness, and protocol application. A proper understanding of the protocol helps the researchers to manipulate them as per their need.

This book is composed of seven parts with 52 protocol chapters. Parts I and II represent the importance, isolation, and purification methods of agriculturally important microbes and include mineral-solubilizing microbes. Part III deals with phytohormones quantitative protocols directly or indirectly associated with microbes. Parts IV and V provide deep insights into protocols for screening agriculturally important enzymes and compounds related to biocontrol activity. Part VI represents assessment methods of soil microbial activity by soil respiration. The final Part VII deals with protocols for selecting microbial strains for inoculant production and quality control ultimately representing commercial biofertilizers production criteria. This book will help postgraduate students, research scholars, postdoctoral fellows, and teachers belonging to different disciplines of Plant Microbiology and Pathology. Moreover, this manual may also serve as a textbook for undergraduate courses like Techniques on Plant-Microbe Interaction/Biological Control of Plant Diseases/Nutrient Use Efficiency.

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Chapter 18

Extraction, Isolation and Culturing of *Mycorrhizal* Spores from Rhizospheric Soil

Satish V. Patil, Bhavana V. Mohite, and Chandrashekhar D. Patil

Abstract

Agriculture is experiencing the time of innovation with soil and its biological interactions. *Mycorrhiza* is a fungus associated with plant roots. *Mycorrhiza* is an ancient living ubiquitous fungus in soil. A decade ago, JL Harley pronounced a sentence "Plants don't have roots, they have mycorrhiza" in order to attract the attention of scientists towards plant-associated fungi in mutualistic association. *Mycorrhizae* increase the absorption of various nutrients, particularly phosphorus along with K, Si, Se, Zn, and Fe, and thus improve the crop productivity. The present chapter is focused on extraction, isolation, and culturing of *Mycorrhizal* fungi.

Key words Micronutrient, *Mycorrhiza*, Spores, Solubilization

1 Introduction

It is well known that the fungus plays various vital roles for mineral metabolism in soil and supplement to various plant. Bacteria and fungi are major contributors for P supplier to plant, besides these they promote nutrient availability, P, K, Si, Se, Zn, and Fe mobilizations through production of organic acids, metal chelators, protein, amino acids, and enzyme production (Fig. 1). *Mycorrhizae* are the nonpathogenic fungi which are associated with plant rhizosphere by mutualistic associations. They cause mild parasitism by invading roots for specific nutrients and provide various nutrients to plants. It is assumed that 90% of plants depend on *Mycorrhizal* supply of mineral nutrients especially phosphorus and iron (Fig. 2c). During some seasonal changes, they also provide nutrients like carbohydrate, sugar, and nitrogenous compounds. There are major two types as per the association, i.e., ecto *Mycorrhiza* and endo *Mycorrhizae*. Ecto *Mycorrhiza* means the fungus which is associated with external root surfaces and endo *Mycorrhiza* is associated with internal root cells of plant growing internally in plant

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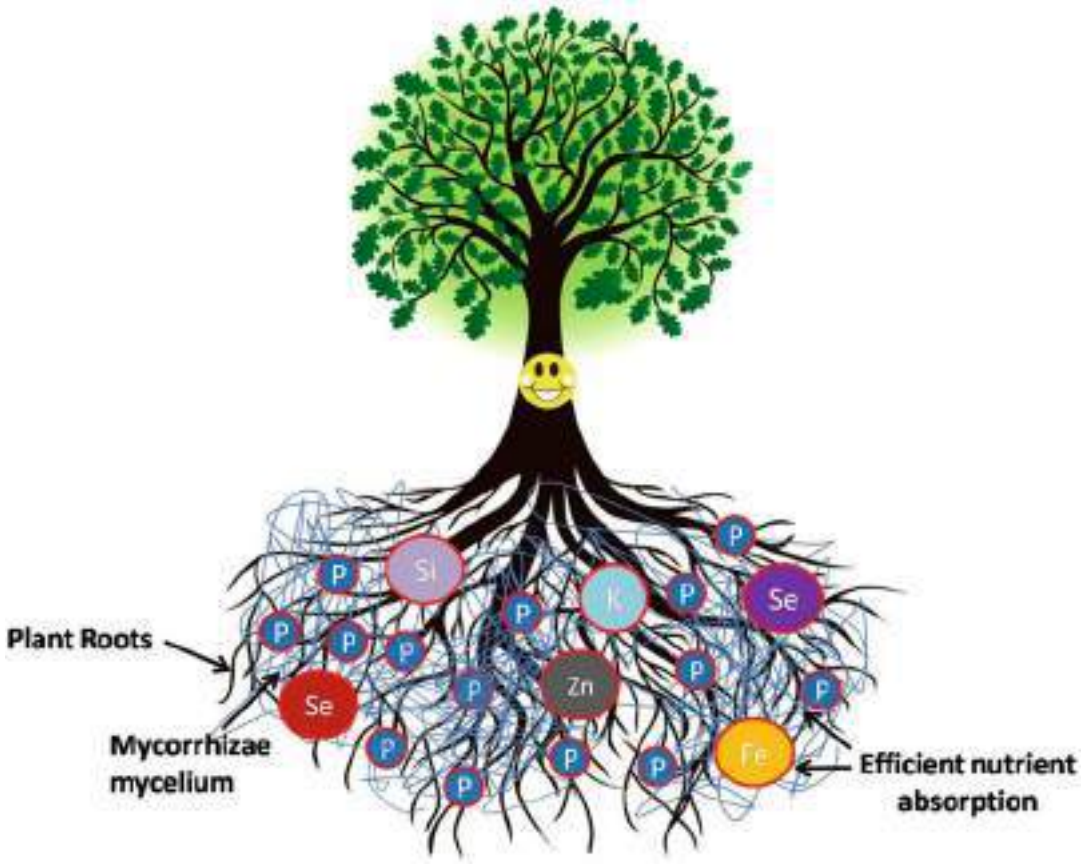

Fig. 1 *Mycorrhizal* plant roots mutualistic interaction

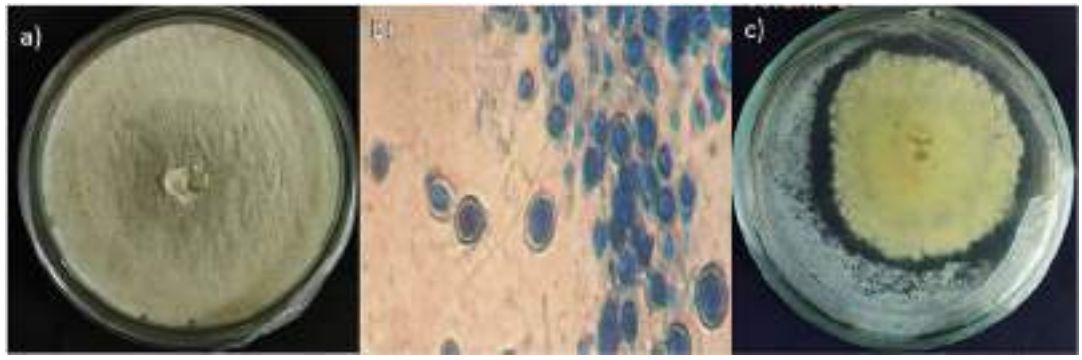

Fig. 2 (**a**) Growth of representative *Mycorrhizal* culture on agar plate (*Piriformospora indica*) (**b**) Microscopic observation of pear-shape *Mycorrhizal* spores (**c**) Zinc solubilization by *Piriformospora indica* (Dr. Satish V Patil Laboratory)

organs. Many times they are plant root specific, e.g., truffles *Terfezia boudieri* with oaks, Larix with larch plants, some Rhizoctonia with orchids. There is vast diversity of *Mycorrhizae* as per crop, plant, and area but generally the *Mycorrhizae* are reported by

detecting or extracting *Mycorrhizal* spores from soil and root samples. Although, there are some *Mycorrhizae* which do not produce always spore, such cultures may be identified by growing with plant tissue culture technique and other advance methods. Even if there are various techniques such as sucrose centrifugations [1] and adhesion floatation technique (AF) [2], capillarity adhesion method for spore extraction from soil and root are reported but wet sieving and decanting is the most practicable method for *Mycorrhizal* spore detection and isolation [3].

2 Principle of Assay

Extraction assay is based on principle that the *Mycorrhiza* are hydrophobic and light weight, and they float on water. These spores are separated by micro mesh sieves (250, 150, 53, and 45μm) and collected from the residue present on the sieves in petri plate and used for microscopic observation and cultivation (*see* **Note 4**).

3 Materials

- Rhizospheric soil/roots, rootlets, etc.
- Sterile containers for soil sample collection.
- Sterile tubes for serial dilution of soil samples.
- Staining dye 0.05% trypan blue in lactophenol.
- *Fragaria vesca L., Festuca ovina L., and Plectranthus* plantlets for cultivation of VAM.

4 Methods

4.1 Collection of Soil Samples and Extraction

1. Collect the 100 g of Rhizospheric or root associated soil samples/roots of specific plants at different locations.
2. Add 100 g soil sample in 1000 ml sterile water in glass container, mix vigorously for 2 min, and keep at 10 °C in refrigerator or in BOD incubator for 8–12 h.
3. After incubation, the supernatant slowly passes through the series of sieves 250, 150, 53, and 45/38μm (*see* **Note 4**).
4. Collect the residue present on each sieve separately in petri plates by washing sieves with sterile distilled water.
5. Observe the collected sieve wash under microscope.
6. For the plant root, dry the root material at 60 °C oven for 10–12 h, and grind the roots in grinder and soak the root

powder in sterile distilled water for 5 h, mix vigorously, and remove floating cellulosic material physically.

7. Allow the remaining water to pass through the sieves and follow above **steps 4** and **5**.
8. Collect the residue present on each sieve separately by washing with sterile water in petri plates.
9. Observe the spore morphology under microscope and identify the type of *Mycorrhizal* spore comparing with standard available cultures.
10. Using the dissecting microscope and micropipette, separate the *Mycorrhizal* spores and inoculate in sterile soil with plantlets of *Arabidopsis thaliana,* white clover *Trifolium repens*, Coleus (*Plectranthus scutellarioides*), in small pots, and allow to grow for 30 days (*see* **Note 3**).
11. Observe the root for *Mycorrhizal* colonization on root cells by root staining methods.
12. The spores collected/identified are inoculated with suitable coculturing plant and after 30 days observed *Mycorrhizal* association/spore, etc., and calculate its efficiency by following methods (*see* **Note 5**).
13. The culturable *Mycorrhiza,* i.e., *Piriformospora indica*, truffles *Terfezia boudieri* may be possible to culture on artificial medium in laboratory (*see* **Note 2**).

4.2 Staining and Determination of Percent of Mycorrhizal *Association*

1. Cut the roots in very small pieces, wash thoroughly under tap water and boil the pieces at 95 °C in 10% KOH for different time 10, 15, 20, and 25–30 min.
2. Then cool the material. Separate it, wash again with tap water then again treat with 1 N HCL for 5 min.
3. Stain the root pieces with 0.05% trypan blue lactophenol reagent. Mount the material on glass slide with fixing reagent, cover the material with coverslip, seal it by applying wax on corner, and observe under 40× (Fig. 2b) (*see* **Note 1**).
4. Measure the segment with spores and calculate the percentage of *Mycorrhizal* association by the following formula [4].

   % *Mycorrhizal* association = Number of *Mycorrhiza* associated segments/Total Number of segments analyzed × 100.

5 Notes

1. The fixative solution for staining the root is acetic acid; glycerol (1:1 V/V).
2. There are few *Mycorrhizae* spp. that are culturable on artificial medium, e.g., *Truffles. p indicus* (Fig. 2a). Other *Mycorrhizae*

fungi are only maintained with seedlings of various plants, e.g., *Trifolium repens*, Coleus (*Plectranthus scutellarioides*), *Arabidopsis thaliana*.

3. The plant specificity was reported for various *Mycorrhizae* sp., hence it should maintain on specific plantlets as per *Mycorrhizae sp.*
4. Glomus species spores generally retain in the 38/45μm sieve. It also catches the majority of spores including large *Gigaspora gigantea* and visible as bright greenish dots under microscope.
5. For better identification of spores, use spore plate photograph from diversity of arbuscular *Mycorrhizal* fungi associated with some medicinal plants in Western Ghats of Karnataka region, India or Distribution of arbuscular *Mycorrhizal* fungi (AMF) in Terceira and São Miguel Islands (Azores)Biodiversity Data Journal 8: e49759 doi: https://doi.org/10.3897/BDJ.8.e49759

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Preface

Agricultural Microbiology is a part of the microbiology branch dealing with beneficial or harmful microbes associated with either plants or soil. This manual focuses on beneficial microbes dealing with soil fertility, microbial degradation of organic matter, soil nutrient transformations, and biocontrol agents. Nowadays, techniques involved in the study of beneficial microbes in agricultural microbiology toward enhancing global agricultural productivity are in trend. This manual covers a wide range of basic and advanced techniques associated with research on the isolation of agriculturally important microbes, identification, biological nitrogen fixation, microbe-mediated plant nutrient use efficiency, and biological control of plant diseases and pests. Introduction to each protocol explains the role/importance of chemicals involved, uniqueness, and protocol application. A proper understanding of the protocol helps the researchers to manipulate them as per their need.

This book is composed of seven parts with 52 protocol chapters. Parts I and II represent the importance, isolation, and purification methods of agriculturally important microbes and include mineral-solubilizing microbes. Part III deals with phytohormones quantitative protocols directly or indirectly associated with microbes. Parts IV and V provide deep insights into protocols for screening agriculturally important enzymes and compounds related to biocontrol activity. Part VI represents assessment methods of soil microbial activity by soil respiration. The final Part VII deals with protocols for selecting microbial strains for inoculant production and quality control ultimately representing commercial biofertilizers production criteria. This book will help postgraduate students, research scholars, postdoctoral fellows, and teachers belonging to different disciplines of Plant Microbiology and Pathology. Moreover, this manual may also serve as a textbook for undergraduate courses like Techniques on Plant-Microbe Interaction/Biological Control of Plant Diseases/Nutrient Use Efficiency.

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Chapter 3

Isolation and Identification of Nonsymbiotic *Azotobacter* and Symbiotic *Azotobacter Paspali–Paspalum notatum*

Bhavana V. Mohite and Satish V. Patil

Abstract

Azotobacter is a renowned nonsymbiotic nitrogen fixer. Since the discovery of *Azotobacter* in 1901, it has magnetized microbiologists' attention for its interesting potential in agriculture for nitrogen fixation as well as synthesis of biologically active substances. It has distinctly enhanced effect on crop production in agriculture by which it deciphers the growing demand of food for ever-increasing population. The exploration of free-living *Azotobacter* sp. along with unique symbiotic *A. paspali* will be an attempt towards augmentation of soil fertility with enhanced crop yield. This chapter will brief the general strategy for isolation and identification of *Azotobacter* with elementary approach.

Key words Cyst, Pigment, Exopolysaccharide, Nitrogen-free medium, Biofertilizer

1 Introduction

Azotobacter belongs to *Azotobacter*aceae family, proteobacteria subclass including nonsymbiotic-free nitrogen fixers and frequently has habitat in soil and water together with sediments [1]. *Azotobacter chroococcum* is the foremost reported species of *Azotobacter* from Holland soil by Beijerinck [2]. Subsequently, various new variety of *Azotobacter* sp. has been reported from soil and rhizosphere.

The *Azotobacter* genus has seven reported species namely *A. chroococcum*, *A. beijerinckii*, *A. vinelandii*, *A. paspali*, *A. armeniacus*, *A. nigricans*, and *A. salinestris* [3]. The utmost quantity of DNA in *Azotobacter* in comparison with other bacteria may be due to larger cells of *Azotobacter* [1]. The mol% GC content *of Azotobacter* is 52 to 67.5. The amount of DNA and quantity of chromosomes is augmented together with ageing.

NifH gene is expansively sequenced gene used for identification of nitrogen fixing *Azotobacter* [4]. The *Azotobacter* sp. has the metabolic potential of fixation of atmospheric nitrogen into ammonia. The three discrete nitrogenase enzymes, molybdenum

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(Mo) nitrogenase, vanadium (V) nitrogenase, and iron-only (Fe) nitrogenases, are the remarkable characteristic to study *Azotobacter* as fascinating nitrogen fixer with its noteworthy agriculture potential.

Azotobacter shows a mixotrophic, autotrophy, or heterotrophic mode of nutrition. The combined nitrogen-free medium with appropriate carbon resource is the ideal prerequisite for the *Azotobacter* growth. The optimum temperature of *Azotobacter* growth is 28–37 °C, but may differ as per the species. The acidic to alkaline pH range beginning from pH 4.8 up to pH 8.5 is applicable for *Azotobacter* growth.

Azotobacter plays a remarkable role among the free-living nitrogen fixing microorganisms as widely distributed in the natural habitat; soil, water, and sediments. The *Azotobacter* has proved as excellent bioinput for crops by nitrogen fixation and affecting the plant growth and yield, producing different plant growth promoting substances as well as stimulating the microflora of the rhizosphere [1].

Most of the *Azotobacter* sp. is from soil, slightly acidic to alkaline soil, but few are from water. The requirement for high phosphorous leads to prevalence mostly in fertile soil. One remarkable sp., *A. paspali,* was isolated from roots system of Bahia grass (*Paspalum notatum* cv Batatais), a tropical grass, due to accessibility of organic substances and appropriate pH by the plant in the rhizosphere. *Azotobacter paspali* grows in the rhizosphere of *P. notatum,* and nitrogen fixed by it may be transferred to Bahia grass and hence improve pasture growth. This restriction to the plant rhizosphere may be due restriction of utilization of wide variety of organic substances. *A. paspali* has the unique antagonistic property against the Gram-positive bacteria which is a beneficial property for life in the rhizosphere. Increases in the nitrogen content of the roots and in the total nitrogen content of the sand plant system were associated with successful *Azotobacter* colonization [5]. The *A. paspali* shows symbiotic highly specific diazotrophic association with *P. notatum* and hence it is interesting to culture and study it.

Azotobacter is straight rods having rounded ends becoming ellipsoidal or coccoid-shaped based on culture age and medium. The dimension is 2 or more × 4μm (diameter × length). *A. paspali* is generally longer (5 × 10μm length) and filamentous (up to 60μm long). The cells are single but can present in pairs or irregular clumps like in *A. paspali*. The morphological type changed according to culture condition. This property along with inability to use several carbon sources, make it a unique species. The increase in carbon: nitrogen (C: N) ratio and culture age [6] causes aggregation of *A. paspali* cells in late logarithmic or stationary phase. *A. vinelandii* and *A. paspali* are the only *Azotobacter* sp. carrying

nif, vnf, and anf genes producing either of the three nitrogenases enzymes based on Mo or V supply in the environment.

Azotobacter is nonmotile or motile with peritrichous flagella. The pigment production and cyst formation are the peculiar characteristics of *Azotobacter* apart from nitrogen fixation potential. The *Azotobacter* sp. undergoes encystment during late stationary stage with production of water soluble or insoluble type of pigments.

1.1 Isolation of the Azotobacter

Isolation of *Azotobacter* sp. is practiced using specific designed medium based on its nutrition category as chemoheterotrophs and potential of fixation of dinitrogen. The enrichment technique is principally based on the potential of nitrogen fixation by aerobic/microaerobic way and utilization of organic substrates as energy source. The enrichment can be carried out by addition of specific stimulatory or inhibitory selective substrate into N_2-free medium such as erythritol or D-arabitol, L-rhamnose, ethylene glycol as carbon source for *A. vinelandii*, *O*-hydroxybenzoate, D-glucuronate or D-galacturonate, and L-tartrate, pH 6 or less for *A. vinelandii*, *A. beijerinckii*, caprylate for *A. armeniacus*, and 35–37 °C temperature for *A. paspali*.

The soil paste plate and silica gel method were conventionally described for isolation of *Azotobacter* sp., in which soil/silica gel is fortified with suitable carbon source and other nutrient elements, seeded with sieved soil and allowed to incubate [3].

The isolation technique comprised of utilization of various nitrogen-free agar medium such as Winogradsky's [7] nitrogen-free media, LG medium [8], Norris medium [9], Ashby's medium [10], and Burk medium [11]. These reported media are fairly similar, merely get differ with a few carbon sources and proportion of micro and macronutrient and minerals (Table 2) (*see* **Note 1**).

Apart from general N_2-free medium, some medium can be designed to make it selective with addition of particular constituent for particular isolation of *Azotobacter* species. For example, for isolation of *A. paspali*: the sucrose medium can be made selective with addition of 0.5% bromothymol blue and using rhizospheric sample from *Paspalum notatum*, for *A. beijerinckii* using α-hydroxyl benzoate, Tartarate and D-galaturonate with maintaining pH 4.9–5.5, for *A. vinelandii* isolation addition of erythritol, butanol, rhamnose, ethylene glycol, 0.1% phenol, and 10% sodium benzoate, for *A. chroococcum* pH need to be maintained at pH 7.0–7.5, for *A. nigricans* addition of citrate, *n*-valerate and for *A. salinetris* Burk medium is fortified with 1.0–2.0% sodium salt [12].

1.2 Identification of the Azotobacter

The organism isolation after the enrichment culturing has increased the possibility of isolation of free-nitrogen fixer. The primary confirmation of *Azotobacter* genus is carried with principal

morphological tests, i.e., cyst formation and pigment production. *Azotobacter* could be differentiated from residual nitrogen fixers based on simple characteristic property of cyst-forming potential.

1.2.1 Cyst Formation

Azotobacter vegetative cells show rods to ovoid shape morphology, and consequently they may also present in larger clumps. In stress condition, the vegetative cells change to round, dormant cell structure referred to as cyst by the process of encystment. The formation of cyst is the leading criteria for taxonomic classification of *Azotobacter* [13]. *Azotomonas* and *Derecio Azomonas* are nitrogen fixers, but do not have the ability to produce cyst. The formation of cyst could be induced by particular carbon sources like ethanol, butanol, β-hydroxybutyrate, and isopropanol as a carbon source [7] (*see* **Note 2**).

1.2.2 Pigment Production

The diffusible and fluorescent pigment production is characteristic property of *Azotobacter* sp. and can be studied in daylight or under ultraviolet light, respectively. The basal agar media of Thompson and Skerman [14] enriched with sodium gluconate could be used for diffusible pigment while Stainer and Scholte medium is used for the nondiffusible pigment. The colonies of *Azotobacter* appear first white, flat, and mucoid, later on become quite glossy, convex, although the type of medium and carbon sources varies the colony morphology [14]. The further incubation allows pigment production (Fig. 1). The identification of *Azotobacter* sp. could be carried out based on pigmentation type as unique type of pigment produced by specific *Azotobacter* sp. (Table 1).

1.2.3 Identification of Azotobacter sp. at Species Level

The cyst formation property on N_2-free medium like Burk's, Ashby's, and Norris proved that the isolate is belonging to *Azotobacter* genus. The species-level identification is relying on range of phenotypic and biochemical investigation. The use of typical carbon source, type and color of pigment, response to particular antibiotics are basis for species-level identification of *Azotobacter* [12] (*see* **Note 3**).

1.3 Azotobacter paspali

The new species of *Azotobacter* named *Azotobacter paspali* [15] was isolated using the silica gel plates, containing Winogradsky's salts and calcium citrate as a carbon source from the rhizosphere soil of *Paspalum notatum*. This name was later changed to *Azorhizophilus paspali* [16]. The unique characteristic for identification of the *A. paspali* sp. is younger filamentous long rods cells (5–10μm length and 1.3–1.7μm in width). It produces red violet water-soluble pigment or yellowish-green fluorescent colonies. Döbereiner [15] examined growth of *A. paspali* on N_2-free modified and Lipman medium [17] having sucrose as solitary carbon source and bromothymol blue indicator.

Fig. 1 (**a**) *Azotobacter* sp. on nitrogen-free medium, (**b**) Different *Azotobacter* sp. pigment production, and (**c**) *Azotobacter* with surrounding biopolymer by negative staining

Table 1
Azotobacter sp. with its specific type of pigment

***Azotobacter* species**	**Type of pigment**
A. vinelandii	Yellow-green, fluorescent, water-soluble pigment
A. beijerinckii	Yellowish or cinnamon pigment
A. paspali	Yellow-green fluorescent or red-violet water-soluble pigment
A. chroococcum	Brown or blackish-brown
A. nigricans	Yellow nondiffusible pigment
A. armeniacus	Diffusible brown-black or red-violet
A. salinetris	Black-brown

A. paspali produces yellow color on a blue background on a sucrose minerals medium containing bromothymol blue indicator, representing the characteristic property of organic acid production (Table 2). After 48 h of incubation, yellow-centered colonies of *A. paspali* are appeared due to the medium acidification and hence resulting bromothymol blue assimilation. *A. paspali* is motile with peritrichous flagella while some strains have curli flagella. *A. paspali* can produce H_2S from thiosulphate and has potential to grow at 14°C.

A. paspali has the unique property of definite rhizospheric association with the wild grass; hence, it is considered to represent the symbiotic nitrogen association. *Azotobacter paspali* affects the growth and development of plant by appreciable increase in weight of roots and shoot [18]. *A. paspali* has specificity for a *Paspalum notatum,* a wild grass, along with some additional *Paspalum* sp., i.e., *P. plicatulu and P. virgatum* [19].

Table 2
Differential characteristics of *A. paspali*

Characteristic	*A. paspali*
Motility by peritrichous flagella	+
Cell morphology Cells in pairs, irregular clumps Long filamentous cell Undulate edged unevenly convex colony with rough surface	+ + +
Water-soluble pigments Yellow-green fluorescent pigment	+
Brown-black to red-violet	+
Nitrogen fixation occurs at pH 5.0–5.5 6.0 6.5–9.5 10	− d + +
Growth at temperature of 9 °C 14 °C 18 °C 32 °C 37 °C	− + + + +
Enzyme production Peroxidase Urease Oxidase	− + +
Production of H_2S from Thiosulphate Cysteine	+ d
Utilization of sole carbon source Fructose, glucose, acetate, pyruvate, fumarate, malate, succinate, α-oxoglutarate, lactate, DL gluconate, acetylmethylcarbinol Sucrose Propan-1-ol Maltose, trehalose, melibiose, raffinose, mannitol	+ + − +
Utilization of sole nitrogen source Ammonia Nitrate Glutamate	+ + −
Nitrate reduced to nitrite	−
Nitrogen fixation genes Nif Vnf Anf	+ + +
Mol % G + C (T_m)2 Genome size	63.2–65.6 4.3–4.6 Mb

Key: +: Positive, −: Negative, d: variable

2 Materials

1. Soil sample from a fertile soil/macerated roots or leaves or other samples.
2. Sterile saline tubes (10 tubes) for serial dilution of the soil samples.
3. Sterile N_2-free medium liquid enrichment medium (Burk's medium, Sergei Winogradsky's N_2-free medium, LG medium, Ashby's medium, Norris medium, Brown's medium, and Döbereiner sucrose mineral medium).
4. Sterile N_2-free medium agar plates (Burk's medium, Sergei Winogradsky's N_2-free medium, LG medium, Ashby's medium, Norris medium, Brown's medium, and Döbereiner sucrose mineral medium).
5. Stain for cyst: violamine/acridine orange/mixture of neutral red, light green, SF yellowish.
6. 0.5 ml of 10% (v/v) glycerol or paraffin oil or in 7% dimethyl sulfoxide in 0.1% phosphate buffer (pH 7.0) for maintenance of culture.

3 Methods

1. Take 1.0 g of collected soil/macerated roots or leaves or other samples, add in 9.0 ml sterile saline, mix well by shaking, and then further dilute up to tenfold, i.e., 10^{-9}.
2. Prepare N_2-free medium (Burk's N_2-free medium, Sergei Winogradsky's N_2-free medium, LG medium, Ashby's medium, Norris medium, and Brown's medium) in pure distilled water in clean 250 ml Erlenmeyer flasks and sterilize it (121 °C for 20 min at 15 psi).
3. The aliquots of diluted suspension (use last 3–4 dilutions, 10^{-6} to 10^{-9}) are incubated in liquid N_2-free agar medium (24–48 h at 30 °C). After the appearance of macroscopic growth, the culture is observed microscopically and cell morphology is observed.
4. The positive cultures are streaked from that liquid medium to the surface of specific N_2-free medium agar plates with selective substance.
5. After incubation of 24–48 h, colonies will appear; further allow it to incubate for 3–5 days to observe the diffusible and fluorescent pigment production and about 2 weeks for cyst formation.

6. Cultures grown for 24–48 h on liquid and solid medium are studied for general morphology by microscopic observations like Gram staining, motility, and cultural characteristics.
7. After incubation of 3–5 days, the plates are observed for pigment production (diffusible pigment in daylight and fluorescent pigment under ultraviolet light (364 nm wavelength)).
8. The cyst may be stained with violamine/acridine orange/mix up of light green SF yellowish neutral red and observed under phase-contrast microscope.
9. The further identification of *Azotobacter* at species level includes biochemical tests using specific compounds/conditions in selective medium as mentioned earlier in text and in Table 2 (for *A. paspali*).
10. The *Azotobacter* isolates can be maintained by subculturing at bimonthly interval at Burk's or Winogradsky's medium with sucrose. The agar grown culture can be maintained by suspending in 0.5 ml of 10% (v/v) glycerol or paraffin oil or in 7% dimethyl sulfoxide in 0.1% phosphate buffer (pH 7.0).

4 Notes

1. In N_2-free Winogradsky's medium, the new cells of varying species are appeared enormously analogous and hence the old age culture should be compared for species-level identification.
2. The exopolysaccharide synthesis is the prominent characteristics of the cyst-forming *Azotobacter*.
3. All *Azotobacter* sp. are very susceptible to streptomycin and kanamycin/neomycin.

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CCA192

MORINGA LEAVES PREPARATIONS AS MIRACULOUS WEAPON TO FIGHT AGAINST MALNUTRITION IN TRIBAL DURING COVID19 BACKGROUND

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In our country around 194 million people go hungry every day. The COVID situation created this scenario more tragic. Most of the populations lack of feasibility and affordability for sufficient and nutritious food to maintain their health. 50% children and women from tribal community are anaemic. The COVID mediated unemployment created a problem of food security among poor, although it will fulfil but still the nutrient insecurity problem remains as it is. In pandamic situation immunity booster is remain the trending topic. Most of promoted advertisement of immunity boosting are merely of commercial interest. So there is urgent need to provide people knowledge of easily available natural green nutritious supplement available in their local area. This paper aims the same using a common plant "Moringa" popular as *drumstick* or s*hevga* as a fruit vegetable all over the India. The miraculous nutritional potential of drumstick plant was already proved by worldwide research, but unknown for nutritional potential of its leaves. It is only known as fruit vegetable, but the Moringa leaves contain almost all essential nutrient growth factors. Hence with help of NASI, Allahabad, we validated locally occurring drumstick (*Moringaoleifera*) leaves nutritional potential and try to aware especially the tribal population about its use in day today food to eradicate common malnutrition problem. We selected some common food and developed common recipes including Moringa leaves. This will make easy, habitual use of Moringa leaves in day today food of tribal but also open new opportunities for entrepreneurs in tribal communities.

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CCA195

SCREENING OF XANTHOMODIN PRODUCING XANTHOMONAS SP. AND ITS APPLICATIONS IN SUNSCREEN AS A NOVEL SUNLIGHT PROTECTING AGENT

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Xanthomonas sp. are responsible for various *diseases* plant diseases like citrus can kerand oily spot disease in *citrus plant*. The pigment may be one of major character of xanthomonas and it may be act as major virulence factor for establishment of infection or pathogen in plant. It was also reported that the pigment protect these pathogens from physical and biological factors like UV light, temperature. By using routing microbiological techniques various bacteria isolated from the infected plant leaves and fruit part on the nutrient agar medium. Yellow pigmented bacterial colonies were consistently isolated from citrus plant leaves exhibiting bacterial streak symptoms. The causative organism was characterized as a aerobic, motile, gram-negative, rod-shaped organisms. The primary study on preferred carbon sources for high yellow pigment production was done on solid agar medium. It was primarily found that the nitrogen source play vital role pigment production while carbon source has negligible role in yellow pigment production. The pigment extracted was primarily characterized and tested for its UV protecting ability. The studies prove that Xanthomodin will be a future significant green source of UV protectant for commercial sunscreen

Keywords**:** Citrus, bacterial canker, *Xanthomonas* sp. EPS, Pigment

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CCA196

SCREENING OF NARINGIN BIOTRANSFORMING MICROBES AND ITS APPLICATION

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Nringin is Flavonoid glycoside especially present in garape, citrus like common fruits. The bitter taste of citrus fruit juice due to two flavonoid compounds specifically Naringin and limnonin . Naringin belongs to the flavonoid family compose of two sugar i.e. α-L-rhamnose and β-D-glucose with naringenine (4,5,7-trihydroxy-flavone).Naringinase s the enzyme responsible for biotransforamation of flvonoid Naringin and produce biologically active and medicinally important product such as narangenn and prunin . Naringinase enzyme also medicinal properties like anti-inflammatory, enhancement of the signaling pathway, anticancer like breast and bladder cancer to induce apoptosis in tumor cells and inhibited proliferation, it's help reduce liver disease. The enzymes are utilized in various new applications in food processing, Pharmaceutical, Agriculture, Lather, Paper industry, etc. The food and beverages industry facing bitter taste problems it's required more intense research debittering . Also most significant challenging factor in citrus fruit juice industry. The bitter taste leads to factor limiting for commercial undertaking proceed citrus fruit juice, Naringinase is the one of solution for this problem.In this investigation we are presenting screening of Naringinase producer microbes from citrus farm and citrus industry waste by using Patil et al 2019 iodine overlay method for Naringinase producer isolates. Effect of Carbon nitrogen sources on naringinase production and antimicrobial and antioxidant activity of crude product of naringin biotransformation

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CCA197

SCREENING AND EXPLORATION RHIZOSPHRIC SELENOBACTERS FOR NANOSELENIUM SYNTHESIS AND ITS VARIOUS APPLICATIONS

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Nanotechnology is the fast emerging and one of the most promising technologies of the modern era. The various types of metallic or non-metallic nanomaterials are replacing many of the conventional methods due to their exceptional and extraordinary properties. Selenium important micronutrients, has biologically important applications in therapeutics, agriculture, as health supplements and also as antimicrobial element.Se has been diversified in its various chemical and ionic forms within the environment. The available inorganic forms of Selenium in the soil areselenate (Se^{6+}), selenite (Se^{4+}) and selenide (Se^{2-}). The least available elemental Selenium (Se^{0}) is very less toxic than its oxyanions; Selenite and selenite. The major organic forms of selenium areselenomethionine and selenocystine which are the aminoacids found in many of the proteins. The transformation of these toxic oxyanions to biologically safe, atomic form becomes important to make it more applicable with least or no toxic effects. This study particularly focuses onisolation and screening *Rhizobium sp*., for selenium transformations. Although variousmicroorganisms have been reported for nanosynthesis, *Rhizobium* is safe non-pathogenic bacterium, reported for various beneficial activities. The organism was isolated from root nodules of different locations and screened for its selenium transformation potential. The isolates did show a red color colloidal suspension formed after 48 – 72 hrs of incubation. The organisms were tested for their maximum selenium tolerance. Their potential antimicrobial and antioxidant properties were also checked. The biotransformation was confirmed by ICP-AES analysis and also characterized by UV-Visible spectrophotometer. This *Rhizobium* assisted nano synthesis method can be commercialized by optimizing the physical factors in future, as it is completely an ecofriendly approach.

Keywords: Selenium oxyanions, bio-nano-factories, Se^{0}Nanoparticles.

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CCA199

SCREENING AND EXPLORATION OF MICROBE BASED BIO ATTRACTANT FOR MOSQUITO: ONE STEP TOWARDS NOVEL MOSQUITO CONTROL METHOD

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The Chemical signals are very important key of successful life cycle of mosquitoes. The use of various chemical cues and signal were detected in various stages of mosquito life cycle. Chemical, visual and physical cues impact on the mosquito's biting and oviposition behavior. In view of the above significance of chemical cues or signals, we screened some microbes from the different preferred sites of mosquito's oviposition and biting. The basic idea behind the work is: some aquatic bacteria, actinomycetes and fungi may be a source of these chemical signals. Routine microbiological techniques were employed for isolation of microorganisms like actinomycetes, yeast and bacteria from the aquatic sites were *Aedes agepty* and other mosquito species eggs and larvae were detected. Besides these on the basis of survey, susceptible individuals for mosquito attracting and biting were analyzed for their skin microflora. The isolated microorganisms were tested for their mosquito attracting potential by simple pot assay in which the food colors were incorporated in sugar/honey solutions along with pure cultures of microbes and their metabolites. The purpose of food color is to determine attracting and feeding mosquito population towards the microorganism and their bioattracting metabolites. The numbers of mosquitoes attracted and feed were analyzed. On this basis of this experiment design, it was revealed that the oviposition is majorly driven by aquatic or marshy place residing Gram negative bacteria, while skin attraction was mainly due to organic and amino acid secreting bacteria like lactobacilli, micrococcus etc.

DNA Barcode: - New Approach for Conservation of Biodiversity

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Abstract

Overexploitation of resources, climate change, global warming, introduction of exotic species and genetically modified organisms, pollution, natural calamities and loss of sensitivity towards natural resources and the most vital anthropogenic activities are all responsible for loss of biodiversity. The diversity of plant species is at considerable risk from human activities that include habitat destruction, the introduction of plants and herbivores outside their native ranges, and anthropogenic climate change. The rates of plant extinctions are hundreds to thousands of times greater than rates of diversification. For the benefit of people and the planet, conservation of biodiversity is essential. It must be achieved through newer and swift technology to have universal acceptance. DNA Bar Coding is found to be novel and worth technique that would involve sequencing the DNA of unknown species and searching for a single sequence that could be easily isolated and used to swiftly differentiate species.

***Keywords**: DNA Barcoding, biodiversity, extinction of plant species*

India is hot spot for biological diversity, Indian Western and Eastern Ghats, Himalaya Nilgiris mountains, Satpuras, Arivalli, Vindhya hills are rich in plant and animal, diversity still in good condition as compared to European countries as we have more than 47,000 species of plants in India. Biodiversity is the precious natural resource of nation and provide the stability of biosphere and supplies food, fodder, fiber, medicine etc. Although the origin of term biodiversity is unclear, what is clear is that biodiversity became the subject of considerable interest in both the popular and scientific literature during the last decade. It is a nonspecific term that is generally agreed to indicate the variability of all living organisms, and at all taxonomic levels, from the species to the ecosystem (Kate and Laird, 1999. and Abell, 2002).

The most useful definition of biodiversity is that given by the International Union for Conservation and Natural Resourses: biodiversity encompasses all life forms, ecosystems and ecological processes and acknowledges the hierarchy at genetic, taxon and ecosystem levels. Biological diversity is usually considered at three different levels: genetic diversity, species diversity and ecosystem diversity. In molecular ecological terms, it can be defined as the number and distribution of different sequence types present in the DNA extracted from the community in the habitat (Dargan and Sarma 2001 and Kumar and Bhatt 2007).

Plant species, both those we know and those we don't, offer a tremendous resource of possibilities that could greatly add to the security of our food. But due to the human activity

Innovative Research Trends in Science and Humanities

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Probiotic Research in Therapeutics

Volume 2: Modulation of Gut Flora: Management of Inflammation and Infection Related Gut Etiology

Springer

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Probiotic Research in Therapeutics

Volume 2: Modulation of Gut Flora: Management of Inflammation and Infection Related Gut Etiology

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Contents

Probiotics for Allergic Airway Infection and Inflammations

13

Satish V. Patil, Bhavana V. Mohite, and Vikas S. Patil

Abstract

Probiotics have expansively reported affecting the composition of the gut microbiota, and it opens promising areas of research for the discovery of probiotics in the prevention or treatment of infectious and inflammatory diseases. Probiotics exert multiple health effects such as immunomodulatory agents and activators of host defense pathways, influencing disease severity, and incidence. The normalization of the properties of unbalanced indigenous microflora by healthy gut microflora constitutes the rationale in probiotics therapy.

The probiotics microbiome is essential for the development of host immune responses, particularly within the context of allergy. The probiotics performance manifests itself in the normalization of the increased intestinal permeability, improvement of the intestine immunological barrier functions, and alleviation of the intestinal inflammatory response.

The effect of probiotics is based on the ability to differentially regulate the production of anti- and pro-inflammatory cytokines as well as the balance between types of T cell responses. Probiotics appear to be a feasible way to decrease the incidence of respiratory tract infections. Probiotics affect the lung

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immune response after the allergic airway inflammation due to an increase of T regulatory-dependent mechanisms. The proper development of bacterial colonization observed to downregulate the hypersensitivity reactions with alterations of the cytokine profile. There is a paucity of data regarding the study of the mechanism of probiotic. There is a need for a mechanism investigation of probiotic action to explore the putative benefit of respiratory disease.

Therefore, the current article focuses on the present scenario of the effect of probiotics on the immune system in allergic airway infections and inflammations.

Keywords

Probiotics · Gut microbiota · Allergy · Airway inflammation · Gut microflora

13.1 Introduction

13.1.1 Probiotics

The World Health Organization (WHO) defined probiotic as "living microorganisms in adequate amount confer the health benefits" (Food and Agriculture Organization of the United Nations, World Health Organization 2002). The phrase "probiotic" is a Greek term and means "for life." Originally it was termed as "substances secreted by one microorganism that stimulate the growth of another" (Lilly and Stillwell 1965). The redefinition by Parker (1974) coined the probiotics as "organisms and substances, which contribute to intestinal microbial balance."

The adapted narration by Fuller (1989) stated as "a live microbial feed supplement, which beneficially affects the host animal by improving its microbial balance." Marteau et al. (2001) provided the most accepted definition as "microbial cell preparations or components of microbial cells that have a beneficial effect on the health and well-being."

The Food Safety Department, World Health Organization (2005) defined probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host." The international scientific community has admitted to this and has become the working definition of probiotics.

The most commonly used probiotics are *lactic acid bacteria* (LAB), particularly *Lactobacillus* and *Bifidobacterium* species, followed by the genera *Enterococcus*, *Streptococcus*, *Propionibacterium*, *Pediococcus*, *Escherichia coli,* and *Bacillus* (Szajewska et al. 2016). Few some yeast species are having potential as probiotics, e.g. *Saccharomyces cerevisiae* and *Saccharomyces boulardii* were utilized for the treatment of gastrointestinal diseases very often (Guarner et al. 2012; Sanders et al. 2013; Schreck Bird et al. 2017; Kerry et al. 2018). However, not all the bacteria can be probiotic, as they need to be strain-specific.

The probiotic produce is in the type of tablets, capsules, powders (which worked as a dietetic complement), and as a food component (e.g., kefirs, kombucha, tempeh, miso, yogurts, or a drug). The dairy products and functional foods are helpful for the restoration of healthy microbiota of the body and almost all adults, as well as

children, consumed it (Reid 2015). Hence dairy probiotic has been commercialized all over the world in different forms. However, allergy and lactose intolerance are the main arrests of dairy probiotics. The milk proteins, casein and whey proteins may act as allergens (Kumar et al. 2015).

Among the food factors, the use of food dyes is also a major reason for food allergy. Various natural and synthetic dyes such as carmine, tartrazine, and so on are added to the food to enhance the aesthetic value but may cause adverse reactions of food coloring allergy (Laura et al. 2019).

Probiotics are the indigenous nonpathogenic bacteria that colonize the mammalian intestinal tract. 10% out of 10^3–10^4 bacteria/ml dwelling in the body are legitimate living bacteria (Sender et al. 2016). The probiotic bacteria colonize initially maternal vaginal and fecal bacteria flora with reductive potential to make an anaerobic condition to favor the development of *Lactobacilli* and *Bifidobacteria*.

13.1.2 Benefits of Probiotics

The gastrointestinal tract is one of the most microbiologically dynamic environments that assume a vital role in the working of the mucosal immune system (MIS). The consumed probiotic stimulates the immune response as well as signaling by intact bacteria or its cell wall structure (Galdeano et al. 2019).

The gut is the site where huge numbers of bacteria from the microbiota and from the intestine which get through food intake coexist with each other. The immune cells are associated with the lamina propria of the villi. This intestinal microbiota does not interrelate straightforwardly with the epithelial cells; however, the maturation and functionality of the immune cells are stimulated by this microbiota through their metabolites (Hooper et al. 2012).

The beneficial effects of probiotics have been widely used in improving the host well-being and for the treatment of diverse infectious and non-infectious pathologies in animal models. Specifically it is included: protection from infection (Park et al. 2017; Acurcio et al. 2017; Mallina et al. 2018), irritable bowel symptoms relief (Hungin et al. 2013), reduction in the gut inflammatory response (Fábrega et al. 2017), cancer prevention (Aragón et al. 2015; So et al. 2017), growth inhibition of *Helicobacter pylori* (Fujimura et al. 2012), and allergies prevention (Velez et al. 2015).

Even though probiotics have shown encouraging results in several health conditions in humans, such as diabetes, multi-drug resistant pathogens, irritable bowel syndrome (He et al. 2017; Abdelhamid et al. 2018; Majeed et al. 2018), extensive research is still essential to include probiotics into human health, nutrition, and regulation of diverse abnormalities.

13.1.3 How Probiotic Function for Immune System?

The primary clause for probiotic microbes is survival in the harsh conditions of the gastrointestinal (GI) tract and stomach of humans. There are various ways by which probiotic microbes modulate the immune system. Figure 13.1 presents a brief of the role of probiotics for the immune system to maintain the human health majorly include: i) Modulation of innate and adaptive immunity, ii) Growth inhibition of pathogenic bacteria, iii) Regulation of anti-inflammatory or pro-inflammatory cytokines, iv) Regulation of the gastrointestinal /mucosal immune system (Baldassarre et al. 2016).

The important properties of probiotics which help to maintain the body to exert the effects are capacity to stick to the epithelial cells, activation of innate and cytokine-mediated immune response by internalization of a fragment of probiotic bacteria inside the immune response stimulating, intestinal epithelial cells (IECs) (Galdeano and Perdigon 2004), strengthening of the intestinal barrier by increasing the number of Goblet cells which reinforce the mucus layer (De Moreno de LeBlanc et al. 2008).

Table 13.1 summarizes the diverse means to promote human health. In recent years, extensive research has been conducted on the role of probiotics in transforming the adaptive and innate immunity as a way to check or treat a wide variety of health conditions (Baldassarre et al. 2016).

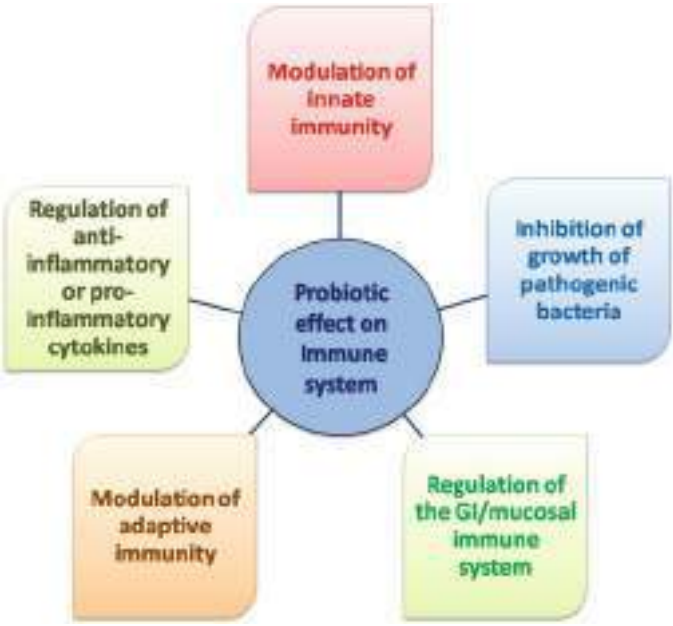

Fig. 13.1 Effect of probiotic on immune system

Table 13.1 Summary of probiotic mechanisms to promote the human health

Sr. No.	Mechanism	Active component	Reference
1.	Inhibiting the growth of pathogenic bacteria through the synthesis of inhibitory compounds such as organic acid, bacteriocins, antimicrobial peptides [29].	Acetic acid, lactic acid lactacin B, plantaricin lysozyme, secretory phospholipase A2, defensins, cathelicidins	Bermudez-Brito et al. (2012); Russell and Diez-Gonzalez (1997); Nielsen et al. (2010); Sankaran-Walters et al. (2017)
2.	Reinforce intestinal barrier integrity in tight junction signaling by amplified gene impression	Actin, zonula occludens-1 (ZO-1), actinin, occludin	Resta-Lenert and Barrett (2003)
3.	Protection of epithelial barrier and increased the tight junction protein expression with activation of signaling pathway	p38 mitogen activated protein kinases (p38 MAPK) and extracellular signal regulated kinase (ERK)	Dai et al. (2012)
4.	Increase in Paneth cells, produce anti-inflammatory metabolites,	Regulatory T cells (Treg) / type 1 regulatory T (Tr1) cells	Liu et al. (2016)
5.	Activation of adaptive immune system	CD4+ regulatory T (Treg) cells, dendritic cells	De Moreno de LeBlanc et al. (2005)
6.	Induction of different cytokines.	Interferon gamma (IFN-γ), tumor necrosis factor-α TNF-α	Jiang et al. (2013)
7.	Increases the phagocytic and microbicidal activity of macrophages	Specific antibody production	Núnez et al. (2013)
8.	Decrease of IgE	Immunoglobulin (Ig) G, interleukin 10 (IL-10) and IFN-γ	Fu et al. (2017); Jerzynska et al. (2016)
9.	Improving lipid profiles, reduce blood glucose and insulin levels	High-density lipoprotein (HDL)-cholesterol	Shah and Swami (2017)
10.	Anti-cancer effect by combination of multiple mechanisms	Anti-genotoxic and anti-gene mutation function, enzyme inhibition	Russo et al. (2014)

Now the probiotics have been commonly considered at therapeutical and clinical research level considering the relationship between the gut microbiome and immune disorders (Kothari et al. 2019), but the clear guidelines for the clinical application have yet to be established. This is particularly significant as the efficiency of probiotic supplementation may be reliant on the strain, dosing, condition, and duration of therapy (Toscano et al. 2017).

13.2 Role of Probiotics in Allergic Airway Infection

The normal healthy microflora constitutes the basis of probiotic therapy. Probiotics commonly mentioned as "good bacteria" or like a replacement for inhabitant stomach bacteria. Although the WHO recognizes probiotics as live microbes, when consumed in adequate quantity as an ingredient of food, it provides a health benefit to the host (Food Safety Department, World Health Organization 2005). At present, any item containing probiotics is viewed as a dietetic complement and is controlled by the principles and guidelines of the Dietary Supplement Health and Education Act of 1994. As indicated by it, the producer can give just common health declare for the manufactured food however it cannot express that any of the element in the product can fix, treat, or avoid illness (Alvarez-Olmos and Oberhelman 2001).

The dysbiosis, an inequity of the microflora constitution has adversely affected the health status. Three subcategory of dysbiosis have been recognized as below: (1) beneficial microbial agents loss, (2) spreading out of potentially harmful microorganisms, and (3) overall microbial diversity loss (Petersen and Round 2014).

Microbial dysbiosis has been concerned for different chronic inflammatory diseases, together with asthma (Sutherland and Martin 2007; Smits et al. 2016), chronic rhinosinusitis (CRS) (Hoggard et al. 2017; Aurora et al. 2013), Crohn's disease (Marin et al. 1983), and ulcerative colitis (Schmitz et al. 1999). The allergic infants reported an augmented number of *Clostridia* and a lower number of *Bifidobacteria* (Goktepe et al. 2005).

Amazingly all these persistent infections found to have altered membrane permeability and distorted functioning of epithelial barrier (Soyka et al. 2012; Steelant et al. 2016).

Probiotics have been publicized for a range of situation such as allergies, respiratory infections, including acute diarrhea, inflammatory bowel disease, and irritable bowel syndrome. This is been a choice to re-establish a healthy immune system (Dorval, 2015). Diverse probiotic strains and the mixing of microorganisms have a wide and differing range of clinical and immunologic potential and can manipulate gut microbiota in human beneficial ways (Table 13.2). The improved presence of probiotic bacteria in the intestinal microbiota has been found to correspond with defense from atopy (Moura et al. 2019). The predominance of hypersensitive ailment allergic diseases such as asthma, atopic dermatitis, and allergic rhinitis has expanded harshly over the past 2–3 decades in numerous nation, and sensitivities/allergies are presently most widely recognized chronic disease among youngsters all through the world (Tang et al. 2015).

The utilization of probiotic live forms could offer advantage to the patient's immunity, prompting improved management of the ailment, along with advanced lung functioning and reduced symptoms. Moreover, another mechanism of working of the probiotics comprised the enhancement in the epithelium membrane obstruction, hindrance of the adhesion of pathogens, binding to the intestinal mucosa, prohibition from pathogenic microorganisms by rivalry, and antimicrobial substance production (Bermudez-Brito et al. 2012).

Table 13.2 Representative studies demonstrating Probiotic effect in allergy

Sr. No.	Strain	Mechanism	Outcome	Reference
1.	*L. plantarum*, *L. lactis*, *L. casei*, *Lactobacillus rhamnosus* GG	Lesser IL-4 and IL-5 discharge	Reduced Th2 responses	Pochard et al. (2002)
2.	*Lactobacillus rhamnosus* GG and *L. bulgaricus*	Induction of IL1b, IL-6, IL-8, and TNF-a	Reduced Th2 responses	Niers et al. (2005)
3.	Lactic acid bacteria	Augmented IFN-g, TNF-a with IL-10	Reduced Th2 responses	Miettinen et al. (1998)
4.	*Lactobacillus rhamnosus* GG and *B. lactis* Bb12	Inducing transforming growth factor-β (TGF- β) secreting Tregs	Suppressed allergic symptoms	Feleszko et al. (2007)
5.	*L. acidophilus* W55	Stimulate functional FoxP3p(C) post-translational modification and Treg from CD25 cells	Supporting the species-specific effects of probiotics	de Roock et al. (2010)
6.	*Microbiota including* Bifidobacteria, lactobacilli,	Induction of mucosal IgA amount in addition to allergic B and T cell immunity	Modulation of allergy	Prescott and Björkstén (2007), Marschan et al. (2008), Galdeano et al. (2011)
7.	*Lactobacillus reuteri*	Reduced airway eosinophils, aryl hydrocarbon receptor (AHR) and TNF-a, IL-5 and IL-13 levels	Attenuate allergic airway disease	Forsythe et al. (2007)
8.	Commensal bacteria	Activation of DC andTh1 response	Stimulation of Th1 cytokines and, suppress Th2 response	Winkler et al. (2007)
9.	Commensal bacteria	Stimulation of mucosal IgA level	Allergen specific B and T cell response	Toh et al. (2012)

Allergic ailment represents a convincing challenge for community well-being concern due to their expanding predominance in evolved and evolving nations. Universally roughly 1 thousand million people are facing allergic symptoms and could be reached to 4 thousand million in the following 3–4 decades (Spacova et al. 2018).

Allergy is defined as a hypersensitive reaction to a particular antigen called an allergen by an immunological reaction (Ring, 2014). The commonly found allergies are against pollen grains, animal dander, mites of dust, or specific foodstuffs. Allergies are caused due to an increase in the amount of IgE (Akdis and Agache,

2014). The repeated exposure to allergen elicits activation of mast cell and basophile cells and release of allergic mediators like histamine and leukotriene resulting in five cardinal signs of allergy that vary from mild symptoms like sneezing but may become serious like difficulty in breathing and hypersensitivity.

The number of studies carried out to study the probiotic as therapy for airway allergy such as a Stockert et al. (2007) in a pilot study investigated the influence of probiotics for asthma suffering kids and discovered improved lung functioning (peak of expiratory flow [PEF]) but no effect on the quality of lives and use of asthma treatment. Furthermore, Chen et al. (2010) observed progress in signs, lung functioning, and immunological criterion in probiotic taking kids. Liu et al. (2016) described the effect of probiotics to improve the curative impact of allergen-definite immune treatment in asthma sufferers. The in vivo trial in rats having airways allergic inflammation when inoculated with *Lactobacillus reuteri*, improvement of inflammation and airway over sensitiveness in the probiotic receiving group of animals was observed (Forsythe et al. 2007; Karimi et al. 2009).

Moura et al. (2019) confirmed the role of probiotics as a complementary therapy for asthmatic children and teenagers. Furthermore, study is suggested to confirm the effectiveness of probiotics in asthma medication, particularly indiscriminate restricted experimental groundwork and ultimate cluster investigation, to assemble supplementary evidence and information on the promising expected advantage of probiotics for asthma sufferers.

There is a growing indication to put forward that each probiotic strain does not have a single exclusive mechanics of activity regardless of common taxonomical rank (Sanders et al. 2018).

The substantial cluster of proof is demonstrating that probiotics amend the type 1 helper T cell (Th1)/ type 2 helper T cell (Th2) (Th1/Th2) parity to forestall the improvement of inflammation infections such as allergy. The gut microbiota is having a vital role in re-establishing Th1/Th2 immunity.

The altered Th2 phenotype prompts an elevated number of IgE and hence activation of a mast cell, which will result in sensitivity to hypersensitivity disorders. The Th2- dominant phenotype of newborn displays higher receptiveness to hypersensitivity diseases. Amazingly, commensal colonization is contributed to this attribute, showing the important function of gut microflora. Commensals likewise assume a job in managing immune cell allocation. Therefore, susceptibility was accounted in adults following intense antibiotic course (Walker and Iyengar, 2015).

Another point of view of the perceptions is demonstrated in the "hygiene hypothesis." This recommends less microbial contact through early stages due to the improved community cleanliness. It is one of the essential reasons for uplifted receptiveness to allergic hypersensitivity. Likewise, these studies set up the role of microflora to affect the allergy immune response (Sharma and Im, 2018).

13.3 The Rationale behind the Mechanism of Probiotics for Allergy

This new strategy is originated from diversified information revealing the pleiotropic impacts of probiotics that incorporate immunomodulation, re-establishment of intestinal imbalance of microbiota just as keeping up epithelium hindrance solidarity (Toh et al. 2012).

Inflammation is an elementary defense mechanism of the immune system against unknown immunogen; however, allergy is a host defensive immunity on recurring presentation to a particular unknown particle as an antigen, yet possibly harmful to the horde. Inflammation is a type of innate immune response against the foreign virulent particles associated with tissue rejuvenation. Probiotics presumably work as immunomodulators and actuator of human defense mechanism, that propose to impact disease seriousness and its rate. Probiotics therapy is established on the idea of typical fine microflora. The probiotic therapy is based on normalization of the properties of unbalanced indigenous microflora by specific strains of the healthy gut microflora. The advancement of mucosal and fundamental resilience depends on immunosuppressant action coordinated by T cells that assuage both Th1 and Th2 responses, mechanisms may incorporate regulation of the useful properties of the microbiota, epithelial cells, DC, and safe cell types.

The superior adhesion properties of probiotic facilitate the maintenance of the mucosal barrier and avoid the absorption of foreign particles and expansion of IgA mediated immune response. The proper development of bacterial colonization observed to downregulate the hypersensitivity reactions with alterations of the cytokine profile.

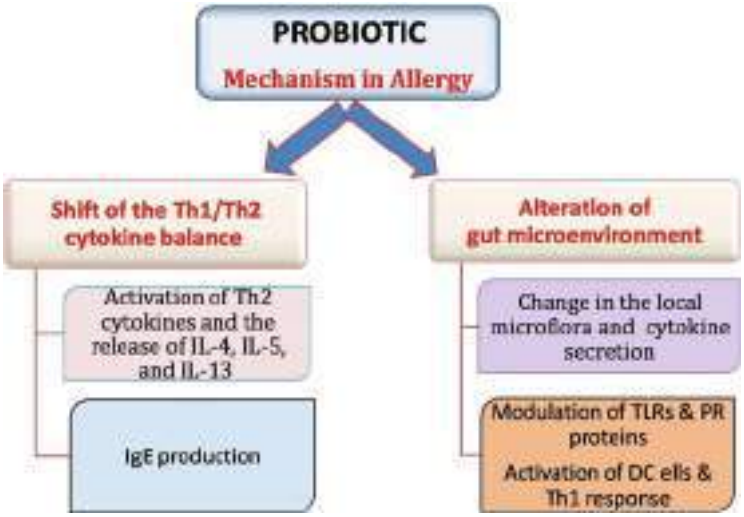

Fig. 13.2 Mechanism of probiotic in allergic reaction

Figure 13.2 describes the foremost activities of probiotic to undertake the airway allergic condition. The probiotic presents in the standardization of the extended intestinal permeableness and distorted gut microbial bionomics, development of the intestinal immunological fence job, and improvement of the response of gut inflammation.

The microbiome is fundamental for the advancement and learning of host immunity, mainly in the framework of allergic diseases. The use of probiotic influences the lung immunity followed by allergic airway infection due to augmentation of T regulatory-dependent mechanisms, however; whether this will impact the lung microbiota ruins to be determined. In reality, there is a need of elucidation of the mechanism of working of probiotic with assumed advantage for respiratory infections but there is paucity of data for airway microbiome composition.

13.3.1 Host Factors

The pathophysiology of susceptible illness, i. e. allergic disease results from an intricate series of actions including various ways of the natural immune response of innate and adaptive type. The allergic immune response involves stimulation of mast and basophil cells by IgE and succeeding allergen exposure resulted in allergic inflammation.

Host-associated factors can impact the working of the operation of the immune response in allergic hypersensitivity conditions and host and microorganisms communication (Laukens et al. 2016). Some vital characters are age, sex, host genetic structure, and microbiological status and can deviate in both human and animal investigation system (Laukens et al. 2016; Martín et al. 2017).

The pathogenic biofilm formation is the major host factor that leads to chronic infections. Biofilm formation is an accounted for about 65% and 80% of all microbial and chronic infections, respectively. Probiotic has the benefit as less cytotoxic than another quorum sensing (QS) suppressing agents and do not create strong pressure for resistance development like antibiotics. Hence probiotic could be an ideal alternative as an anti-virulent agent (Barzegari et al. 2020).

Probiotic prevents QS, biofilm formation, co-aggregation, and the survival of biofilm pathogens by interfering with biofilm formation and its quality. This is accomplished by decreasing the pH, competing for the adhesion sites with pathogens, and production of various antimicrobial agents like bacteriocin, hydrogen peroxide, and organic acids (Vuotto et al. 2014).

13.4 Allergy Prevention Studies with Probiotics

Current studies on meta-analysis of probiotics indicated a direct helpful impact on preliminary eczema impediment (Cuello-Garcia et al. 2015; Zuccotti et al. 2015), particularly to subsequent nativity to maternal and child to whom probiotics are administered. The probiotic will reduce the frequency of allergic sensitization with

perinatal intercession, which is not at all the condition for pre- or postpartum cure only (Zhang et al. 2016). Nevertheless, the support of probiotic for the avoidance of allergic airway disease is rare. There is no noteworthy outcome on the breathless incident or asthma improvement (Azad et al. 2013).

Lactobacillus probiotics strain is found to modulate the pro-inflammatory cytokines such as TNF-α, IL- 6, IL 1–10, and IL 1β by activating the macrophage (Rocha-Ramírez et al. 2017).

Probiotic consumption could decrease the occurrence of respiratory tract infections. Aerosol delivery of probiotic diminishes tumor seeding in the lung and improves chemotherapy against exploratory metastases. Probiotic seems to defeat commensal microbes incited tolerance encouraging the maturation of resident antigen presenting cells.

The prevention or repairing of "leaky" epithelial barriers could serve for the pro-inflammatory response. The epithelium barrier is the primary defensive physical obstacle of the individual for the entry of detrimental particles like any pathogen, irritants, and allergic compounds (Koch and Nusrat, 2012).

Eventually, probiotics can influence the inflammatory response by contrasting the basis of pro-inflammatory motivation related with low-quality endotoxemia. Besides, probiotics and some of their emitted metabolic products can straightforwardly influence key pro-inflammatory pathways by acting as ligands for innate immune system receptors. Intercellular junctions, for instance, tight junctions (TJs), adherence junctions (AJs), and desmosomes contribute to the construction and continuation of the physical barrier.

The probiotics have an advantageous impact on epithelium barrier malfunction which is widely considered for the digestive tract. The example may include *Lactobacillus plantarum* MB452 which elevate the articulation of TJ-related genes by in vitro testing in well abdominal epithelial cells (IECs) (Anderson et al. 2010).

Related encouraging impacts were confirmed in case of probiotic strains such as *Lactobacillus rhamnosus* GG (Orlando et al., 2014), *L. plantarum* MB452 (Resta-Lenert and Barrett 2003), *Streptococcus thermophiles* ATCC19258, and the gram-negative probiotic strain *Escherichia coli* Nissle (Ukena et al., 2007; Zyrek et al. 2007) on abdominal epithelium barrier intactness and TJ expression. Moreover, certain Lactobacillus strains show the potential to elevate epithelium barrier integrity through the stabilization of AJs expression (Hummel et al. 2012).

In particular, to mention, the tested lactobacilli strain enhances the E-cadherin and b-catenin and diminishes the ample protein kinase C expression in T84 human abdominal epithelium cell line. Protein kinase C is the enzyme responsible for the disassembling of adherens junctions (AJs) (Hummel et al. 2012).

Several barrier-rebuilding characteristics of probiotics have also been verified in diverse in vivo models (Laval et al. 2015). There are at present scarce reports in the airways, relating the dictatorial characteristics of probiotics on the epithelium lining. The oral medication with *L. rhamnosus* CRL1505 could circumvent the polycytidylic acid [poly (I:C)]-induced improved permeable nature of the bronchoalveolar-capillarity barrier for in vivo experimentation, as find out by albumin levels in the lungs (Zelaya et al. 2014). This progress was associated to diminish

the activation and synthesis of pro-inflammatory cells and cytokines in the lungs (Zelaya et al. 2014). Alike results were reported by nasally managed *Lactococcus lactis* NZ9000, which could neutralize *S. pneumonia* prompted permeable nature of lung tissue (Medina et al. 2008).

The in vitro studies reported dose reliant augmentation in epithelium obstacle functioning and reduction in epithelium permeability by prompting Calu-3 lung epithelium cells with the artificial bacterial lipopeptide Pam3CysSK4. This is caused due to improved articulation of the TJ proteins claudin-1 and ZO -1 and a lessen articulation of occluding.

Even though asthma is customarily viewed as a Th2-type inflammatory situation, it has been perceived as a clinically varied illness. The microflora composition of the gut and respiratory system is related to asthma incidents, as indicated by several reports. But it is not yet satisfactorily explained how disturbance of microbiota influences sensitivity to allergic asthma. It is projected that some metabolites formed during the fermentation of dietary fibers like short-chain fatty acids (SCFAs) by commensal suppress allergic airway responses (Trompette et al. 2014).

The Th2 response in the lungs is suppressed by higher serum SCFA, mainly propionate amending DC progenitors by G-protein fixed receptor in reliant way in the bone marrow. Butyrate is the foremost potent immune regulatory metabolite among the SCFAs. Histone deacetylase (HDA) inhibition is the mechanism of action for the butyrate and propionate function, with improvement in the acetylating status of histone in the Foxp3 site (Furusawa et al. 2013; Arpaia et al. 2013) and inducing tolerogenic DCs to augment Treg generation(Arpaia et al. 2013).

The Clostridiaceae family bacteria *Lachnospiraceae* and *Ruminococcaceae* are too recognized for the synthesis of SCFAs by fermented dietary fibers in the colon and thus sustaining epithelial integrity and homeostasis. But how this will helpful for humans, it needs to be confirmed by clinical trials (Sharma and Im, 2018).

13.5 Recent Advances: Clinical and In Vivo Status

In recent years, several experimental studies have investigated the capability of probiotic bacteria to improve the virulent traits of hypersensitivity disorders.

The animate models can be utilized in support of the probiotic impact and their systems of activity. This is found unrealistic in humans inferable from obscure dangers and moral concerns. The impact of such components should take into account during the experimental preliminary plan. The information exploration will encourage the advancement of superior probiotic intercessions and reinforce the proof for probiotic application in the prevention and cure of human beings ailment.

The effect of the human being genotype has likewise been proposed to assume a vital function in the result of probiotic medications, incorporating these acted with regard to allergic diseases. Individual hereditary contrasts and inclination towards inflammatory diseases ought to be thought about while surveying the impacts of probiotics in a clinical setting. The age of an individual and the influence of their gut

microflora should take into consideration for the human being testing. All around elegant study and strong in vivo and in vitro investigation are thus essential to advance definite choice of probiotic species for anticipation and management of allergic illness (Spacova et al. 2018).

To date, in any case, a large portion of the study on probiotic has concentrated on the microflora only as opposed to the interaction between host and microbiota. Additionally, accessible information discards the significance of mycobiome and virome. The existing screening system is centered on the cytokine production efficiency and capability of microbes by using the cell lines or ex vivo isolated peripheral immune cells, even though they do not symbolize phenotypically to gut cells. It is a requirement to develop high-performance screening procedures to ensure the particularity and sufficiency of picked probiotics. The majority of the commercial probiotic preparations are a combination of different bacteria with distinct colony forming units (CFUs). The purpose is learning of the consumer about the period for the viability of a specific strain and number of bacteria in specific dose.

Consequently, experimental testing should be extended to incorporate distinct geological areas. Considering this, it is advantageous not to execute meta-analyses on shared records when diverse strains of bacteria were utilized since the impact can vacillate drastically between the strains. The use of probiotic strains ought not to be permitted except the security and effectors compounds of the probiotics are very well cleared (Sharma and Im, 2018).

13.6 Safety Considerations and Contraindications

Immunomodulatory action may rely upon strain-specific characters so ideal strain might be presented. Probiotics are viewed with a safe, rare short term side effect (Ciorba, 2012). Isolated instances of bacteremia or fungemia have been related to probiotics, though inhabitants information additionally shows that there is no across the board danger of these complications (Snydman, 2008). Microorganisms that are "generally regarded as safe" incorporate species of *Lactobacillus* and *Bifidobacterium* and definite yeast strains. Other bacteria, such as *Enterococcus* and *Streptococcus* strains, are not generally considered as safe, however they are utilized as probiotics (Snydman, 2008). Itself alert ought to be practiced in prescribing probiotics to these populaces. Studies examining probiotics are comparatively short in length, limiting the long term security information and the ability for the real unfavorable circumstance. To make the firm ends, an additional experimental trial examining the safety of probiotics must be led.

The inconsistent outcome may result from the contrast in the cogitation plan, readout, and patient understanding. One significant impediment for an absolute meta-analysis of probiotic studies is the implementation of diverse probiotic species and strains, mainly *Bifidobacterium* or *Lactobacillus* or combination of that (Zuccotti et al. 2015). The administered probiotic doses also change significantly among the study from 10^7 to 10^{10} or more (CFU)/day, and treatment duration may also vary from a while to quite a while (Zuccotti et al. 2015). Nonetheless, the

outcome can vary among experimental set up in any event, though utilizing a similar probiotic strain and a similar direction routine because of the hidden possible significance of host-associated parameters. Along these lines, clinical studies heterogeneousness stays a significant hindrance to the conceptualization of validation-based rules on probiotic execution in allergic hypersensitivity (Forsberg et al. 2016).

Probiotics are susceptible to environmental surroundings such as moisture, heat, light, and oxygen. Customers should take precaution for storing probiotic containing product and adhered to the guidelines shown on the item label. One specific impediment restraint is the inability to indicate probiotic bacterial used for the study, depiction study duplication troublesome. Furthermore, numerous consumer diet complement exclude the particular bacterial strain or dosage of a probiotic on the mark, which makes it difficult for the drug specialist to advocate a product, in any event when a lesson is properly directed to deliver viable outcomes. Albeit numerous experimental testings bolster the protected use of probiotics, more exploration is expected to decide the long lasting safety of these items.

13.7 Future Directions

In current circumstance where the ebb and flow proof was created from hardly any preliminaries with serious extent of heterogeneity, routine utilization of probiotics as an added substance on treatment in subjects with unfavorably susceptible aviation route ailments cannot be suggested.

But the probiotic consumption emerges as a practicable way to diminish the frequency of respiratory tract diseases. Probiotics can affect together innate and adaptive immunity. Knowledge-based strategies supported with experimental data can be applied for successful clinical trials such as selection of optimal probiotic strain, microbe-derived compounds, the duration of regimens, administration forms, doses, and long follow-up time, as well as identification of potential early biomarkers of treatment efficacy. Recently scientist from Ireland, UK, and the USA propose the microbiome, live biotherapeutic product as a predictor of COVID-19 outcomes, for targeted immunomodulation in COVID-19 infection like prevention of virus attachment on host cells as well for prevention or treatment such as use of specific *Lactobacillus* strain as immunostimulatory adjuvant for intranasal vaccination, genetically engineered antigen producing organism. Consequently probiotics has great scope for the allergic airway infections which needs to determine.

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RAMAN-2021

CERTIFICATE

Participation

International E-Conference on Recent Advances in Material Science and Nanotechnology

Date : 7 th to 9 th Feb 2021

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This is to certify that **S. S. Khandare, M. M. Shukla, M. G. Ingale** have presented a research paper entitled ***'Green Synthesis of Silver Nanoparticles Using Apple and Banana Peel Extract, Their Characterization and Optimization'*** in the RAMAN-2021 held during 7th to 9th Feb 2021, Organized By Department of Physics, Arts, Commerce and Science College, Maregaon, Maharashtra, India In Association with Department of Physics, P. N. College, Pusad, Maharashtra, India

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ABSTRACT BOOK

INTERNATIONAL E-CONFERENCE ON RECENT ADVANCES IN MATERIAL SCIENCE AND NANOTECHNOLOGY

(RAMAN-2021)

7th - 9th February 2021

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ABSTRACT

Since last decade, green synthesis of metal nanoparticles such as silver nanoparticles is emerging as a new path to stand against various infections. The present study aims to synthesize silver nanoparticles by a green biological route, using an extract derived from apple (AE) and banana peel waste (BPE), which acts as a reducing and capping agent for reduction of Ag^{+} into Ag^{0} derived from silver nitrate ($AgNO_3$) showing development of reddish-brown and yellowish-brown colour respectively. Process of synthesis was optimized using several parameters. Optimum concentration of $AgNO_3$ was found to be for AE: 1.25mM; BPE: 0.75 mM, concentration of extract for AE: 500 µl; BPE: 200 µl, pH was for AE: 9.0 and BPE: 9.0, temperature for AE: 50° C and BPE: 50° C and incubation period for AE: 96 hr; BPE: 24 hr for optimum synthesis of silver nanoparticles. Characterization of the synthesized nanoparticles with UV-Visible spectroscopy reveals a characteristic absorption of surface plasmon resonance (SPR) peak at 422 nm and 422.4 nm respectively. Fourier transform infrared spectroscopy (FT-IR) affirmed the role of AE and BPE as reducing and capping agent of silver ions.

Keywords:- Silver nanoparticles, Green synthesis, Characterization, FTIR.

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I. INTRODUCTION
Nanotechnology (sometimes abbreviated to "nanotech") is the study of manipulating matter on an atomic and molecular scale. Generally, nanotechnology deals with structures sized between 1 to 100 nanometre in at least one dimension, and involves developing materials or devices within that size (Kahn et al.,2006). Nanomaterials are leading circumference of the rapidly developing field of nanotechnology. They are attracting gradually because of their unique physicochemical properties, determined by their dimensions, shape, composition and crystallinity. They were employed for the treatment of water, in catalysis, field of medicine and biotechnology etc. Among synthetic nanomaterials so far produced, the metallic nanoparticles (NPs) have distinctive properties like conduction of electricity, catalysis, high stability for chemicals and antimicrobial activities (Muzaffar and Tahir et al.,2018).Among all metal nanoparticles, Silver nanoparticles (AgNPs) are important materials that have been studied extensively, such nanoparticles possess unique electrical, optical as well as biological properties and are thus applied in catalysis, bio sensing, imaging, drug delivery, nano device fabrication and in medicine (P. K. Jain, Huang, El-Sayed, and El-Sayed et al.,2008; Nair and Laurencin et al.,2007). As Silver is a nontoxic, safe inorganic antimicrobial agent that is capable of killing about 650 types of disease causing microorganisms there is an

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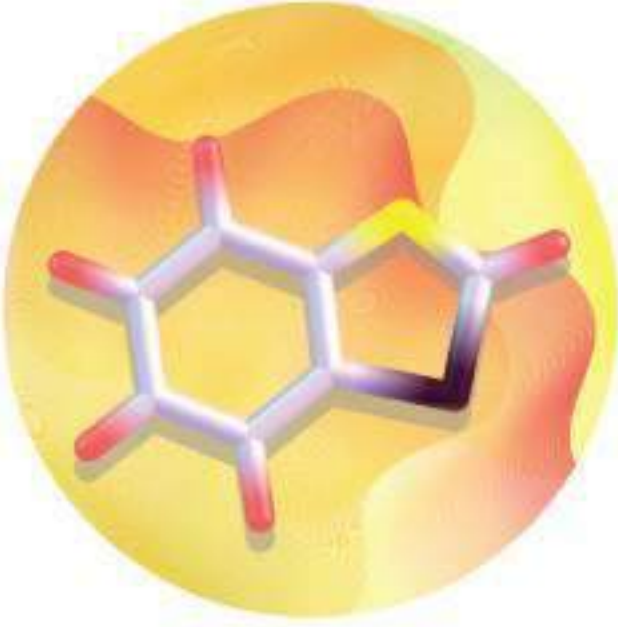
Benzothiazole

Preparation, Structure and Uses



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Chapter 3

ESIPT INSPIRED BENZOTHIAZOLE FLUORESCENT MOLECULES

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ABSTRACT

Excited state intramolecular proton transfer (ESIPT) based 2-substituted benzothiazole fluorescent moleculeshavegainedconsiderable attention in the pastfew yearsas ausefulmolecule in high-tech and classical application. It was due to its desirable unique photo-physical properties induced due to the proton transfer in an excited state. The photo-physical

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DECOLOURIZATION AND DEGRADATION OF TEXTILE DYE "DIRECT BLUE 15" BY BACTERIAL CULTURES SCREENED FROM TEXTILE INDUSTRY

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Abstract:

The textile industries use different types of dyes in their processing units which are liberated in natural water bodies through wastewater which causes serious damage to the environment. Chemical and physical method to treat effluent to remove color is expensive hence we study the biological method to degrade Direct Blue 15 dye. Here we isolate and identify four dye decolorizing bacteria by using selective enrichment culture in Bushnell-Haas (BH) medium amended with co-substrate glucose, yeast extract, and mgL-1 Direct Blue 15 dye. The isolates were identified as *Staphylococcus*spp., *Pseudomonas* spp., *Acinetobacter* spp.and *Bacillus* spp. Among these, *Pseudomonas*spp. was found to be the most efficient dye degrader with 72 % dye degradation efficiency. Percent dye degradation efficiency shown by *Staphylococcus, Bacillus,* and *Acinetobacter* was 66 %, 64%, and 28 % at optimum temperature of 30^0C and pH 7 respectively. As compared to individual bacteria, enriched bacterial consortium was found to degrade the dye more efficiently with 88 % dye degradation efficiency.

Keywords:Direct Blue 15 dye, Decolorization, Degradation, Textile industry

I. Introduction:

The textile industry plays an important role in the world economy as well as in our daily life, but at the same time, it consumes large quantities of water and generates large amounts of waste water. Dyes have been extensively used in many textile industries worldwide. Dyes are compound that absorb light with wavelength in the visible range, i.e., 400 -700nm. These are composed of a group of atoms called chromophores which are responsible for the dye color [1].The chemical reagents used in the textile sector are diverse in chemical composition ranging from inorganic to organic molecules [2].During the process of dyeing 10-15% dyes remains unused and is discharged in the water bodies with improper treatment. Globally the concentration of these dyes in the water bodies constitutes around 2, 80,000 tons per year. Besides forming toxic compounds these also create anaerobic conditions and unavailability of light to the aquatic life [3].Few of the dyes alone or in combination with hazardous chemicals may turn carcinogenic and may cause various health hazards [4].Dyes are recalcitrant by design and not readily amendable to common treatment methods, imposing a challenge for closed water systems therefore the treatment of dyes is a serious concern. Extensive research in the field of biological azo dye decolourization has shown promising results[5]. Several methods are being employed for removal of these dyes from water bodies. For these purpose biological and nonbiological systems are in effect. The biological systems are more preferred as these are eco-friendly and economical[6].

In the biological methods, the microbes such as bacteria, fungi and algae are being used for thewastewater treatment, which could be a viable option as low-cost and eco-friendly technology. There are various microorganisms found in the contaminated environment, have potential to decolorize and even completely mineralize many dyes from the wastewater efficiently under certain environmental conditions have been reported by various researchers[7].Among synthetic organic dyes, azo dyes are the most widely used, and they account for 60–70% of the total consumption of dyes. Furthermore, it was proven that most azo dyes and their metabolites can generate toxic, carcinogenic, mutagenic, and teratogenic effects on human health and the environment [8, 9 & 10]. According to the survey of Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers (ETAD) out of 4000 kinds of dyes, diazo Direct dyes demonstrated highest toxicity[11]. The intermediate aromatic amines and acrylamides generated in the metabolism process of benzamine-derived azo dyes have serious carcinogenic effects [12 & 13]. Therefore, azo-dye wastewater is a major concern, and the removal of azo dyes is extensively explored.Thus, the present study was aimed to isolate and characterize indigenous bacterial strain, capable of decolorizing Directblue 15 azo dye commonly used in dyeing industry of Wardha, Maharashtra, India. Additionally, effect of various environmental parameters on decolorization of the dye by isolated bacterial strains was studied.

II. Materials and Methods:

Sample Collection

The dye effluent was collected from a dyeing industry located in Gol Bazar, Wardha city. The effluent samples were collected in sterilized can and transported to the laboratory and were stored at 4^0C till further analysis.

Enrichment, isolation and screening of dye decolorizing bacteria

The isolation was carried out in Bushnell-Haas medium. The medium composition was KH2PO4 - 0.1%, K2HPO4 - 0.1%, MgSO4.7H2O - 0.02%, CaCl2.2H2O - 0.002%, NH4Cl - 0.1%, NH4NO3 - 0.1%, NaCl - 0.01%, and FeCl3-6H2O - 0.005% and the pH wasadjusted to 7.0. For enrichment of dye decolorizing bacteria, 10 mL of the effluent sample was inoculated in 90 mL sterilized Bushnell-Haas medium(BH) supplemented with filter sterilized 100 mgL^{-1} Direct blue 15 azo dye. After 3 days of incubation (37^oC, 150 rpm) 10 mL enriched broth was transferred to fresh dye supplemented BH medium and incubatedat the same condition for another 3 days. Such repeated sub culturingwasperformed for 3 times for the enrichment of putative dye decolorizingbacteria. Serial dilutions of the enriched broth were madeup to 10^{-6}dilutions and aliquots from each dilution were platedon BH agar medium amended with 100 mg L^{-1} of dye mixtureand incubated at 37^0C for 48 h. After incubation, morphologicallydistinct colonies were picked up and purifiedthrough repeated streaking on the same medium. The purified bacterialisolates were maintained in nutrient agar slants as pure culture and preserved for long term[14].

Identification of the selected isolates

Bacterial isolates able to grow profusely at 100 mgL^{-1} of dye concentration were considered as promising dye decolorizer and subsequently, characterized based on their morphological,cultural, physiological and biochemical characteristics[15]. The isolates were then tentatively identified by comparing the test results with Bergey's Manual of Determinative Bacteriology [16].

Dye decolorization assay

Decolorization assay was performed by inoculating the isolates in dye containing BH medium followed by the centrifugation of the broth afterevery 24 hrsandabsorbance of the supernatantwas recorded at 600 nm against a blank.

Analysis of decolorization efficiency

For dye decolorization experiment, Erlenmeyer flasks containing 50 mL of sterilized BH medium (pH 7.0) amended with Direct Blue 15 dye to a final concentrationof 100 mg L^{-1} were inoculated with 10% (v/v) inoculums of each isolate as well as the developed consortium and incubated for 3 days (at 37^0C with 150 rpm). Control was maintained without inoculation. After 24, 48 and 72 hrs of incubation the culture broth was centrifuged (10,000g, 15 min at 4^0C) and absorbance of the supernatant was recorded at 600 nm. The decolorization activity in terms of (%) decolorization was calculated according to the following formula given by Chen et al. [17]

$$\text{Decolourization (\%)} = \frac{\text{Initial} - \text{final}}{\text{Initial}} \times 100$$

Effect of pH and temperature on the decolorization of dyes

In order to study the effect of pH and temperature, the sterilized BH medium was amended with 100 mg/L of filter sterilized Direct blue 15 dye. The medium was maintained at different pH: 5,6, 7, 8. A volume of 1 mL of overnight culture was inoculated in the flasks and incubated in a shaker at 37^0C. Theeffect of temperature was studied by inoculating overnightculture and incubating in a shaker at 20^0C, 30^0Cand 40^0C. The measurement of decolorization of the total dye concentration was performed at an interval of 24 h for 3 days.

III.Results and Discussion:

In present studyselected bacterial isolates and developed consortium were tested for their ability to decolorize a commonly used textile dye Direct blue 15. In order to isolate dye decolorizing bacteria, the effluent sample was inoculated in BH agar medium amended withDirect blue 15. A total of 4 morphologically distinct bacterial colonies were isolated and screened out by repeated sub culturing method on dye amended BH agar medium. During screening process growth of isolates on dye supplemented BH agar medium in the form of white colonies considered as a positive result for screening of potent dye decolorizers[18].The selected bacterial isolates were characterized on the basis of their cultural, morphological, physiological and biochemical characteristics as presented in Table 1. All these characteristics were then compared with the Bergey's Manual of Determinative Bacteriology and the isolates were provisionally identified as *Psedomonas* sp., *Staphylococcus* sp., *Bacillus* sp.and *Acinetobacter*sp.After preliminary screening all these isolates were individually tested for their dye degradation efficiency for three days. Among the four isolates, *Pseudomonas* was found to be the most efficient dye degrader species with percent dye degradation efficiency of 72 % after three days at 30^0 C optimum temperature and pH 7. While percent dye degradation efficiency for *Staphylococcus*, *Acinetobacter* and *Bacillussp.* were found to be 66 %, 64 % and 28 % respectively. On the other hand enriched bacterial consortium could efficiently degrade the dye up to 88 % at optimum temperature of 30^0 C and pH 7 in three days. Temperature and pH found to influence dye degradation efficiency greatly.At 20^0C *Pseudomonas*sp. showed 36 % dye decolorization. Decolozization efficiency of *Pseudomonas* sp. increased significantly to 72 % at 30^0 C. But at40^0 Cdecolozization efficiency of *Pseudomonas* sp.was reduced to 61%. Dye degradation efficiency of consortium was also found to increase from 42 % to 88 % after increase in temperature from 20^0 C to 30^0 C. Hence temperature plays crucial role in dye degradation. Similar effect of temperature was also found on other isolates and 30^0 C was found to be the optimum temperature for all the isolates as well as for consortium. As far as the pH is concerned, 7 pH was found to be optimum for all the isolates as well as consortium. Increase or decrease in pH than 7 results in decrease in percent dye degradation. As compared to individual isolates, bacterial consortium showed significant hike (16 %) in dye degradation.

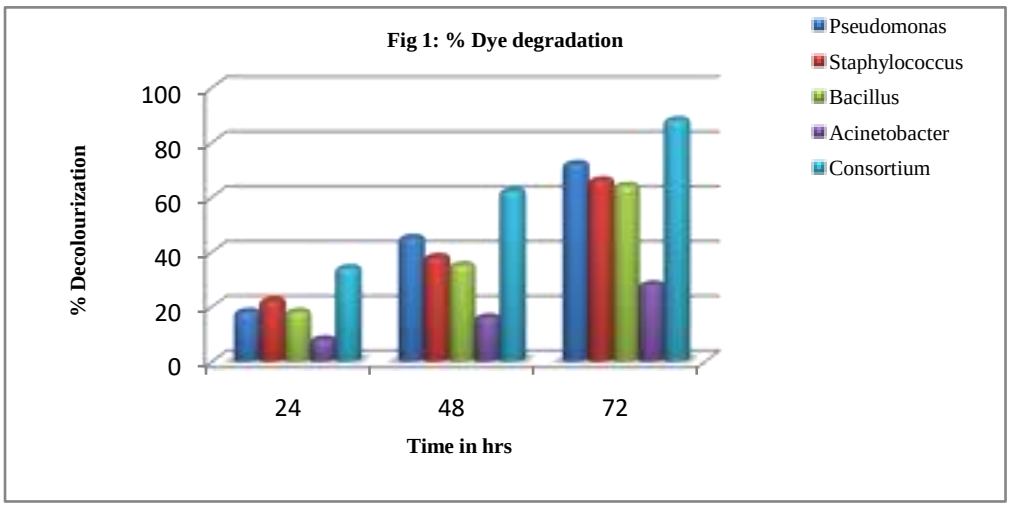
Fig 1: % Dye degradation
% Decolourization
100
80
60
40
20
0
24
48
72
Time in hrs
Pseudomonas
Staphylococcus
Bacillus
Acinetobacter
Consortium

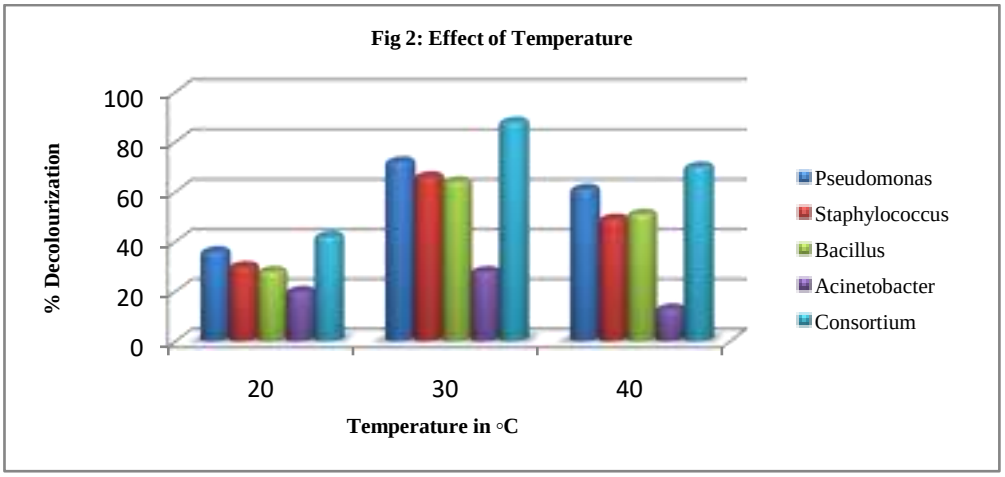
Fig 2: Effect of Temperature
% Decolourization
100
80
60
40
20
0
20
30
40
Temperature in ◦C
Pseudomonas
Staphylococcus
Bacillus
Acinetobacter
Consortium

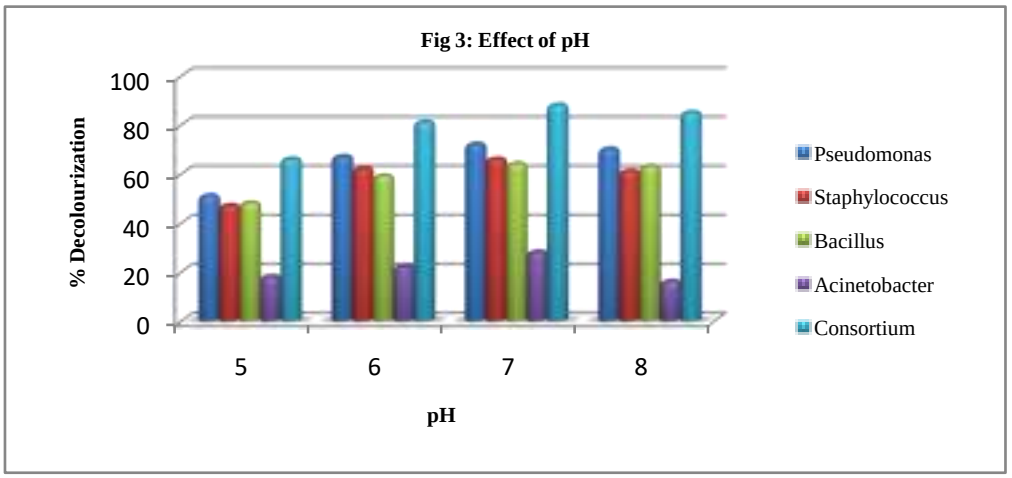
Fig 3: Effect of pH
% Decolourization
100
80
60
40
20
0
5
6
7
8
pH
Pseudomonas
Staphylococcus
Bacillus
Acinetobacter
Consortium

Table 1: Morphological and biochemical characteristics of isolates.

Characters	Test	Pseudomonas sp.	S. aureus sp.	Bacillus sp.	Acinetobacter sp.
Morphology	Gram character	-	+	+	-
	Shape	Rod	Cocci	Rod	Cocobacilli
	Motility	Motile	Non Motile	Motile	Non Motile
Biochemical tests	Indole	-	-	-	-
	MR	-	-	-	-
	VP	-		-	-
	Citrate utilization	-	-	+	+
	Nitrate reduction	-	-	+	-
	H2S production	-	-	-	-
	Catalase	+	-	+	+
	Oxidase	-	-	+	-
	Urease	+	+	-	-
Sugar fermentation	Lactose	-	+	+	-
	Mannitol	-	-	-	-
	Glucose	-	+	+	+

Conclusion:

The dye decolorizing abilities have been studied over a three days period where we found that 72 hr was most suitable to achieve maximum degradation. The most efficient species were *Pseudomonas* and *Staphylococcus*with 72 % and 66 % dye degradation at optimum temperature of 30^0 C and pH 7 respectively in three days. On the other hand enriched bacterial consortium could efficiently degrade the dye up to 88 % at optimum temperature of 30^0 C and pH 7 in three days. As compared to individual bacteria,consortium showed significant hike (16 %) in dye degradation.

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MODIFICATION AND CHARACTERIZATION OF STARCH AS AN ADHESIVE

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Increasing demand of energy globally and scarcity of petroleum resources has shifted focus of chemical industries to look for alternative raw material resources. Bio-based adhesive are attracting more and more attention in various fields due to their improved environmental footprint and independence from petroleum resources. Though synthetic chemical based resins have better bonding properties, it shows some drawbacks, which are harmful to humans as well as environment. Starch is produced by plants as a way to store the chemical energy that they produce during photosynthesis. The main goal of this study was to use cornstarch and arrowroot starch in the production of environmentally sound adhesives. Arrowroot & corn starch are environmentally friendly products, they can be modified as adhesive. Starch is an ideal material for manufacturing of wood-composite adhesives due to low cost, high free hydroxyl content, easy processing and treatment.

MODIFICATION AND CHARACTERIZATION OF STARCH AS AN ADHESIVE

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Abstract : Increasing demand of energy globally and scarcity of petroleum resources has shifted focus of chemical industries to look for alternative raw material resources. Bio-based adhesive are attracting more and more attention in various fields due to their improved environmental footprint and independence from petroleum resources. Though synthetic chemical based resins have better bonding properties, it shows some drawbacks, which are harmful to humans as well as environment. Starch is produced by plants as a way to store the chemical energy that they produce during photosynthesis. The main goal of this study was to use cornstarch and arrowroot starch in the production of environmentally sound adhesives. Arrowroot & corn starch are environmentally friendly products, they can be modified as adhesive. Starch is considered as a ideal material for manufacturing of wood-composite adhesives because of its low cost, high free hydroxyl content, easy processing and treatment.

I. INTRODUCTION AND LITERATURE SURVEY:

Adhesives play a fundamental role in many modern technologies and adhesive failure have catastrophic consequence. It is necessary to understand the factors responsible for the production of a good durable adhesive bond. Starch is produced from wheat, corn, sweet potato, rice etc. But the corn starch is having a good adhesive and film forming properties. Since starch is renewable, cheap, non-toxic, easily available, biodegradable and hence it has more demand in adhesive industry.

Pure starch powder has white colour with no taste and odour which is insoluble in cold water or alcohol. It is a polymeric carbohydrate, consists a large number of glucose units joined by glycosidic bonds. It consists of two types of molecules; the linear and helical amylose or the branched amylopectin. Depending on a source, starch contains 20 to 25% amylose and 75 to 80% amylopectin [1].

Starch derivatives are used in various industries as thickeners, gelling agents and encapsulating agents for papermaking, as wet-end additives for dry strength, surface sizes and binders, as adhesives (bag, bottle labeling, laminating, envelopes etc.), for warp sizing in textiles, and for glass fiber sizing [2]. Arrowroot becomes thick at a lower temperature than flour & not affected by acidic ingredients as well as freezing [3].

The most commonly available industrial starches are waxy cornstarch, regular corn starch, high-amylose corn starch type V, and high-amylose corn starch type VII, with amylose concentrations of 0, 28, 55, and 70% respectively [4].

Adhesives are substances that are able to make things adhere or stick together without deformation or failure through a process called adhesion. Renewable and biodegradable starch adhesives are topic of interest for research because of its environmental freindliness. Adhesives prepared from starch are most extensively used in corrugated board industry because of its abundant supply, low cost, renewability, biodegradability, and ease of chemical modifications [5].

Properties of good adhesives:

An adhesive is considered to be good if it is able to give complete bonding with good drying rate in presence of adequate heating [6-15] · To realize this, an adhesive needs to have following properties :

1. Appropriate viscosity
2. High initial tack
3. Solids in range of 20-33%
4. Consistency in batch glue properties
5. Fast setting

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RESEARCH ARTICLE **OPEN ACCESS**

Scatchard analysis of analgin with BSA at various pH and molecular modeling study.

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ABSTRACT

Study of the binding of drug with plasma protein by the acoustical properties shows simple and effective method. Analgin is a pain reliever and antipyretic drug. We studied binding of analgin with plasma protein by ultrasonic, FT-IR and molecular modeling techniques. In the present study, we used ultrasonic method for the study of the binding of analgin with BSA which is the novel method for study of binding of analgin with BSA. Study of interaction of analgin with BSA shows successful binding with BSA. Binding of BSA with analgin further confirmed using FT-IR spectroscopy and molecular modeling study. Effect of pH on the binding of analgin with BSA was also studied. The values of the association constant calculated from the Scatchard plot at varying pH 3, 4 and 5 are 0.5012, 0.4994 and 0.5014 respectively. Study of interaction by FT-IR spectroscopy gives the changes in peak positions of amide bands. The amide I changes from 1635 to 1642 cm^{-1} and amide II 1538 to1556 cm^{-1}. It shows the secondary structure of BSA changes on binding with analgin. binding interaction of analgin with BSA was further confirmed by using molecular modelling study. The energy value obtained (-213.34) shows that the analgin efficiently binds with BSA.

Keywords Ultrasonic study, FT-IR spctroscopy, Scatchard analysis, association constant, molecular modeling study.

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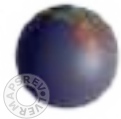

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RESEARCH ARTICLE **OPEN ACCESS**

Thermo acoustical studies of molecular interaction of N-[[(2S)-1-ethylpyrrolidin-2-yl] methyl]-2-methoxy-5-sulfamoylbenzamide at different temperature.

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ABSTRACT

Density(ρ),Viscosity(η) and Ultrasonic Velocity (U) of an alcoholic solution of N-[[(2S)-1- ethylpyrrolidin-2-yl]methyl]-2-methoxy-5-sulfamoylbenzamid drug(NSB) 2.5mM,5.0mM and 10mM were measured at 300, 305 and 310K. The resulting data were used to calculate various acoustical parameters ,acoustic impedance (Z), adiabatic compressibility(β), Intermolecular free length (L_f), Wada's Constant (W), Rao's Constant (R), free volume (V_f) , were calculated which provides valuable information regarding drug-alcohol interaction

Keywords NSB, Acoustical parameters, Inter molecular interaction, drug-alcohol interaction.

INTRODUCTION

Ultrasound refers to such high frequency sound waves that they can't be heard. Now a day's Ultra-Sonic technology due to non-destructive nature [1–3], is used in a variety of applications in medicine, biology, industry, materials science agriculture, oceanography, sonochemistry etc. Ultra-sound waves have been used extensively as chemical additives for order to improve the production yield of produced foods, and also useful in the preparation of biomaterials, protein microspheres, polymer and polymer surface modifications, etc. for material chemistry [4-7].

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CHAPTER

19

Azotobacter

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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
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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

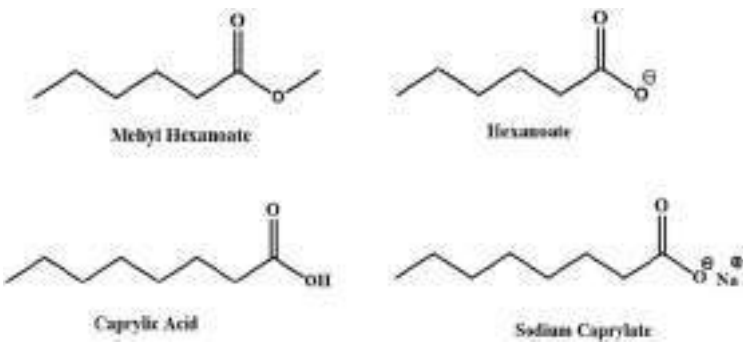

FIGURE 19.1 Structure of unusual carbon sources for *Azotobacter* differentiation.

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

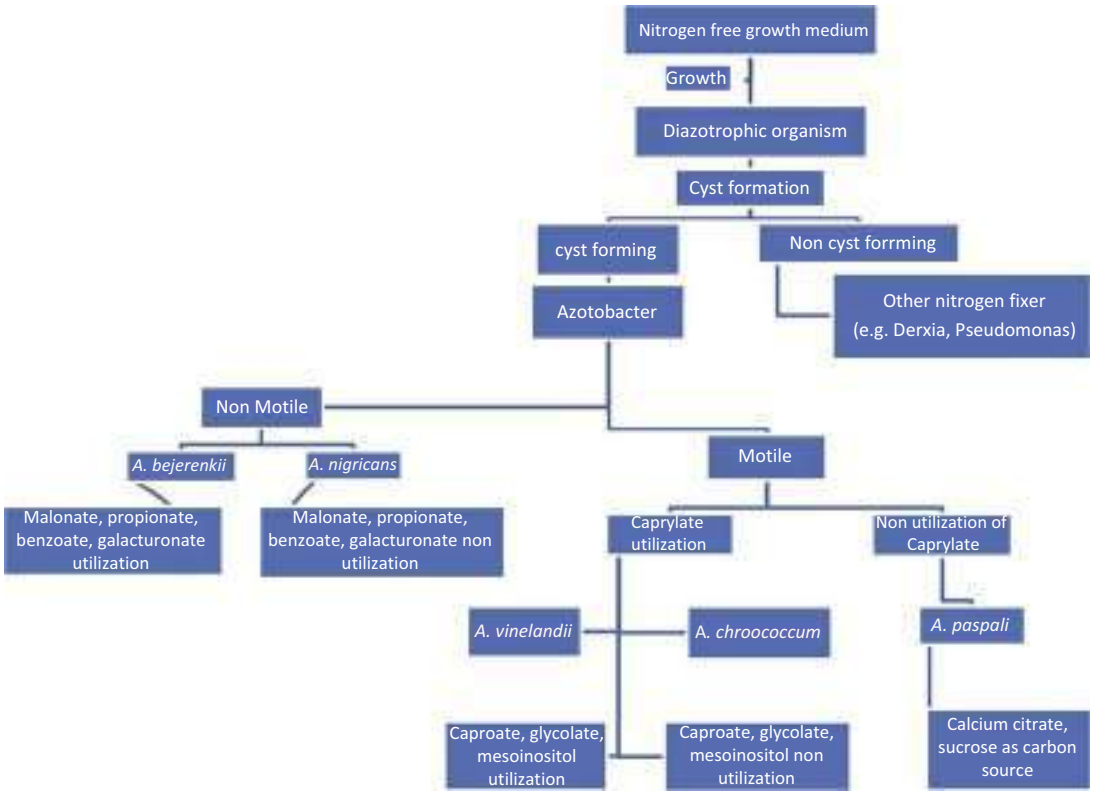

FIGURE 19.2 Key for identification of *Azotobacter* sp. based on important morphological and biochemical characteristics.

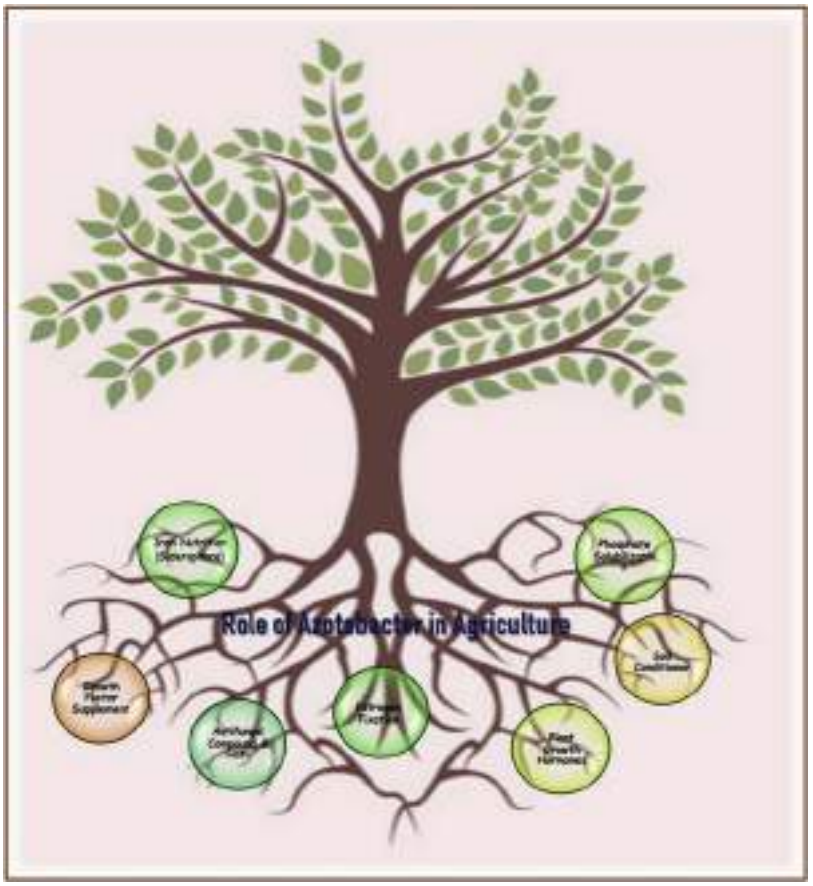

FIGURE 19.3 Role of *Azotobacter* in sustainable agriculture.

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),

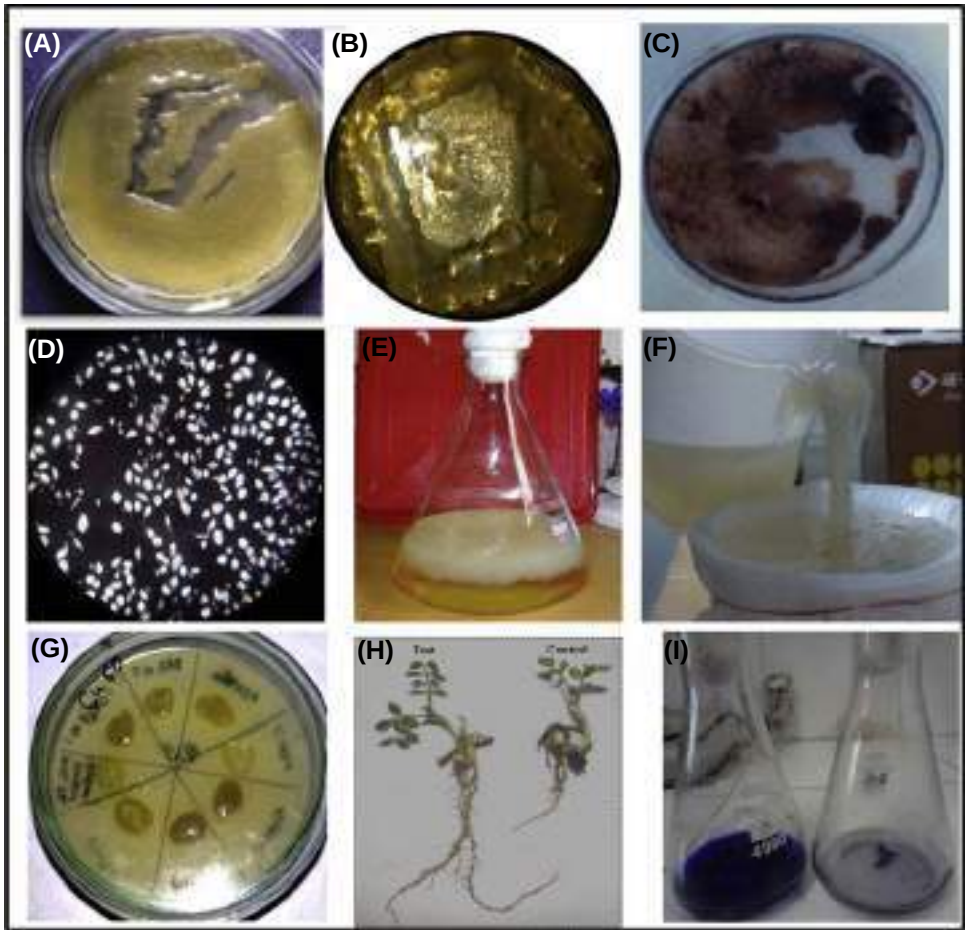

FIGURE 19.4 *Azotobacter* biopolymer and its explored applications (At KBC NMU laboratory), (A), (B), and (C) Different *Azotobacter* sp. on nitrogen-free medium, (D) *Azotobacter* with its biopolymer sheath by negative staining, (E) and (F) *Azotobacter* biopolymer production and recovery; (G), (H), 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.

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BAJAJ COLLEGE OF SCIENCE (AUTONOMOUS) WARDHA

A TEXT BOOK OF
CONCISE CHEMISTRY

(B.Sc. II Year, IV Semester Students)

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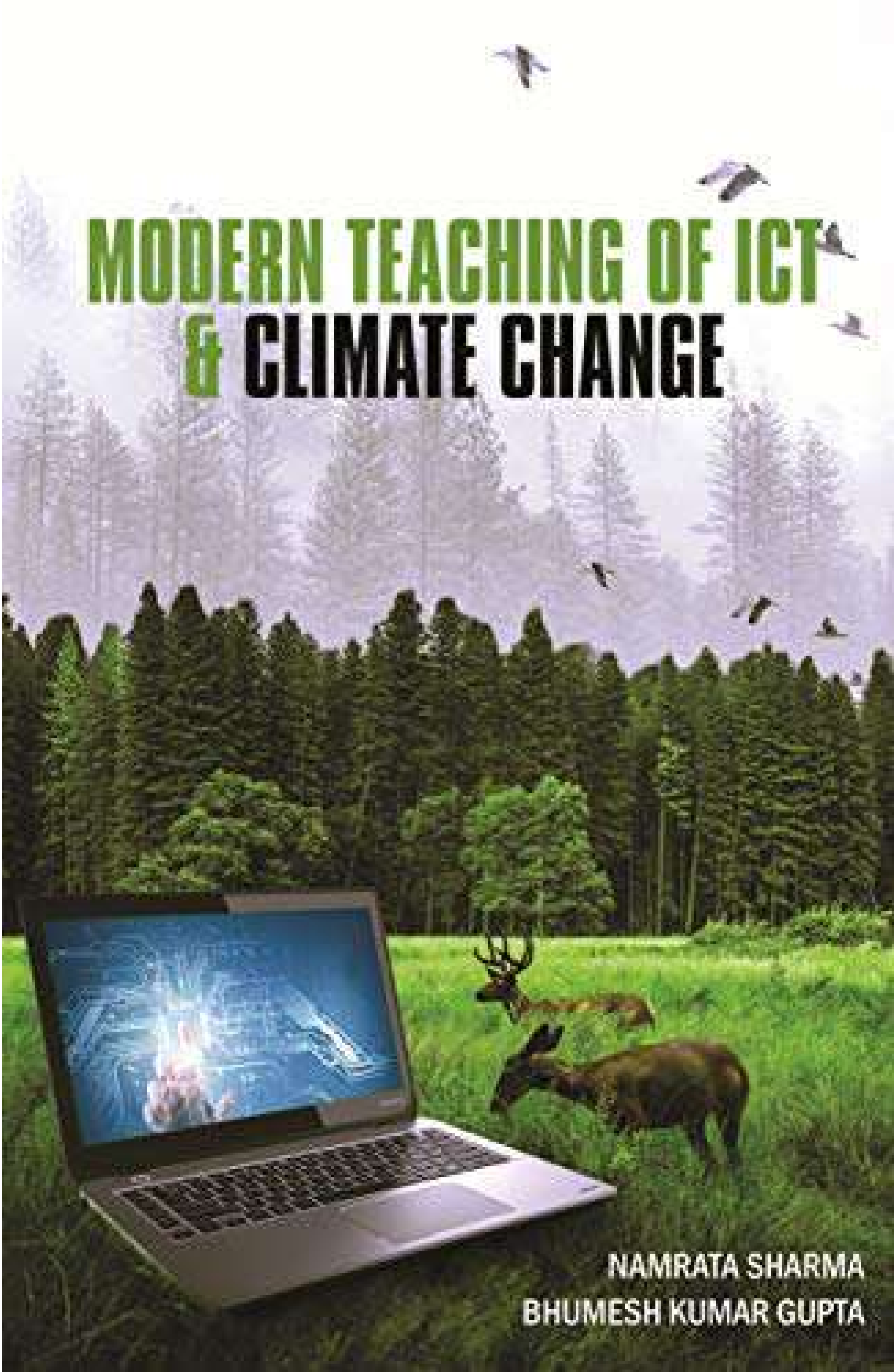
MODERN TEACHING OF ICT & CLIMATE CHANGE



NAMRATA SHARMA
BHUMESH KUMAR GUPTA

Modern Teaching of ICT & Climate Change

This edited book contains chapters/articles contributed by participants of 121st Orientation Programme

Chief Editor
Namrata Sharma, Bhumesh Kumar Gupta

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By

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CONTENTS

ENVIRONMENT AND SUSTAINABLE DEVELOPMENT THROUGH HIGHER EDUCATION

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Abstract:

Sustainable development has become an important concept for securing future of the world. Indian problems such as growing population, migration of people from rural area to urban area, increasing use of resources is endangering the passing of environmental benefits to next many generations to come. The higher education is the tool and solution for sustainable development in India as the awareness about the environmental hazards can be communicated to the lemans by the method of education. India or other countries of world is working on sustainable development through higher education since 1972. This paper is to review the progressive development of environment and sustainable development through higher education. Many findings have been used to see the progressive development. It is suggested that the serious approach is to be made in order to preserve natural resources for future generation and education can be the best solution.

Keywords: Sustainable development, Higher education, Environment

1. Introduction:

Sustainable development has become an important concept for securing future of the world and India. (Sathaye, Shukla, and Ravindranath 2001) Indian pr4oblems such as growing population, migr ation of people from rural area to urban area, increasing use of resources are endangering the passing of environmental benefits to next many generations to come (Sathaye et al. 2001). Thus sustainable development is very quintessential need of today 's India. But it is very difficult to attain a sustainable development without considering the higher education. (Banga Chhokar 2010; Chhokar 2010; Dutz 2007) Higher education is the tool and solution for sustainable development in India as the awareness about the environmental hazards can be communicated to the lemans by the method of educating the one who belongs to the people and society

most (Misra 2012; Srivastava and Rehman 2006) Hence it is essential to know about the education first and then the Sustainable develo pment will be discussed. Also the various initiatives by India and rest of world will be discussed. At the last the benefits and shortcoming of higher education in sustainable development will be characterized.

2. Education

Education in India is mostly categ orised in two different categories as Basic education and the Higher education.

a. Basic Education:

The basic education starts from the kinder garden to the 10 th standard in India. In India primary education is free as per the 86th Amendment of the Constitution of India. The amendment was made by then Prime Minister of India Shri. Atal Bihari Vajpeyi. As per this amendment primary education is made free and compu lsory for children of one age groups 6 to 14 years as their fundamental right under "Sarva Shiksha Abhiyan". (MHRD, 2015).(Kainth 2006; Rao 2009) In 1963, the NCERT major criterion was to make aware the student from their childhood age about the sensible use of natural resources. The syllabus consists of units like Air, Water and Weather; Rocks, Soils and Minerals, which clearly deals with environmental study. The Kothari commission (1964 -66) sugges ted that basic education should contain details of Environmental education and coordination of it with the life needs and desire of the people and the nation. (Agarwal 2007; Tilak 2018) The report recommended that the aims of teaching scien ce should build a proper understanding of the concepts, principles, facts, and procedure of the physical biological environment. The Review Committee (1975) recommended that there should be one book for environmental studies for classes III, IV and V . (Agarwal 2007) The courses for environmental studies was designed in a way that it include both the natural and social environment. Thus a balance was made between one society and one environment. According to the National Curriculum Framework of 2005 (Research and (India) 2005) at the primary stage, EVS was given special importance.

b. Higher Education

The higher education consists of the education which students acquire during under graduation, post graduation, doctorate and post doctorate

studies. The Supreme Court of India had acknowledged the significance of environmental education at all levels and it was made mandatory to have a course on Environment at the undergraduate level to sensitise the youth towards the environmental issues and concerns. As per the Supreme Court direction, the University Grants Commission introduced six months of compulsory environmental course in all the universities and colleges during the academic year 2004-05. (Rajagopalan 2015)

3. Environment and Sustainable Development

To find the solution we must first know what sustainable development actually is sustainable development? We will have to strike balance between the different types of needs of Indian people and one environment. The distribution should be in a way that the need does not a profound impact on one environment. Thus sustainable development can be defined as economic development that is conducted without depletion of the natural resources. In other words Sustainable development is development that meets the need s of the present, without upsetting the ability of future generations to meet their own needs. Sustainable development and social development go hand in hand hence proper linkage between Sustainable development and social development is needed. Sustainable development comprises of the need of the current generation and the future generation in an extent to which are environment can be used for the development of social development of current generation so that future generation will not get affected by the use of the environment. Education is a factor that can influence sustainable development in a broader extend. Higher Education can be a path for proper functioning of our proper development of human being along with conserving the same for the future gener ation. The sustainable development ideas were formulated in chronological order as follow(Kumar, Kumar, and Vivekadhish 2016; Misra 2012; United N ations 2016):

1. In 1972 the first coin was tossed about sustainable development which was discussed in UN conference on human environment held at Stockholm.
2. It was in 1987 a report "our common future" was published by UN world Commission on human environm ent and development. This report also called as Brundtland report was the first to define sustainable development

which says "meeting the need of present without compromising the ability of future generations to meet their own needs".

3. Although the conference was held in 1972 it was in 1990 when universities of different parts of the world where requested by the International Association of Universities (IAU) to promote sustainable development by the method of Higher Education.
4. In 1992 Brazil hosted UN Earth summit the main objective of the summit was to give a thought on the environmental problems and how to resolve by multidisciplinary approach mechanism.
5. A major development took place in 1993 when Kyoto declaration on sustainable development was adopted by IAU.
6. Hereafter there were several other meetings such as in year 2000, the chief of various countries gathered in UN to discuss about environmental concern.
7. The major improvement was done in 2002 on sustainable development in a conference held at Johannesburg by declaring decade 2005 -2014 as decade for education for sustainable development. UNESCO was the agency which was looking after sustainable development and higher educationKyoto declaration (Ganapati and Liu 2009; Suthaye et al. 2001; Sotamaa 2009) was a major breakthrough to find the linkage between higher education and sustainable development. This declaration was mainly promising the following points:

1. The socio-economical benefits combined with eco-cultural benefits
2. Accommodating sustainable development with the higher education and research
3. Suggest a different way of thinking
4. Development is directly related to the human race.
5. To deliver welfare education as to create sensible citizen with respect to sustainable development.
6. To form various collaborations between different institution for sustainable future

The Kyoto declaration had helped to encourage the rural population to know about sustainable development. It also wants local universities to provide information on sustainable development to the local rural people. Thus universities can help to remove the misunderstanding of the relation b etween

cultural myths and sustainable development. This will be helping local people to overcome the myths and superstition regarding the misuse of resources which are in great danger. The Kyoto declaration also realised that the available resources on earth should be categorised in different types depending upon the danger they are facing due to the over utilisation of resources. This data was shared with universities so that they can interpret and find solutions by the method of project and research wor k in higher education, thus Universities can create a method to overcome these problems. Thus Kyoto declaration has helped universities to research in sustainable development and find the reaction of their research on society. It is emphasising on consider ation of International Association of universities (IAU) and implementation of the declaration done by IAU. The IAU had emphasized on universities to work on environmental literacy that is to find a way to convey environment and sustainable development to public at large. These will develop relation and cooperation between the different segments of society and will increase collective handwork for safeguarding the natural resources for future generation.

The Kyoto declaration recommendation for universities is as follow:

a. Each University will have their own Action Plan which will ensure that the institutions working under it will be committed to follow the principle and practices of sustainable development and in return it should communicate the same to its s tudents, employee and public which is directly or indirectly attached to the instructions.
b. It should not only educate others but also follow the sustainable development practices in its own campus.
c. It should engage several teachers in their academic staff to teach sustainable development.
d. It should encourage their staff and students to protect the environment and follow the procedures adopted by the University in accordance with the environmental and sustainable policies of the university and the institutions.
e. It should try to implement interdisciplinary (intra -university) work for the functioning of program related with sustainable development.
f. To improve ethical obligation of the stakeholders who are working on sustainable development.
g. University students and staff should work hand in hand and they should overcome the forces which lead to environmental misuse.

h. University can also promote Inter University collaborations for the betterment of the environment by utilising the best available facilities of each university.

i. University can build a partnership with various other Institutions which are involved in developing innovative Technologies which will help in making environment better than previous.

4. Sustainable Development Goal (SDG)

The SDG (Le Blanc 2015; Bongaarts 2016; Rasul 2016; United Nations 2016)term was tossed when leaders of 193 countries came together in 2015 for the betterment of the future. After analysing the present scenario of the food shortage, water scarcity due to low rainfall, the unnecessary wars, poverty etc everywhere in the world. Hence to overcome this situation and to provide peoples around the world a better future, these countries created a plan called the Sustainable Development Goals (SDGs). This set of 17 goals is designed to get solution for poverty, health, hunger, etc and protect the adverse effects of climate change. Although it seems to be an adventurous plan but previous data of 15 years shows that due to the collective efforts of leaders around the word, the poverty has decreased to 50 percent in 2015 from 2000. Hence, all leaders around the world started to work on the SDGs one among them is United Nations Development Program (UNDP). UNDP is one of the leading organizations working to fulfill the SDGs by the year 2030. The 17 Goals as per their Goal Number are as follow:

1. No Poverty

This goal was set to end the extreme poverty by 2030. Although the world poverty was halved during 2000 to 2015 but as per survey around 80 crore peoples are still living in extreme poverty all over the word. The extreme poverty is considered when the livelihood of an individual is around Rs. 100 per day ($1.25). Hence the goal is to overcome this by 2030.

2. Zero Hunger

This goal is set to end hunger by 2030 by confirming food security for each individual. Also, improvement of nutrition value and promotion of sustainable agriculture through organic farming is considered

3. Good Health and Well-being

This goal is set to ensure the healthy lives to all classes and age of the human being.

4. Quality Education

The goal 4 is to bring equality in quality education. Everyone is liable for the learning throughout life. Hence every individual should have an environment for quality education throughout life.

5. Gender Equality

The goal is to achieve gender equality and empower the girls and women in every society around the globe.

6. Clean Water and Sanitation

The goal is to ensure sustainable development in water management and provide sanitation to each and every person of the world.

7. Affordable and Clean Energy

To ensure affordable, modern, reliable and sustainable energy to all is the primary target of this goal.

8. Decent Work and Economic Growth

This goal is set to promote sust ainable economic growth for all that is everyone should get productive employment and decent work.

9. Industry, Innovation and Infrastructure

The goal is to build durable infrastructure, promote sustainable industrialization and innovation.

10. Reduced Inequality

The goal is reduce inequality within countries and support the developing countries and under develop countries to make up with the developed countries.

11. Sustainable Cities and Communities

The goal is to make cities sustainable for the safety and living values.

12. Responsible Consumption and Production

The goal is to make the utilization of any commodity in an equal manner. Hence emphasis is given on responsible consumption as well as responsible production.

13. Climate Action

There is a need of urgent action on the climate change to sustain the available resources for the future generations. The goal is set to take control on the fast accelerating climate change.

14. Life below Water

The sea is explored at a very high rate, hence the goal is set to conserve oceans. Also one goal is to ensure sustainably use the oceans, seas and marine resources.

15. Life on Land

To protect, restore and encourage sustainable use of earthly ecosystems is one of part of this goal. It is also emphasizes on management of forests, prevent desertification, and the most important one is to halt and reverse land degradation and halt biodiversity losses.

16. Peace and Justice Strong Institutions

Peace is the need for sustainable development of the society and it can be achieved through the equali ty in justice delivery for all and equality in institutions at all levels

17. Partnerships to achieve the Goal

The last goal is to strengthen the means of implementation and refresh the global partnership for sustainable development.

5. India and SDG's

The Univ ersity ranking agency, Times Higher Education (THE) of United Kingdom released a major ranking to put various universities of the world in order of the extent of work done in the field of SDG. This ranking has considered the 11 SDGs which are having a dire ct impact of higher education on it. The SDGs which are considered to evaluate universities are Goal 3 to 5, Goal 8 to 13, Goal 16 and 17. The universities of Japan had the most representation in the world ranking list followed by the United States and Ru ssia. The rankings are given on the overall of 11 SDGs as well as individual SDGs. The list has been topped by different nations in different categories. But considering India with rest of the countries it is found that the contribution of India is very le ss. India is not standing even in top 100 of any of the 11 SDG (Kumar et al. 2016) categories. A joint report of NITI Aayog and United Nation was released in December 2018 which shows the SDG India Index for the Indian states and Union Territories in executing 2030 SDG goals. The report had emphasized on the critical progress of India o n SDG in the next decade as the population is increasing with an accelerated rate and also due to the high economic growth. Due to this reason it has become important to implement the SDG in a firm way. But the situation found in the ranking shows a very d ifferent picture, the institutions which are representing India on a global platform such as Central universities, NITs, IIMs and even IITs do not find any place in the the University Impact rankings . But on the other hand some private institutions have found their places in this ranking which are:

1. JSS Academy of Education and Research, TamilNadu
2. Amrita Vishwa Vidyapeetham
3. Annamalai University
4. Christ University, Bengaluru
5. Jamia Millia Islamia
6. KIIT University
7. KLE University
8. Manipal Academy of Higher Education
9. Pondicherry University
10. PSG College of Technology

This is a big question for the developing India whether our leading institutes are in reality accountable campuses that propagate sustainable development in higher education.

6. Conclusion

Sustainable development is a very important factor for the preservation of the environment for the future generation. This idea is to be percolated in the minds of the common people so that the excessive amount of utilization of the natural resources could be made un der control. Along with the environmental studies in basic education, higher education it should also be given a higher concern in environment and sustainable development. Higher education is the only way to create awareness about the sustainable developme nt. International community had already considered higher education as an important facture in achieving Sustainable Development Goals (SDGs). But India is par away from progress done by the international community. Hence India needs to develop a strong policy for the environment and sustainable development through higher education.

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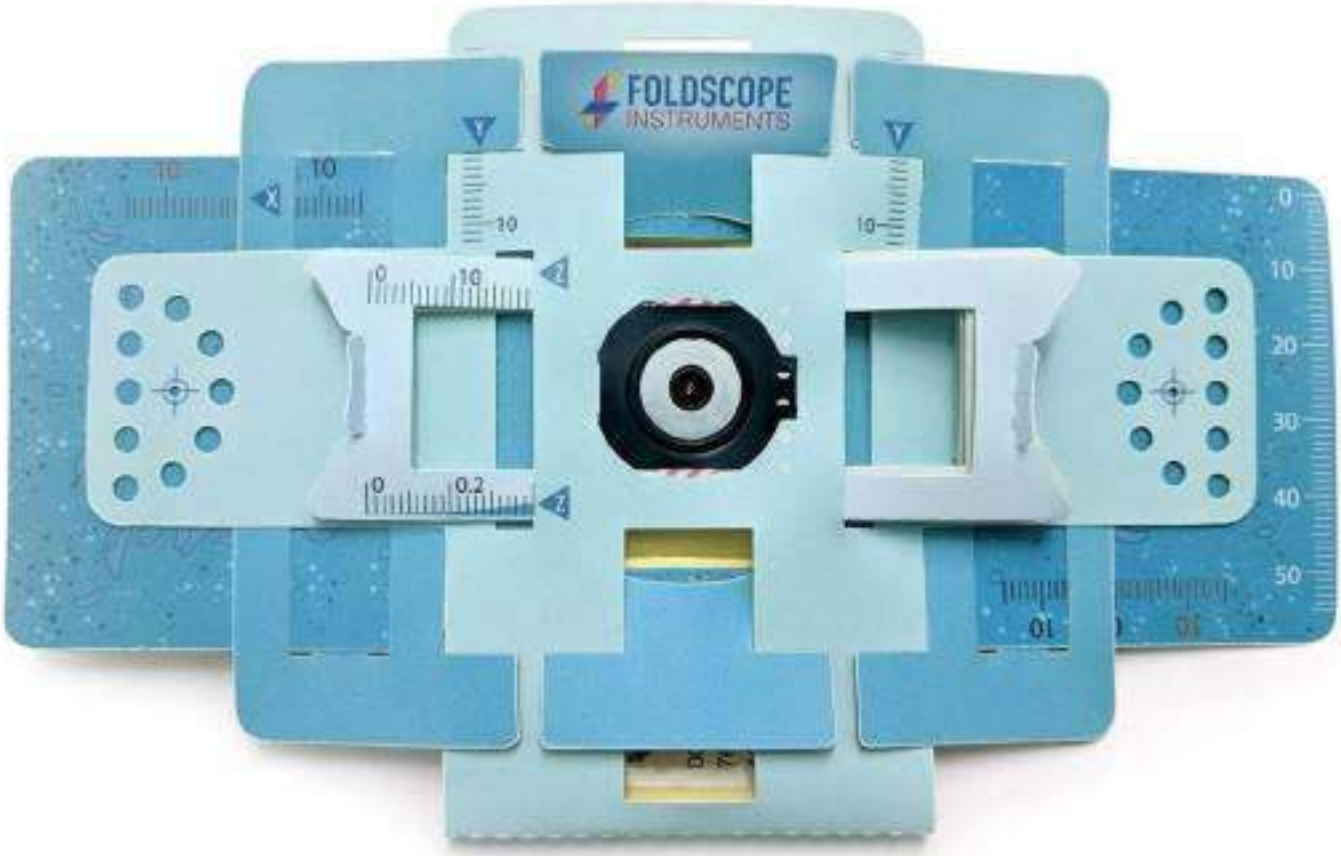
FOLDSCOPE AND ITS APPLICATIONS



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PREFACE

A Foldscope, a low-cost science tool, is an optical microscope that can be assembled from simple components, including a sheet of paper and a lens. It was developed by Dr. Manu Prakash and designed to cost less than US$1 to build. It is part of the "frugal science" movement which aims to make cheap and easy tools available for scientific use in the developing world. The Department of Biotechnology (DBT), Government of India and the Prakash Lab at Stanford University, USA signed an agreement to bring the Foldscope to India to encourage curiosity in science. It is being used as a teaching tool for the students in biology, chemistry, physics and many other streams. Keeping these facts in the background, the editors and authors of the book have tried to compile their research and review outlook about Foldscope usage and its various applications. The aim of this book is to facilitate the adoption of Foldscope as an educational and research tool by students, teachers, scholars, scientists and the general people. Many authors who are also Project Investigators and recipients of the Foldscope research grant acknowledge Department of Biotechnology, Government of India. The authors hope that this book will not only provide pleasant reading but also practical knowledge which can be utilized by the user of this book in the area of Foldscope microscopy.

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Dhananjaya Pratap Singh
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Microbial Interventions in Agriculture and Environment

Volume 1 : Research Trends, Priorities and Prospects

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Dhananjaya Pratap Singh
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Editors

Microbial Interventions in Agriculture and Environment

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Contents

New Age Agricultural Bioinputs

14

Bhavana V. Mohite, Sunil H. Koli, Hemant P. Borase, Jamatsing D. Rajput, Chandrakant P. Narkhede, Vikas S. Patil, and Satish V. Patil

14.1 Introduction

Nitrogen-based biofertilizers are significant bioinputs, but according to current environmental changes and ever-increasing food demand, it is the need of time to popularize more efficient bioinputs for soil. These bioinputs will help to fight against problems like an unpredictable monsoon, global warming, and decreasing soil fertility, and indiscriminate use of agrochemicals.

Besides chemical fertilizers, organic soil conditioners, the application of phosphate solubilizers, nitrogen fixers, and *Trichoderma*, *Verticillium*, *Metarhizium* like versatile biocontrolling agents are the common strategies of soil conditioning. In the past 50 years, there is tremendous work published on nitrogen fixers and phosphate solubilizers. The results of these findings directed to the exploitation of common biofertilizers like *Azotobacter* and *Rhizobium* as a nitrogen fixer and other organic inputs. In addition to above, phosphate, zinc, sulphur, potassium solubilizers are a

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significant part of current agricultural practices. Although these practices proved beneficial to uphold soil fertility and other agronomical problems like pest attack and plant susceptibility to various infections, physiological problems due to the change in the atmosphere need some novel strategies or additional bioinputs.

There are various significant bioinputs like the application of 1-aminocyclopropane-1-carboxylic acid (ACC) enzyme and phytase producing microorganisms and bacterivorous flora. These are which were reported, but unfortunately remain as neglected practices by Indian farmers. The following three major bioinputs are need of time to use as new soil bioinputs in modern agricultural practices:

1. Use of ACC oxidase and deaminase producer bioinputs
2. Use of phytase producer
3. Use of bacterivorous soil microbes

The central idea of this chapter is presented in Fig. 14.1, which represents the ability of major modern agricultural bioinputs.

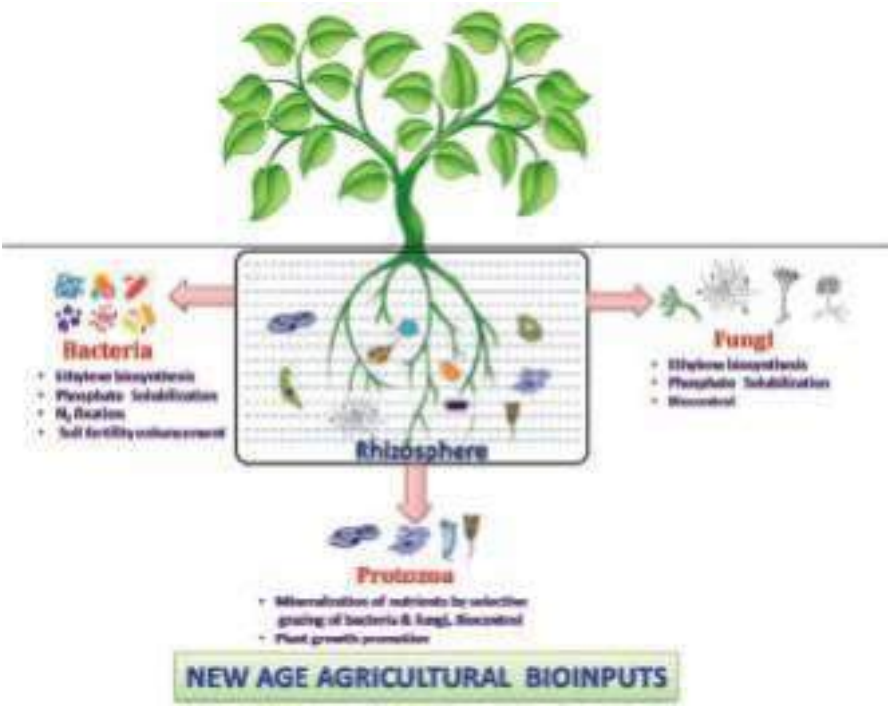

Fig. 14.1 Schematic representation for the new age agricultural bioinputs

14.2 Application of ACC Oxidase and Deaminase Producer Bioinputs

14.2.1 ACC and ACC-Degrading Enzymes

The Yang cycle produces 1-aminocyclopropane-1-carboxylic acid (ACC) and ACC oxidase and deaminase (ACCO and ACCD) (Yang and Hoffman 1984). Shang Yang unlocked the mystery of freshness of fruit, flowers, defoliation, and ripening of fruits by proposing a continuous biochemical cycle known as the Yang cycle. The Yang cycle biosynthesizes ethylene in plants. Ethylene is important in host–pathogen interactions, seed germination, flowering, and fruit ripening. It establishes the central role of methionine in ethylene synthesis. Yang's study proved the genesis of S-adenosylmethionine as a transitional compound which is further converted into ACC and then ethylene (Fig. 14.2).

ACC is the signaling molecule of a plant, easily transported through intra- and intracellular tissues over short and long distances.

ACC is a cyclic α-amino acid with a three-membered cyclopropane ring merged to an α-carbon atom of the amino acid (Fig. 14.3) and chemical formula $C_4H_7NO_2$ with a molar mass of 101.0 g/mol^{-1}. ACC is considered an essential intermediate that regulates ethylene biosynthesis. The enzyme ACCO is a member of the oxidoreductase class, which is responsible for the transformation of

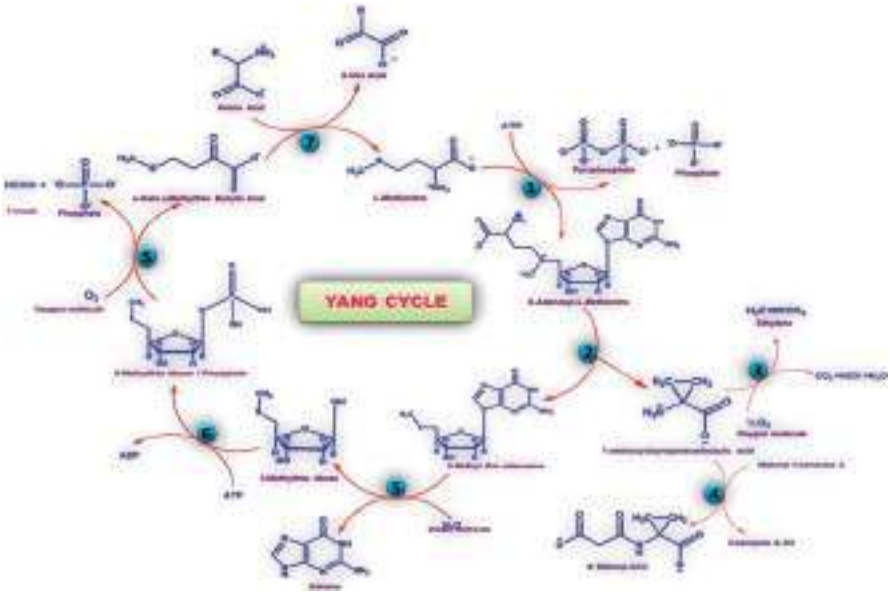

Fig. 14.2 Yang cycle for ethylene biosynthesis. Cycle path: (1) SAM synthetase, (2) ACC synthase, (3) ACC oxidase, (4) ACC N-malonyltransferase, (5) MTA nucleosidase, (6) MTR kinase, and (7) transaminase, (S) spontaneous reaction

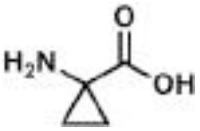

Fig. 14.3 Chemical structure of ACC

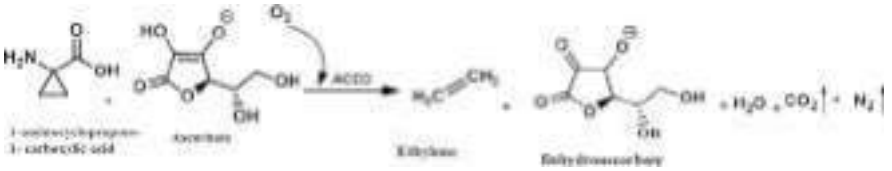

Fig. 14.4a Transformation of ACC to ethylene with ACCO

1-aminocyclopropane-1-carboxylate to ethylene with carbon dioxide, water, and other by-products (Fig. 14.4a).

In drought stress conditions, ethylene synthesis is rapidly increased (Morgan and Drew 1997). Ethylene is the one of the marker compounds of drought conditions and is also known as stress ethylene. Nitrogen fixation and nodulations are influenced by the various effects of high ethylene synthesis through water and temperature stress, like reduction of transpiration rate by closing stomata to regulate the abscisic acid pathway (Tanaka et al. 2005; Tamimi and Timko 2003; Penmetsa and Cook 1997; Guinel 2015). Hence, if the ACCO is regulated, then the natural synthesis of ethylene is regulated. Various researchers advocated that various rhizospheric microbes also control the ethylene level in a plant by deaminating ACC diffused through root cells and seeds (Finlayson et al. 1991; Penrose and Glick 2001; Penrose and Glick 2003).

14.2.2 Aminocyclopropane-1-Carboxylic Acid Oxidase (ACCO)

Aminocyclopropane-1-carboxylic acid oxidase is an enzyme recognized to fight against the consequences of drought in plants. It was well documented that drought affects various biochemical, morphological, and physiological activities of plants, e.g., turgor pressure, transport of soil nutrients, nutrient transport to root, nutrient diffusion through root mass, and a run of water-soluble nutrients such as silicon, manganese, and sulphate. Besides these, it leads to oxidative stress, which causes a decrease in chlorophyll synthesis, membrane deterioration, and protein degradation in plants (Hsiao 2000; Selvakumar et al. 2012; Sgherri et al. 2000; Rahdari et al. 2012).

14.2.3 Aminocyclopropane-1-Carboxylic Acid Deaminase (ACCD)

ACCD is the enzyme synthesized in the cytoplasm of bacteria. It is a multimeric sulfhydryl enzyme having a monomeric subunit with molecular weight of 35–42 KD (Glick et al. 2007). ACCD catalyses ACC conversion and produces α-ketoglutaric acid and ammonia (Fig. 14.4b). It was reported that D-serine and D-cysteine (D-amino acids) also act as a substrate for ACCD. Previously, the optimum temperature and pH for ACC deaminase were reported as 30–35 °C and 8.5 (Jacobson et al. 1994; Honma and Shimomura 1978; Jia et al. 1999). But currently, there is significant research going on to screen a versatile ACC deaminase producer who has a broad temperature and pH range (Xuguang et al. 2018). Various bacteria were reported for the production of ACCD, e.g., *Enterobacter cloacae*, *Pseudomonas putida*, *Pseudomonas* sp., *Alcaligenes*, *Hansenula*, *Rhizobium*, *Sinorhizobium* sp., *Pseudomonas chlororaphis*, *Rhizobium leguminosarum*, and *Bacillus subtilis* (Klee et al. 1991; Glick 1995; Belimov et al. 2007; Tittabutr et al. 2013; Ma et al. 2004; Duan et al. 2009). Similarly, some fungi and yeast were also reported for ACCD production, e.g., *Penicillium citrinum* (Minami et al. 1998; Jia et al. 1999).

Glick (1995) described the role and importance of some plant growth–enhancing *Rhizobacterium* in the management of drought pressure and various physiological activities of plants. Glick (1995) illustrated that ACC is produced in more quantity during drought stress and exudated outside of the root cells. The plant growth-inducing bacteria around the roots are recognized for its versatile activity and utilize the ACC exudate by ACC deaminase, and to keep the balance in internal and external ACC level, internal ACC is transported outside of the root. This process reduces the amount of ACC required for the biosynthesis of ethylene inside plant cells. Hence, if such ACCD-producing *Rhizobacterium* is present around the rhizospheric area of vegetation in a drought condition, ethylene production is suppressed, further leading to restrain inhibitory stress; ethylene causes defoliation, inhibition of root elongations, and nodulation transpiration (Glick et al. 2007). The presence of ACCD-producing microbes in soil proved their significance in a variety of plant growth–promoting activities, e.g., the existence of ACCD producer enhances the nitrogen fixations by inducing the normal process of root nodule organization in drought or temperature stress conditions.

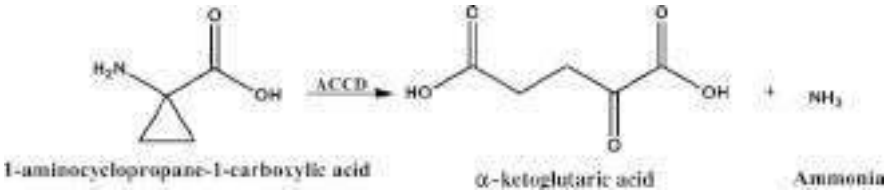

Fig. 14.4b Conversion of ACC to ethylene with ACCD

14.3 Application of Phytase Producer

14.3.1 Importance of Phosphorous

Phosphorous (P) is the next main macronutrient required for plant growth after nitrogen. It accounts for about 0.2% of dry weight of a plant. It makes vital biomolecules like nucleic acids, ATP, and phospholipids, and ultimately plant growth is inhibited without the supply of this nutrient. It also has a role in the regulation of the metabolic pathway and enzyme-catalyzed reactions. Phosphate affects germination and seed maturity and eventually plant development. Plant development comprising of root, stem, and stalk is dependent on phosphate. Phosphate has a role in the formation of seed and flower, which ultimately has an effect on crop development and yield (Khan et al. 2009). It has a remarkable function in N fixation in legumes, energy metabolism, membrane synthesis, photosynthesis, respiration, enzyme regulation, crop value, and abiotic and biotic stress resistance. No atmospheric source of phosphate could be made available to plants (Ezawa et al. 2002), and soils normally contain trace quantities of available phosphate (predominantly as HPO_4^{2-} and $H_2PO_4^-$) that is readily available for plant uptake. Phosphate addition in the soil in the form of fertilizers fulfills the plant requirement (Richardson et al. 2009). The unavailability of phosphate in soluble form is a vital factor (Xiao et al. 2011) that restricts the agricultural production worldwide (Ramaekers et al. 2010). Both organic and inorganic phosphate accumulate in soil and consequently not available for plant consumption. Inorganic phosphate is fused through chemical adsorption and precipitation, while immobilization of organic phosphate occurs in soil organic matter (Sharma et al. 2012).

Even phosphatic fertilizers fail due to their conversion to an insoluble form like calcium phosphate and aluminum phosphate (>70%) (Mittal et al. 2008). Phosphate is available in low quantity in soil (1.0 mg kg^{-1} soil); additionally, it becomes unavailable by reacting with reactive metals like Al^{3+} in acidic, calcareous, or normal soils (Gyaneshwar et al. 2002; Hao et al. 2002). Crop plants can, therefore, make use of only a little bit of phosphorus, which eventually results in reduced crop performance (Reddy et al. 2002). The high percentage of an insoluble type of phosphate leads to eutrophication, while frequent use of phosphate causes soil infertility and rapid depletion of nonrenewable phosphate reserves. The outcome of this event would be the lake's biological death i.e. cyanobacterial blooms, hypoxia, and death of aquatic animals due to depleted bioavailable oxygen and buildup of nitrous oxide. (Vats et al. 2005). In the plant, a range of morphological and physiological changes was observed due to deficiency in phosphate, which consecutively affects plant growth, productivity, and survival (Tran et al. 2010), and hence are a significant pin down for the agriculture industry worldwide.

Hence, effective phosphorous utilization is crucial for the sustainable expansion and prevention of undesirable environmental effects (Scholz et al. 2015). The translation of a phytate–phosphate compound in the soil in crop accessible orthophosphate would mitigate phosphate-related obstacles.

14.3.2 What Is Phytate?

Phytate is a significant storage compound of phosphorus in seeds. Eighty percent of the total seed phosphorus is made by phytate, which accounts for 1.5% of seed dry weight (Raboy and Dickinson 1987). The myo-inositol hexakisphosphate is a phosphate salt of myo-inositol having all six hydroxyl groups substituted by phosphate residues (Fig. 14.5). The myo-inositol 1,2,3,4,5,6-hexakis (dihydrogen) phosphate is commonly called myo-inositol hexakisphosphate, or phytate, which is a collection of the organic form of phosphorus compounds found widely in nature. The prefix "hexakis" designates that the phosphates are not internally connected and the compound is formed by a polydentate ligand, which binds with more than one metal atom coordination site. Each phosphate group is in ester form within an inositol ring and binds entirely with 12 protons (Bohn et al. 2008; Cao et al. 2007).

Phytate usually presents as a salt of monovalent and divalent cations (Fe^{2+}, Mn^{2+}, K^{+}, Mg^{2+}, and Ca^{2+}) and formed in seeds at the stage of ripening. In phytic acid, the negatively charged phosphate sturdily binds with positively charged metallic cations resulting in an insoluble complex and restricting the accessibility of nutrients. Phytic acid and its derivatives are accountable for various cellular events such as signaling, RNA export, endocytosis, DNA repair, and vesicular cell trafficking (Bohn et al. 2008; Frias et al. 2003). In plants, phytate is the prime storage type of inositol phosphate. The plant root has 30% phosphorus fractions, while seeds and cereal grains have 80% phosphorus (Lott et al. 2000; Turner et al. 2002; Haefner et al. 2005). Two pathways are considered for the biosynthesis of phytate: lipid-dependent and lipid-independent. The synthesis of phytic acid starts from myo-inositol via a series of phosphorylation steps. In the former route, phytate is attained by the successive phosphorylation of Ins(1,4,5)P3 (inositol 1,4,5-triphosphate) and Ins(1,3,4)P3 (inositol 1,3,4-triphosphate). The subsequent compound is released from PtdIns(4,5)P2 (phosphatidylinositol 4,5-biphosphate) by the effect of a specific phospholipase C. The intracellular location of the intermediates of phytic acid biosynthesis is not fully explored.

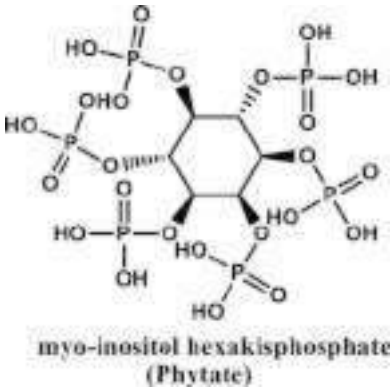

Fig. 14.5 Structure of phytate

Organic phosphate in rhizosphere has a high affinity to soil particles by precipitation and adsorption and hence it creates deprived accessibility to the plant as it cannot be desorbed (Menezes-Blackburn et al. 2013). Phytic acid is degraded in seed germination by a precise assembly of enzymes called phytases.

14.3.3 Phytase Enzyme

Phosphorus deficiency results from the phytase secretion of a variety of plant roots (Minggang et al. 1997). The distinct phosphatases phytases (myo-inositol hexakisphosphate phosphohydrolase) sequentially hydrolyze the phosphomonoester bonds from phytic acid, thereby liberating lower inositol phosphates and inorganic phosphate (Singh et al. 2011). These catalysts commence phytic dephosphorylation at various positions on the inositol ring, and it produces diverse isomers of lower inositol phosphates (Turk et al. 2000).

14.3.4 Structure and Mechanism of Action of Phytase

Phytase (myo-inositol hexakisphosphate phosphohydrolase) is a homodimaeric enzyme (EC 3.1.3.26 and EC 3.1.3.8) (Hegeman and Grabau 2001; Guimarães et al. 2004). Phytases carried out the subsequent release of inorganic phosphorus from phytic acid. Phytases act hydrolytically to break the phosphate ester bond of phytate and release inositol phosphates and phosphorus with other essential nutrients, which are required for plant absorption (Angel et al. 2002) (Fig. 14.6). Phytases are involved in the dephosphorylation of inositol-6-phosphate and high-order inositol hexakisphosphate hydrolyze sequentially to form lower-order esters like inositol monoesters (Hayes et al. 1999; Vats and Banerjee 2004). The inositol penta- and hexakisphosphate (phytate) hydrolyzing enzymes are of interest because they constitute a high percentage of the whole organic phosphate (Turner et al. 2002).

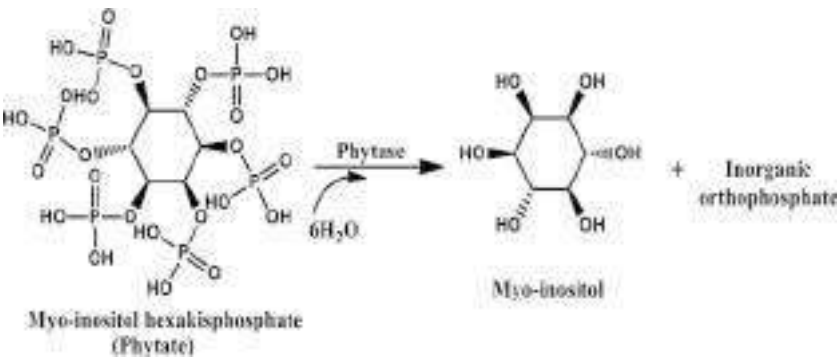

Fig. 14.6 Phytase action on phytate

The phytase protein has substrate binding and catalyzation conserved domains. The substrate binding domain is present at the N-terminal with RHGxRxP conserved sequence for substrate binding. The C-terminal catalyzation theme comprises of particular HD components. The "pocket" structure is framed by the connection of residues in the motif (Mullaney et al. 2000). The substrate restricting site with RHGxRxP arrangement responds with the substrate and frames the chemical substrate complex. The phosphate groups are then released from the substrate by the HD element (Li et al. 2010).

Phytate hydrolysis occurs in two stages: the nucleophilic attack and protonation. The histidine in the dynamic site of the catalyst caused a nucleophilic assault to the fragile phosphoester bond of phytate and caused the protonation by the aspartic acid of the leaving cluster (Li et al. 2010). The ß-propeller alkaline phytases lack the RHGXRXP sequence motif, and hence it needs calcium thermostability as well as enzyme activity to produce the IP3 (inositol triphosphate) (Kim et al. 1998a; Mullaney and Ullah 2003).

Phosphatases cause hydrolysis of 60% of the total organic phosphate. The highest quantity of phosphate was released by phytases from phytate (Bünemann 2008). The release of orthophosphate from soil natural phosphate is effective in microbes as well as in plants. Plant phytases have been distinguished in roots and root exudates during the early stage of seed germination; they frequently show a poor action, making them inefficient for hydrolyzing soil phytic acid as well as phosphorous usage (Hayes et al. 1999; Richardson et al. 2009) and thus suggest that the microbial catalyst demonstrates superior, effective liberation of phosphorous (Tarafdar et al. 2001).

14.3.5 Categorization of Phytases

Phytases are assembled by their enzyme action, pH action, and the initiation site of dephosphorylation of phytate. They are categorized into 3-phytases (EC 3.1.3.8), 5-phytases (EC 3.1.3.72), and 6-phytases (EC 3.1.3.26) on account of the initial hydrolysis position of phytate according to IUPAC-IUBMB (Bohn et al. 2008), which were subsequently alienated into alkaline and acid phytases (Jorquera et al. 2008). The three-dimensional structure and catalytic mechanism cause classification into four classes: histidine acid phytases (HAP) (EC 3.1.3.2), cysteine phytase or purple acid phosphatase (PAP) (EC 3.1.3.2), beta-propeller phytase (BPP) (EC 3.1.3.8), and protein tyrosine phosphatase (PTP)-like phytases (Li et al. 2010), which have recently been characterized (Lei et al. 2007). HAPs and BPPs are the most well-known and contemplated phytases. Various bacterial, fungal, and plant phytases have a place with the HAP family, while BPP has all the earmarks of being the prevalent phytase in *Bacillus* species (Greiner et al. 2007; Huang et al. 2009). These two most important categories have a different catalytic activity that results in distinct end products. While HAPs catalyze the hydrolysis of PA in myo-inositol and Pi, BPP activity results in the creation of the inositol-triphosphates – either Ins(1,3,5)P3 or Ins(2,4, 6)P3 (Greiner et al. 2007; Kerovuo et al. 2000).

As per the optimum pH, acid phytases, for the most part, incorporate HAP, PAP, and PTP-like phytases, though alkaline phytases include just BPPs from *Bacillus* species (Singh and Satyanarayana 2015; Tye et al. 2002). Alternatively, carbon position of dephosphorylation initiation resulted in phytases grouping into 3-phytase (myo-inositol hexakisphosphate 3-phosphohydrolase), 6-phytase (myo-inositol hexakisphosphate 6-phosphohydrolase), and 5-phytase (myo-inositol hexakisphosphate 5-phosphohydrolase).

The categorization of phytase into EC 3.1.3.8, EC 3.1.3.26, and EC 3.1.3.72 (myo-inositol-hexaphosphate phosphohydrolases) was organized on the background of protein sequencing, and successive dephosphorylation (George et al. 2007) of P occurs at three and six positions, correspondingly. The labeling basis is the three- and six-bond position of myo-inositol 6-phosphate. The 3-phytases (EC 3.1.3.8) are present in filamentous fungi like *Aspergillus* sp. and 6-phytases (EC 3.1.3.26) are found in plants, e.g., wheat.

14.3.6 Reserve of Phytase

Phytases can be formed by microorganisms, plants, and animals. Wheat, rice, soybeans, barley, peas, corn, and spinach are examples of plant sources. Microorganisms like bacteria, fungi, and yeast are the real source of phytase found in the blood of vertebrates such as fish and reptiles (Gupta et al. 2015; Bohn et al. 2008). Among the phytases from microorganisms, attention is focused on *Aspergillus* sp. because of its high production and extracellular activity (Gupta et al. 2015). To circumvent this obstacle the sole strategy is the application of phytases which hydrolyze the phytate and increase availability of P to plants. Commercially available phytase addition is costly and time-consuming, and hence the maintenance of rhizospheric phytase producer is important. Another engineering approach involves incorporation of genes behind phytase production from microbes into transgenic plants. However, there is a range of constraints for phytase engineered crop plants like loss of seed viability, yield, vulnerability for ecological pressure, and rejection of genetically modified organisms (GMOs) (Reddy et al. 2017).

14.3.7 Microorganisms Producing Phytase

Phytases of microbial origins are of rigorous significance among plants, animals, and microorganisms owing to the ease of genetic manipulation and large-scale production (Adhya et al. 2015). Microorganisms are the key drivers in the soil, which regulates phytate mineralization. The occurrence of microorganisms in soil rhizosphere may balance plants inability to procure P directly from phytate. In microorganisms, bacteria, yeast, and fungi have been effectively researched for extracellular phytase action (Pandey et al. 2001). A single phytase cannot address the issues of business and ecological applications (Bakthavatchalu et al. 2013). Microbial

phytases are investigated mainly from fungi of a filamentous type such as *Aspergillus ficuum* (Gibson 1987), *Mucor piriformis* (Howson and Davis 1983), *Aspergillus fumigatus* (Pasamontes et al. 1997), *Cladosporium* sp. (Quan et al. 2004), and *Rhizopus oligosporus* (Casey and Walsh 2004). Phytase production by different bacteria has been described, viz., *Bacillus* sp. (Kim et al. 1998b; Choi et al. 2001), *Citrobacter braakii* (Kim et al. 2003), *Pseudomonas* sp. (Richardson & Hadobas 1997), *Escherichia coli* (Greiner et al. 1993), *Raoultella* sp. (Sajidan et al. 2004), and *Enterobacter* (Yoon et al. 1996). The anaerobic rumen bacteria, mainly *Selenomonas ruminantium*, *Prevotella* sp., *Megasphaera elsdenii*, and *Mitsuokella multiacidus* (Richardson et al. 2001b) and *Mitsuokella jalaludinii* (Lan et al. 2002), have also been investigated for phytases. The γ-proteobacteria group possesses the phytase production potential among the majority of soil bacteria. Fungi have extracellular phytases, while bacteria produce cell-linked phytases. *Bacillus* (Choi et al. 2001; Kerovuo et al. 1998; Kim et al. 1998a; Powar and Jagannathan 1982; Shimizu 1992) and *Enterobacter* (Yoon et al. 1996) are the only bacterial genera having extracellular phytase activity. The phytase activity of *Selenomonas ruminantium* and *Mitsuokella multiacidus* (D'Silva et al. 2000) is outer membrane linked, while *Escherichia coli* produces the periplasmic phytase enzyme (Greiner et al. 1993).

B. subtilis is as a competent of phytase producer owing to its nonpathogenic and safe nature for industrial-level phytase production. This microorganism has numerous additional advantageous properties like organic acid production and antibiosis for phosphate solubilization in the soil. Currently, *Aspergillus* and *E. coli* are the commercial phytase producers. Among the various organisms reported, the inhabitant *E. coli* enzyme demonstrates the maximum phytase activity.

Phytases from bacterial sources are a genuine option in contrast to fungal enzymes because of their specificity to the substrate, protection from proteolysis, and effective catalytic action (Konietzny and Greiner 2004). *Bacillus* phytases are exceptionally effective due to its higher thermal stability and neutral pH. The *Bacillus* phytase has stringent specificity for a substrate for the calcium–phytate complex effective for application in the environment (Farhat et al. 2008; Fu et al. 2008). Nevertheless, owing to inefficient enzyme production methods for *Bacillus* sp., it could not be produced at commercial scale as only a few strains have been significantly commercialized for phytase production (Zamudio et al. 2001). *Lactobacillus sanfranciscensis* is the main sourdough lactic acid bacteria that demonstrated a significant level of phytate degrading action (De Angelis et al. 2003). The HAP are specifically produced from *Aspergillus* sp. like *A. terreus*, *A. ficuum*, and *A. niger* (Wyss et al. 1999), while the alkaline phytases are produced from *Bacillus amyloliquefaciens* (Idriss et al. 2002) and *Bacillus subtilis* (Kerovuo et al. 2000). Escobin-Mopera et al. (2012) had purified phytase from *Klebsiella pneumoniae* 9–3B. Rhizobacteria can mineralize phytate and may enhance P uptake of plants in soils (Patel et al. 2010). A better and substitute resource of phytase is continuously searched by screening new organisms that may produce novel and effective phytases. The ultimate aim is to produce phytase cost-effectively with optimized conditions for industrial application.

14.3.8 Why Do Bacteria Produce Phytase?

Bacterial phytase production is an inducible complex regulatory mechanism. Phytase synthesis control is different in various bacteria. Phytase production is not a condition for balanced bacterial growth, but it is the response to an energy or nutrient constraint. Phytase formation takes place when bacterial cells face environmental variations prior to the commencement of growth or when actively growing culture faces a stressful condition. The metabolic regulation by signal transduction is also a mechanistic role (Zamudio et al. 2002).

14.3.9 Parameters Affecting the Activity of Phytases

The soil environment presents extreme difficulties like denaturation, degradation, adsorption, and dilution to extracellular chemicals (Wallenstein and Burns 2011). The constancy of extracellular and intracellular enzymes is variable. Stability is portrayed more in extracellular than intracellular proteins and is credited by glycosylating disulfide bonds that alter thermal soundness, an expansive pH scope of action, and some protection from proteases. Some are stabilized by binding with humic substances and clay minerals (Quiquampoix and Burns 2007). Biological and physicochemical procedures influence phytase action. The former causes changes in enzyme creation rates leading to isoenzyme generation and changes in microbial network synthesis, while the latter causes changes in absorption desorption responses, substrate dissemination rates, and enzyme degradation rates (Wallenstein et al. 2009). Essential elements influence the action of enzyme include the amount and kind of substrate (Fitriatin et al. 2008), type of solvent, pH, temperature, the existence of an inhibitor and activator, the quantity of the enzyme, and the reaction product (Sarapatka 2002).

14.3.9.1 Effect of Substrate on Phytase Action

Phytase action shifts with various substrates. The different substrates include 1-naphthyl phosphate, 2-glycerolphosphate, glucose-6-phosphate (Escobin-Mopera et al. 2012), 2-glycerolphosphate, fructose-6-phosphate, calcium phytate, sodium phytate, p-nitrophenyl phosphate, ß-glycerol phosphate, adenosine-5′-monophosphate (AMP), guanosine-5′-triphosphate (GTP), adenosine-5′-diphosphate (ADP), adenosine-5′-triphosphate (ATP), and nicotinamide adenine dinucleotide phosphate (NADP) (Farouk et al. 2012; Bakthavatchalu et al. 2013). Phytases are categorized as substrate particular and nonparticular acid phosphatases (Rossolini et al. 1998; Rodríguez and Fraga 1999).

14.3.9.2 Effect of pH on Phytase Action

The activity of phytases relies on the pH and temperature. Plant phytases have less pH and thermal stability than microbial phytases. The optimum pH for phytase activity is 5.0–8.0, hence classified as acid or alkaline phytases, respectively (Konietzny and Greiner 2002). The optimum pH for fungal phytases is 4.5–6.5 with

80% activity; for example, *Rhizoctonia* sp. and *F. verticillioides* have an optimum pH of 4.0 and 5.0, respectively (Marlida et al. 2010). The optimum pH for bacterial phytases is 6.0–8.0 (Kerovuo et al. 1998; Kim et al. 1998a). Acidic phytases have an optimum pH range from 4.5 to 6.0 (Konietzny and Greiner 2002), and pH 8.0 is the optimum for alkaline phytases in legume seeds (Scott 1991), lily pollen (Baldi et al. 1988), and cattail (Kara et al. 1985; Scott 1991).

14.3.9.3 Effect of Temperature on Phytase Action

Temperature is the most indispensable factor of enzyme action, influencing both enzyme generation and degradation rates by microorganism. The ideal temperature of phytate-degrading enzyme fluctuates from 35 to 77 °C. Predominantly plant phytases have the greatest action at lower temperature compared to microbial phytases (Konietzny and Greiner 2002). The ideal temperature for plant phytases ranges from 45 to 60 °C (Johnson et al. 2010). In general, metabolic rate of enzyme producing life forms increases with temperature over the range 5–40 °C. In this way, temperature supposes a more vital job in the rate of extracellular enzyme activity when contrasted with enzyme kinetics itself.

14.3.9.4 Effect of Soil Type on Phytase Action

The action of phytase in soil is additionally influenced by physicochemical properties of the soil, which incorporates soil compose, organic matter content, nitrogen content, C/N proportion, and aggregate P content (Djordjevic et al. 2003). The soil performance of phytase fluctuates with soil compose, and the movement of phytase lost expeditiously is dependent on three differentiating soil nature. The initial fate of phytase is confined by adsorption in the soil. The degradation and magnitude of phytase adsorbed continue as before for a wide range of soil arrangements. The highest adsorption was recorded at low pH, and it becomes nearly equivalent to zero when pH is adjusted to 7.5. The adsorption bestows defense to phytase degradation in the soil, but also limits loss of enzyme activity in the adsorbed state.

14.3.10 Mechanism of Phytase Activity

Microorganisms can enhance the capacity of a plant to acquire P through various mechanisms, and the important one is phytase like enzyme production (Richardson and Simpson 2011). The purified crystalline form of phytase has different catalytic properties with specific diverse mechanisms. The principal action of all portrayed phytases depends on the enzymatic hydrolysis of the bonds among inositol and phosphoric acid deposits. Enzymatic hydrolysis of bonds happens among inositol and phosphoric acid deposits whereupon the component of activity of all phytases is based. The results of this arrangement of responses are six-fold alcohol and phosphates (Mukhametzyanova et al. 2012). Microbial phytases decay fresh plant build-ups in the soil prompting the release of phosphorus from organic compounds. There are various arrangements alongside differing rates of responses by which the phosphoric acid deposits are discharged through microbial hydrolysis of phytate

(Mukhametzyanova et al. 2012). The histidine acidic phytases catalyze the release of phosphates in neighboring free hydroxyl group, after the dephosphorylation of a first phosphate group. For the most part, plant phytases display a difference in transitional myo-inositol pentaphosphate development among the first phase of the response. In the course of the first venture of hydrolysis, microbial 6-phytases frame a different set of intermediates. The acid phosphatases with phytate hydrolyzing properties hydrolyze glucose-1-phosphate in *Enterobacteriaceae* (Greiner and Sajidan 2008). Alkaline phosphatases in lily pollen, *B. subtilis*, and reed mace formed myo-inositol triphosphates as end products (Greiner et al. 2007; Greiner and Sajidan 2008; Mukhametzyanova et al. 2012).

14.3.11 Importance of Microbes for Phosphorous Mobility with Phytase

Soil microorganisms, particularly the higher plant rhizosphere, are exceptionally powerful in discharging P from natural pools of aggregate soil P by mineralization and inorganic complexes through solubilization (Hayat et al. 2010).

Mineralization results from the transformation of organic P, for example, phytate to plant-accessible inorganic P, by microorganisms through their expressed enzyme phytase (Ariza et al. 2013). Phytases have been recognized in roots and root exudates in plants (Li et al. 1997; Hayes et al. 2000; Richardson et al. 2000). Despite the fact that it is accounted for the enzymatic action in root exudates, it is not sufficient for efficient use of natural phosphorous (Brinch-Pedersen et al. 2002; Richardson et al. 2000). The addition of exogenous phytase into the media resulted in phytate availability for plant growth (Hayes et al. 2000; Idriss et al. 2002; Unno et al. 2005). The addition of exogenous phytase (Idriss et al. 2002; Richardson et al. 2001b; Singh and Satyanarayana 2010; Hayes et al.2000) or expression of phytase gene of microbial origin in plant (Richardson et al. 2001a; Li et al. 2007a, b, 2009) resulted in growth of plant with phytate as solitary source of phosphate. The current research is targeted on the genetic expression of phytase genes in the plant for organic P utilization from the soil. The graphic demonstration of the function of microorganisms in phosphate solubilization is described in Fig. 14.7.

The action of plant phytases comprises just a little extent of the aggregate phosphatase reaction and is viewed as insufficient for guaranteeing adequate phosphate securing (Richardson et al. 2000; Findenegg and Nelemans 1993; Hayes et al. 2000). Bacterial phytases are effective for growth and yield of the plant. The limitation of plants to extort P from soil phytate could be overcome by treatment with phytate-degrading bacteria, like biofertilizer. Microbial phytase plays a very important role for the availability and mobility of phosphorous in soil because of its agronomic and ecological value for the growth of the plant as suggested by the recent scientific research. The long-term phosphorous deprivation in plants could be met by phytase from microorganisms; hence, the use of microbial phytase on an industrial scale is very appealing nowadays (Jorquera et al. 2008). The fungal extracellular phytase-treated seeds support the plant phosphorus nutrition in high phytate

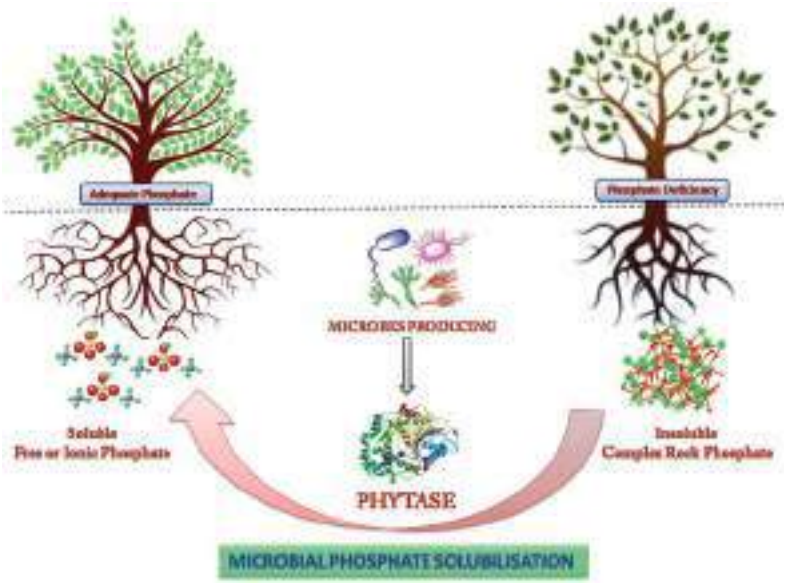

Fig. 14.7 Role of phytase from microorganisms in phosphate solubilization

content soil (Tarafdar 1995). The enrichment of soil with phytase from bacteria like *B. amyloliquefaciens* and *Bacillus mucilaginosus* advances the development of corn and tobacco, respectively (Li et al. 2007a, b; Idriss et al. 2002). Phytases from bacteria also release the vital soil micronutrients by phytate chelation and make it available to the plant. The purified microbial phytase or phytase-producing microbial strains could be functional as an effective and eco-friendly way to increase bioavailable soil phosphorus and limit the wide utilization of inorganic phosphate fertilizers.

14.3.11.1 Transgenic Plants for Phytase

Gene for phytase from a microorganism is integrated into plants like tobacco with a phyA gene from *A. niger* constituting phytase as soluble proteins in tobacco seeds. Genetically modified plants produce extracellular phytase from roots, which showed significant improvement in P nutrition in the soil, with higher phytate content or artificially modified for phytate (George et al. 2004, 2005). Thus the phytase from a microorganism is the critical element, and their existence in the rhizosphere helps the plant to recover from its inability to use the unavailable phytate.

Phytases have developed to be a valuable key to supportable agribusiness. It gives an approach to stop the revenue costs that turn out to be superfluously high because of the expansion of phosphorus manures. Broad research on phytase utilizing biotechnological applications will unquestionably give efficient arrangements towards practical agribusiness and ecological insurance in the coming years.

14.4 Use of Bacterivorous Microbes from Soil

14.4.1 Bacterivorous Protozoan

It was an accepted truth that soil microbes provide essential functions supporting soil fruitfulness and plant well-being. Recent evolution in molecular techniques like molecular sequencing resulted in a boom in studies of various microflora like an insect, animal gut, lakes, ponds, and terrestrial flora. However, all these studies cover bacteria and fungi only and neglect other trophic levels. But most attempts to use these bacteria and fungi as bioinputs in natural soil have been reported unsuccessful.

For the past 50 years the terms "biofertilizer" and "PGPR bacteria" only represent nitrogen fixer and phosphate and growth hormone producer. However, the truth is there is still no confirmation that these added bioinputs sustain soil fertility. The accepted truth is that these fungal and bacterial bioinputs have significant selective pressures of predation and not resource availability. These predators are bacterivorous and fungivorous protist. Protists massively consume bacteria as well as other soil microbes like fungi and yeast, and unicellular algae and release various micronutrients, growth-promoting substances, and different assimilable nitrogenous compounds and mineral (Ekelund and Rønn 1994).

Although various soil protozoans and nematodes are reported for their bacterivorous role, very few reports exist discussing the function of protozoans in the development of crop plant or soil richness (Bonkowski and Brandt 2002; Bonkowski 2004). The size of most soil protozoan ranges from 10 to 100 μm in diameter, but their weight is negligible. It was assumed that the biomass of total protozoan in soil is equal to the biomass of all other clusters of soil animals together except earthworm (Schaefer and Schauermann 1990; Schröteret al. 2003). In the biological energy coordination, the soil organic cycle plays an important role, which involves anabolic and catabolic steps of energy investment and energy escape or lost. Protozoans are major engineers which motion this organic energy cycle in the soil. Protozoa drive this cycle continuously where there is sufficient water available like moisture-containing intersoil capillaries, pore spaces, and fissures. Besides these, protozoans account for significant respiration of soil. It was noted that they contribute to 15–70% of the entire soil respiration. These indicate that protozoans are a vital component of the soil. The soil protozoans majorly include ciliates, flagellates, and naked and testate amoebae (Fig. 14.3). Although these protozoans have an extensive array of food assimilation and enzyme syntheses like a higher animal, they are not capable of synthesizing some vitamins and cofactors, and hence they depend on some microbial population for it.

Ciliates are one of the group including protozoan, which are identified for its extraordinary bacterivorous capacity (Sherr et al. 1987); owing to their large size. Algae, fungi, and small animals are foods for these ciliates (Bernard and Rassoulzadegan 1990; First et al. 2012). They have various habitats like freely swimming in the water, crawling on surfaces, and physically attached to surfaces by very flexible spring-like stalk, e.g., *Paramecium*, *Euplotes*, and *Vorticella* (James

and Hall 1995). There are some ciliates, which have special cilia for swimming and hairs for predation known as membranelle, which help for catching massive bacteria or prey in food vacuoles. Ciliate feeding rates are very high; it was recorded that single ciliates can digest 1254 bacteria h^{-1} (Iriberri et al. 1995).

Flagellates are another member of protozoans bearing one or more flagella having a different size from 2 to 20 μm. They are versatile in nature like swimming freely or attaching to solid surfaces by trailing flagellum or stalks. Flagellates using these flagella either create feeding current or exploit it to put the water and prey in the oral furrow and at the base of the flagellum where the pseudopodia ingest the prey. Flagellates show selective grazing as per their size. They prefer smaller-size organisms as significant prey. It was reported that bacteria are more susceptible to flagellate grazing than other microbes having size >2.4 μm. Chrzanowski and Šimek (1990) reported that flagellate bacterial grazing rate varies from 2 to 300 bacteria h^{-1} (Davis and Sieburth 1984; Eccleston-Parry and Leadbeater 1994a).

Amoebas are widely occurring protozoans and are very normal in water, soil, and other habitats. They are abundant in the soil, i.e., 103–107 g^{-1} of dry soil, with varying size <10 μm. Amoebas play a very important function in the cycling of various minerals and minute supplements such as nitrogen and phosphorus, particularly in shallow levels of nutrient environments (Goldman et al. 1985; Eccleston-Parry and Leadbeater 1994b). Amoebae, ciliates, and flagellates together selectively nurture on bacteria and control bacterial soil population (Table 14.1). They act as an essential constituent of the "microbial loop" (Azam et al. 1983). They are well recognized as Rhizopoda amoebae because they use their cytoplasmic protrusions, i.e., pseudopodia, for locomotion and nourishment. Amoebae are of two types, naked amoebae and shelled amoebae (testate amoebae).

Naked amoebae have no perfect shape but show three major morphological forms, i.e., floating, active form with extended lobose; fan-shaped, slug-like pseudopodial form trophozoites; and smaller and dormant form called cyst, an unusual rounded form (Page 1988; Griffiths 1970). Typical examples of naked amoeba are *Amoeba*, *Acanthamoeba*, *Vannella*, and *Vampyrella.*

Testate amoebae secrete the siliceous shell around the body. These testate are species-specific architectures. The testate shell amoebae designate the nutritional category of the living environment. The aperture is at one side of a shell, which is used for feeding or catching of different preys (Jassey et al. 2012). The dominant victims of amoebae are bacteria; the intake rate of the amoebic cell was reported to be 0.2–1465 bacteria h^{-1} (Heaton et al. 2001; Huws et al. 2005).

14.4.2 Role of Protozoans as New Bioinputs

Various studies indicated that protozoans majorly preyed upon bacteria. Bacteria, unicellular fungi, yeast, algae, and cyanobacteria were assumed as a nutritional capsule. In addition to nitrogen and carbon sources, these nutritional capsules are enriched with micro- and macronutrients in addition to various growth factors (Table 14.2). It was formerly confirmed that the nitrogen and carbon content of a

Table 14.1 Bacterivorous capacity of various protozoans

Types	Example	Bacterivorous capacity (bacterial cell h^{-1})	References
Amoeba			
Naked	*Saccamoeba*	0.2–1465	Heaton et al. (2001) and Huws et al. (2005)
	Acanthamoeba		
	Euglypha cristata		
	Hartmannella		
	Cf. *Mayorella*		
	Cf. *Polychaos*		
	Vannella		
	Vampyrella		
Shelled	*Arcellinid testate*		
	Euglypha cristata		
	Arcella gibbosa		
	Difflugia		
	Foraminifera		
	Nebela		
Flagellates	*Giardia intestinalis*	2–300	Davis and Sieburth (1984) and Eccleston-Parry and Leadbeater (1994a)
	Peltomonas hanelisp. nov.		
	Apusomonas australiensis sp.		
	Cetcomonar crassicauda		
Ciliates	*Paramecium*	20–1254	Iriberri et al. (1995)
	Vorticella		
	Balantidium coli		
	Oxytricha trifallax		
	Stentor roeselii		

Table 14.2 Elemental composition of bacteria and fungi

Element	Bacteria (% dry weight)	Fungi (% dry weight)
Carbon	50–53	40–63
Hydrogen	7	–
Nitrogen	12–15	7–10
Phosphorus	2.0–3.0	0.4–4.5
Sulphur	0.2–1.0	0.1–0.5
Potassium	1.0–4.5	0.2–2.5
Sodium	0.5–1.0	0.02–0.5
Calcium	0.01–1.1	0.1–1.4
Magnesium	0.1–0.5	0.1–0.5
Chloride	0.5	–
Iron	0.02–0.2	0.1–0.2
References	Luria (1960)	Lilly (1965)
	Aiba et al. (1973)	Aiba et al. (1973)
	Herbert (1956)	

fungal and bacterial cell are 10–15% and 50–63% by dry weight of fungi and bacteria, respectively. Similarly, bacterial and fungal mass sufficiently contain valuable micronutrients such as phosphate, potassium, sulphur, calcium, and iron (Luria 1960; Herbert 1956; Aiba et al. 1973). All protozoans are well characterized for their enormous feeding habits on other microbes such as bacteria and other microbes. Different soil bacterial flora assimilated the atmospheric nitrogen with organic and inorganic matters from the soil and locked in their cells, which are not freely accessible for the plants. The enormous grazing activity remobilized this immobilized nitrogen and released ammonia, which is ultimately utilized by the plant (Goldman and Caron 1985). Griffith and Bardget (1997) proved that the nitrogen requirement of protozoans is comparatively less, and they make about 60% of ingested nitrogen available to plants in the form of ammonia. Hence after the ingestion of bacteria by a protozoan, nitrogen is not only released but also various nutrients like 50–63% carbon, 2.0–4.5% phosphorus, and 0.02–0.5% iron (Table 14.3). Bonkowski (2004) reported the essential function of protozoa in sustaining soil productiveness and plant health.

Protozoa provide all essential nutrients by mineralizing complex material in bacteria during feeding. They also control the structure and activity of bacterial loops of soil and root-associated communities (Sieburth and Davis 1982; Bonkowski and Brandt 2002). Krome et al. (2010) reported that selective predation of bacteria promotes the production of various plant growth hormones. Besides offering different mineralized nutrients, it was proved that protozoans also increased the nutrient assimilation rate by altering the root morphology. Bonkowski and Brandt (2002) reported that when the *Acanthamoeba castellanii* was inoculated in the rhizosphere, it induces the extensive fibrous and fine root, suggesting that protozoans play an important role like plant growth hormones (Krome et al. 2010). Jousset et al. (2010) also proved that protozoans not only stimulate growth but also play a noteworthy function in pathogen suppressions by encouraging other bacterial soil flora for antibiotics like chemicals. Similarly, it induces iron chelating organic molecule production, which makes iron unavailable for plant pathogen growth and multiplication (Levrat 1989; Mazzola et al. 2009; Müller et al. 2013; Mellano et al. 1970).

Nielsen et al. (2002) proved that bacteria such as *Pseudomonas* and *Bacillus* produce various antipathogenic compounds such as phenazines, DAPG (diacetyl phloroglucinol), and cyclic lipopeptides like tensin, amphisin, and viscosinamide, but Mazzola et al. (2009), Jousset and Bonkowski (2010), and Weidner et al. (2017) revealed that protozoan grazing pressure induced the making of such antipathogenic

Table 14.3 Performance of protozoans for phosphatases, ACCD, and tryptophan

Sr. no.	Bacterivorous organism	Phosphatase (IU/h)	ACC deaminase activity (μM of α-ketoglutarate/mg/h)	Tryptophan (μg/h)
1	*Acanthamoeba* sp.	16.20	0.161	15
2	*Paramecium* sp.	18.40	0.093	17
3	*Amoeba* sp.	11.20	0.218	11
4	*Tetrahymena* sp.	14.00	0.187	07

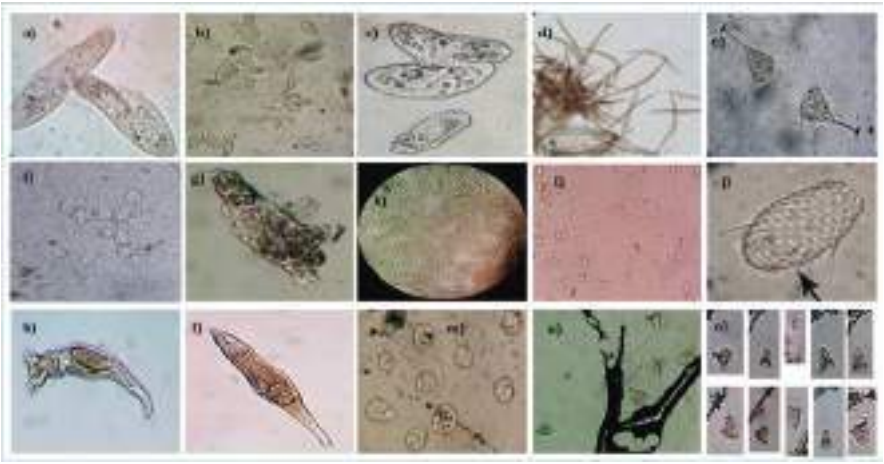

Fig. 14.8 Bacterivorous animals of soil cultured at School of Life Sciences, KBC NMU laboratory (**a**–**c**) *Paramecium* sp., (**d**) *Spirostomum* sp., (**e**) *Suctoria* sp., (**f**, **g**) *Acanthamoeba* sp., (**h**, **i**) cyst of amoebae, (**j**) testate amoebae, (**k**, **l**) Rotifer, (**m**) *Actinosphaerium* sp., (**n**, **o**) *Vorticella* sp.

fungal and bacterial compound. Recently in our laboratory studies at KBC North Maharashtra University (KBC NMU), Jalgaon, we have isolated and cultured various important agricultural bacterivorous animals, viz., *Paramecium*, *Amoeba*, Rotifer, and Vorticella (Fig. 14.8). It was revealed that *Acanthamoeba castellanii*, *Paramecium caudatum*, *Spirostomum*, and *Amoeba* spp. have the potential to produce various enzymes like phytase, phosphatase, and ACC deaminase. All these enzymes previously assumed the essential character of plant growth–promoting bacteria (Zahir et al. 2004). In laboratory-grown culture studies, it was discovered that *Paramecium* and *Acanthamoeba* efficiently utilized ACC and phytate and phosphate. Similarly, *Suctoria* sp. and *Spirostomum* were also investigated to use phosphate, phytic acid, and ACC like substrate at low concentrations (Table 14.3). *Amoeba* sp., *Acanthamoeba,* and *Paramecium* sp. were also found to be the producer of metabolic products such as amino acids like tryptophan, which was previously reported for a vital role in the stimulation of auxin production (Krome et al. 2010).

Sayre (1973) reported the potential of *Amoebae* as a future potent nematicidal agent. At KBC NMU laboratory, the cultured *Amoebae* sp. was also established to have an extraordinary potential of controlling invasive plant nematodes. Nematodes are the root-knot disease-causing agents of tomato and brinjal, i.e., *Meloidogyne incognita* and *Meloidogyne javanica.* It was observed that amoeba had 50–65 egg ingestion rate per amoeba per 24 h of both *Meloidogyne incognita* and *Meloidogyne javanica* and the 10–20 juvenile and 6–7 adult nematode ingestion per amoeba in 24 h.

14.5 Conclusion

Currently, nitrogen fixers, phosphate solubilizers, mycorrhiza, and biocontrolling agents like *Trichoderma* sp. are the most popular bioinputs throughout the world, even though it is necessary to recommend the utilization of other microbial bioinputs like ACCD, phytase producing microorganisms, Zn, K, S mobilizers. Besides that, latest studies proved the extraordinary potential of protozoa as the real new age bioinput, which proved their beneficial power for plant growth development, soil fertility augmentation, and biocontrol of soilborne pathogen. Recent advances in protozoans as bioinput will open a new avenue for plant–microorganism interaction research to solve current agricultural problems. The microbes present in the soil employ different strategies, and these beneficial belowground microbial interventions influence the plant beneficially. The character of these new age agricultural bioinputs is noteworthy for soil and plant well-being through nutrient fixation, solubilization, mineralization, and mobilization that are eventually accountable in the agroecological perspective. Such modern biological inputs in agriculture will help to achieve the future food demand of a growing world population and address the global problem of food security and malnutrition. So there is much more to do with nature's gift microorganisms which have tremendous metabolic flexibility and potential functionality.

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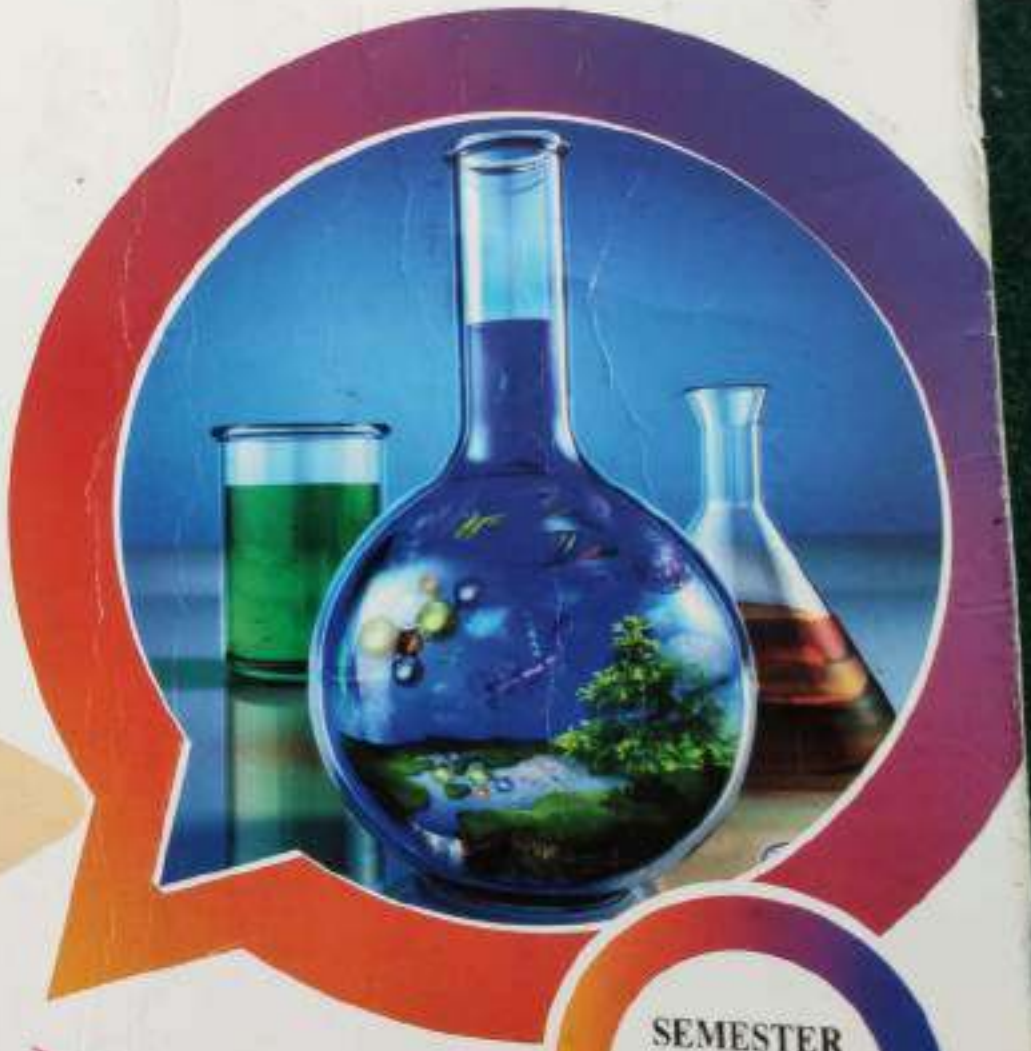

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Thermoluminescence glow curve analysis of RE doped $LiMgBO_3$ phosphor using GCCD function

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Thermoluminescence Glow Curve Analysis of RE Doped $LiMgBO_3$ Phosphor Using GCCD Function

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Abstract. The sol-gel synthesized Eu^{3+}, Dy^{3+} and Tb^{3+} doped $LiMgBO_3$ was studied for its TL response. The phase purity of the sample is checked using powder XRD pattern. The TL studies were performed by irradiating samples with γ-ray from ^{60}Co irradiation source in the dose range 10Gy to 1kGy. The samples have shown a linear dose response over the said range. The complex glow curves were analyzed using GCCD function given by Kitis taking general order kinetic into consideration. The activation energy and order of kinetics were calculated using the curve fitting method and frequency factors using Chen's formula.

INTRODUCTION

Boron-based materials are very important for radiation dosimetric applications due to their tissue equivalent absorption coefficient, low cost, thermal stability and neutron sensitivity[1–4]. Boron-based materials show interesting results of thermoluminescence when exposed to ionizing radiations. Improvement in the TL characteristics of borates was reported for recently developed rare earth doped mixed lithium calcium borates (LCBs)[5,6]. Previously luminescence properties of Lithium borate and Magnesium borate in both microcrystalline and nanocrystalline form has been studied. Recently researchers studied lithium magnesium borate phosphor and was found to have the lowest Z_{eff} value and suitable for a dosimetric application. Earlier very few reports were found for LiMgBO3 [7]. Recently the thermoluminescence properties of rare earth (RE= Tb, Gd, Dy, Pr, Mn, Ce, Eu) doped Lithium magnesium borate, synthesized by simple solid state diffusion technique, were studied [1]. The optical properties of Lithium magnesium borate glasses doped with Dy^{3+} and Sm^{3+}were reported by Alajerami for their potential application as laser material [8]. The intense red emitting Eu^{3+} doped $LiMgBO_3$ and the effect of co-doping by Bi^{3+} on photoluminescence properties were reported by Liang *et. al.*[9]. The present study reports the TL response and glow curve analysis of sol-gel synthesized rare earth (RE) doped $LiMgBO_3$ phosphor.

EXPERIMENTAL

The starting materials were $LiNO_3$, $Mg(NO_3)_2$, H_3BO_3, citric acid, ethylene glycol and RE_2O_3 (RE=Eu, Dy, Tb) all of AR grade procured from Loba Chemie. $RE(NO_3)_3$ salts were freshly prepared by a reaction of RE_2O_3 with dilute nitric acid. Lithium, magnesium, europium, dysprosium, and terbium citrates were prepared from appropriate mixtures of nitrates with citric acid and ethylene glycol in aqueous media. After being evaporated for several hours in an 80 °C water-bath, the solution becomes a pale yellowish polymeric gel. The gel was dried at 150 °C for 12h, and then the precursor was calcined on a high-temperature muffle furnace at 600 °C temperatures for 2 h.TL glow curves were recorded using a Harshaw TLD reader (Model 3500) fitted with a 931B photomultiplier tube (PMT). The heating rate for TL measurement is kept at 5 °C/s.

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RESULTS & DISCUSSION

XRD

To check the phase purity of the doped and undoped materials, XRD pattern was recorded on Rigaku diffractometer with Cu-Kα radiation (λ=1.5406 Å) at 40 kV tube voltage and 75 mA tube current with the step size 0.02° of 2θ.The XRD patterns of as-prepared $LiMgBO_3$, $LiMgBO_3:Eu^{3+}$, $LiMgBO_3:Dy^{3+}$, $LiMgBO_3:Tb^{3+}$ phosphorswere matched well with the standard JCPDS card no. 79–1996 ($LiMgBO_3$). $LiMgBO_3$ crystallized in the hexagonal crystal structure with space group C2/c (15). Also, it has been observed that the addition of small amounts of RE^{3+} (activator) did not alter the crystal structure of the host lattice, which suggests that the activator ion was fully incorporated in the host lattice. The calculated lattice parameters for pure $LiMgBO_3$ were approximated to be a = 5.163 Å, b = 8.885 Å, c = 9.914 Å, β = 91.22°and V = 454.787 $Å^3$.

Thermoluminescence Studies in Eu, Dy, and Tb Doped $LiMgBO_3$

For taking thermoluminescence (TL) measurements 5 mg of each sample is exposed to 10 Gy of γ-ray dose from ^{60}Co irradiation source at room temperature. The heating rate of 5 °C/s is chosen for TL glow curve measurements. The TL glow curves for $LiMgBO_3:Eu^{3+}$ for various concentrations of Eu^{3+} ions is shown in Fig. 1(a). The glow curve shows two well-separated peaks at 146 and 381 °C. With the increasing concentration of Eu^{3+} ions the intensity of both the peaks increases, the peak at 381 °C saturates early and shows decrease in intensity above 0.2 mol%, whereas the intensity of glow peak at 146 °C continues to increase till 0.5 mole% and further increase in concentration it saturates (Inset of Fig. 1(a)). The TL glow curves for Dy^{3+} doped samples (Fig. 1(b)) shows the main glow peak at 140 °C and a shoulder near 112 °C. The intensity of TL signal increases up to 0.5 mol% and a further increase of Dy^{3+} ions decreases the TL intensity (inset of Fig. 1(b)). The TL glow curves for Tb^{3+} doped $LiMgBO_3$ (Fig. 1(c)) shows a clear complex nature. For the lower concentration of Tb^{3+}ions, the glow curve shows approximately four peaks which on increasing concentration decreases and mainly two overlapped glow peaks are clearly seen. The TL intensity increases with increasing concentration of Tb^{3+} ions and reaches a maximum at 0.5 mol% (inset Fig. 1(c)). Further addition of more Tb^{3+} ions decreases the TL intensity and two well-resolved peaks can be observed. The glow peak with maximum intensity (0.5 mol%) has the main peak of 191 °C and a shoulder near 161 °C. From the analysis of the glowcurve, it can be seen that the Dy^{3+} doped samples are having the highest intensity and Eu^{3+} doped samples showthe least intensity. Thus $LiMgBO_3:Dy^{3+}$is proved to be more sensitive than the other two. For further investigation of signal fading, dose-response and kinetic parameters, samples with the highest TL intensity are chosen.

The stability of traps can be examined by keeping irradiated samples for longer duration and taking TL measurements over certain intervals also known as TL fading. TL fading is taken over a period of 15 days. The TL signal of Dy^{3+} doped material fades about 40%, whereas the Eu^{3+} doped material fades about 35%. The Tb^{3+} doped material shows least fading with 5%. Thus it can be concluded that the traps in Dy^{3+} and Eu^{3+} doped materials are quite unstable and traps in Tb^{3+} doped materials are stable. Thus irradiation memory of Tb^{3+} doped materials can be sustained for a longer time than the other two. For dose-responsestudies, the phosphor materials are exposed to γ-ray dose of 10 Gy to 1 kGy. All the materials show linear TL response over the said range and the glow curve structure remains invariant under changing the dose. This is the key feature for any tissue equivalent low Z dosimetric material.

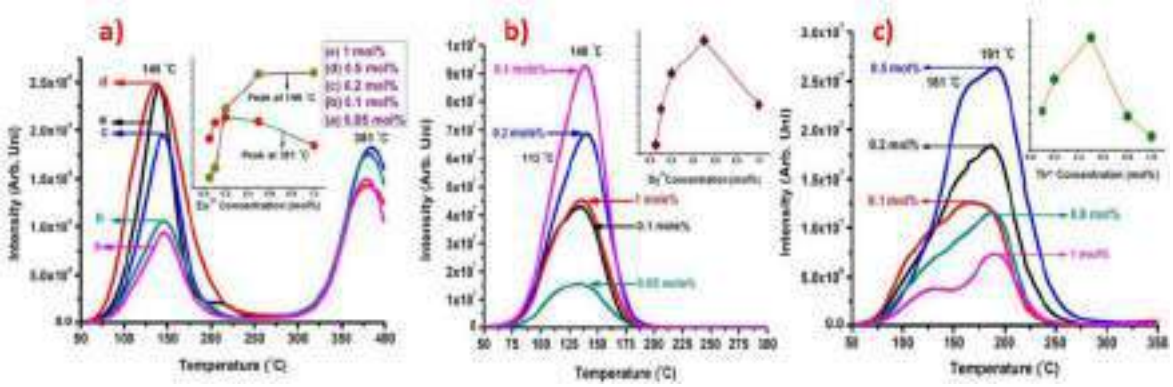

FIGURE 1.TL Glow curve for a) $LiMgBO_3$:xEu^{3+}, b) $LiMgBO_3$:xDy^{3+} and c) $LiMgBO_3$:xTb^{3+} (x= 0.05, 0.1, 0.2, 0.5, 1 mol%) exposed to 10 Gy γ-ray from ^{60}Co.

TL Glow Curves Analysis and Determination of Kinetic parameters

For the analysis and determination of kinetic parameters of TL glow curves, many authors have suggested several methods[10–14]. Most of the methods requirea well resolved single glow curve. But for complex glow curve analysis, these methods can't be directly applied. The first step is to separate these individual glow peaks from the complex glow curve. Currently, no experimental methods are available to completely separate out individual glow curve. But combining some experimental and some theoretical processes could be useful also known as glow curve convolution de-convolution (GCCD)[13,15]. The process of GCCD requires first the approximation of maximum peak temperature (T_m) and maximum intensity (I_m) of a glow curve that has to be separated from the complex glow curve. Both can be obtained experimentally by using the process of thermal cleaning. The explanation of the thermal cleaning process can be found elsewhere [16]. We have used modified efforts for the thermal cleaning process. For this process, we have irradiated about 100 mg of all three samples with 50 Gy gamma-ray dose and taking 5 mg of each sample 20 batches are made each batch having a set of three irradiated materials. Each batch is kept for 10 s at a different temperature from 60 to 250 °C with a difference of 10 °C. We have stopped to only 250 °C as 2 out of three samples show the absence of any possible glow peak above 250 °C, only in the Eu^{3+} doped sample a glow peak at 381 °C is seen which is taken as an individual glow peak. The samples annealed at different temperature are taken for TL measurements immediately. All the glow curves for each sample are collected and carefully examined. From the analysis of the data, some rough estimation of T_m and I_m is made. This data is further used to generate a theoretical glow curve by using the GCCD function for general order kinetic (GOK) given by Kitis [13]. The GCCD function for GOK is fitted into an excel spreadsheet keeping activation energy (E) and order of kinetic (b) as variable parameters. The solver program in excel is used to minimize the figure of merit (FOM). The fitting method by GCCD function in excel spreadsheet with solver program is explained in detail by Afouxenidis *et. al.*[17]. The GCCD equation for GOK to fit the data is given in Eq. (1) and FOM can be calculated using Eq. (2). The frequency factor (s) is calculated using Eq. (3) given by Chen[14].

$$I(T) = I_m \times b^{b/b-1} \times \exp\left(\frac{E}{kT} \times \frac{T-T_m}{T_m}\right) \times \left[(b-1) \times \left(1 - \frac{2kT}{E}\right) \times \frac{T^2}{T_m} \times \exp\left(\frac{E}{kT} \times \frac{T-T_m}{T_m}\right) + Z_m\right]^{-b/b-1} \quad (1)$$

Where, $Z_m = 1 + (b-1)\frac{2kT_m}{E}$ and $k = 8.617 \times 10^{-5} eV/K$ =Boltzman's constant

$$\text{FOM}(\%) = 100 \times \frac{\Sigma|I_{exp} - I_{fit}|}{\Sigma I_{fit}} \quad (2)$$

$I_{exp} and\ I_{fit}$ experimental and fitted TL intensity.

$$s = \frac{\beta E}{kT_m^2} \exp\left(\frac{E}{kT_m}\right)[1 + (b-1)\Delta_m] \quad (3)$$

TABLE 1. Kinetic parameters for various deconvoluted glow peaks of a complex glow curve.

Phosphor	Glow Peak Temperature	Activation Energy	Order of Kinetics	Frequency Factor
$LiMgBO_3:Eu^{3+}$	111 ^{0}C	0.87 eV	1.2	8.83×10^{10} s^{-1}
	140 ^{0}C	0.92 eV	2	4.90×10^{10} s^{-1}
	181 ^{0}C	1.02 eV	2	5.61×10^{10} s^{-1}
	381 ^{0C}	1.6 eV	2	4.33×10^{11} s^{-1}
$LiMgBO_3:Dy^{3+}$	116 ^{0}C	0.92 eV	1.2	2.89×10^{11} s^{-1}
	141 ^{0}C	1.04 eV	1.4	1.57×10^{12} s^{-1}
$LiMgBO_3:Tb^{3+}$	111 ^{0}C	0.81 eV	2	1.26×10^{10} s^{-1}
	154 ^{0}C	0.87 eV	1.2	5.05×10^{9} s^{-1}
	194 ^{0}C	1.11 eV	2	2.62×10^{11} s^{-1}

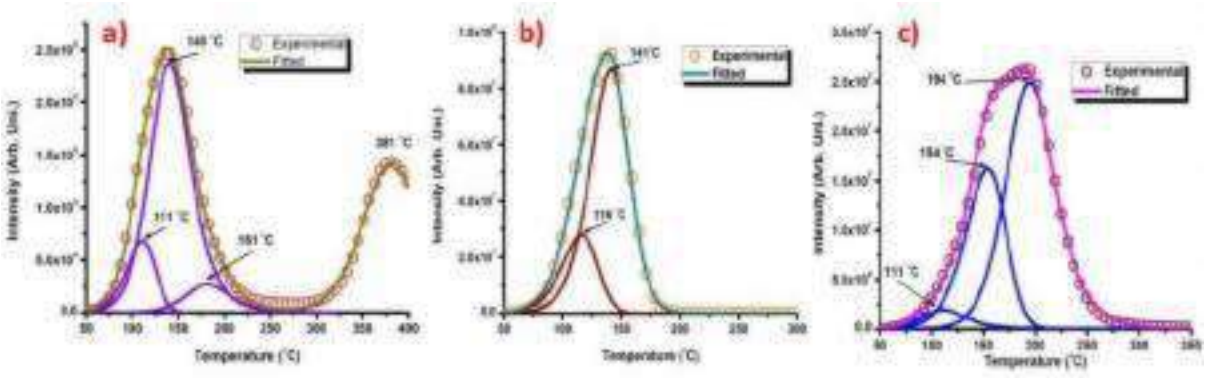

FIGURE 2. Experimental and theoretically fitted TL glow curves a) $LiMgBO_3:0.5Eu^{3+}$, b) $LiMgBO_3:0.5Dy^{3+}$ and c) $LiMgBO_3:0.2Tb^{3+}$ exposed to 10 Gy γ-ray from ^{60}Co using GCCD function.

The obtained results are shown in Fig. 2 (a), (b) and (c) and the values of kinetic parameters are tabulated in Table.1. Form the fitting data it can be seen that the Eu^{3+} doped sample has four glow peaks at 111, 140, 181 and 381 °C, Dy^{3+} sample have two glow peaks at 116 and 141°C and Tb^{3+} samples have three glow peaks at 111, 154 and 194 °C. The FOM for Eu^{3+}, Dy^{3+},and Tb^{3+} doped samples are 3.81%, 3.25%,and 3.05% respectively, which are well below 5% and thus can be considered as the good fit.

CONCLUSIONS

Eu^{3+}, Dy^{3+},and Tb^{3+} doped $LiMgBO_3$wassuccessfully prepared using sol-gel synthesis method. The XRD studies show the pure phase of $LiMgBO_3$ with a small amount of MgO phase. The addition of RE impurity in the host lattice didn't affect the actual crystal structure apart from alteration in lattice parameters. TL studies show that the TL glow curve of Eu^{3+} doped $LiMgBO_3$ has total four glow peaks, three of them are in shallow trap region and one in the deep trap region. The Dy^{3+} doped $LiMgBO_3$ has two glow peaks, both in shallow trap region. The Tb^{3+} doped samples show three glow with two in shallow trap region and one in deep trap region.

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An Intelligent Controller for Greenhouse Temperature Control Using Fuzzy Logic

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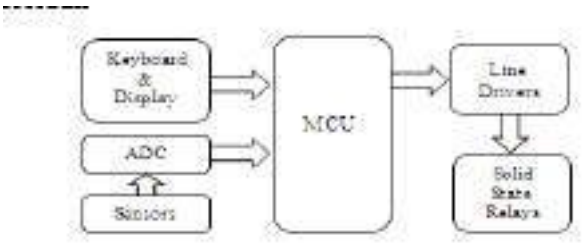
Abstract –

The present paper deals with the design of an intelligent controller for greenhouse to control temperature inside the greenhouse. A fuzzy logic is used to control the temperature. This controller is designed to handle two inputs, two outputs and 27 fuzzy rules; also inside and outside temperature can be monitored using LCD. The temperature inside the greenhouse can be set by the user as per the need of the crop in different seasons during the lifecycle of the plant. The PWM outputs are generated to control temperature according to the set point value.

Keywords – Intelligent Controller, FLC, Fuzzy Inference, Fuzzy Logic, Greenhouse, PWM

Introduction:

The greenhouse is a structure that is covered with a material that is transparent to the visible portion of the electromagnetic spectrum, which is utilized in the growth of plant. The performance of the greenhouse is best when temperature is not too hot and not too cold. It is necessary to maintain suitable temperature at growth stage of several plants. So, with the controlled environment in the greenhouse it is possible to increase the quality and quantity of crop produce per unit land in minimum possible time [1]. So, with the controlled environment in the greenhouse it is possible to increase the quality and quantity of crop produce per unit land in minimum possible time. Automation in greenhouse is very important for successful management of the greenhouse crops [2][3]. Fuzzy control has been widely applied in industrial controls and domestic electrical equipment [4]. The automatic learning of fuzzy rules is a key technique in fuzzy control. In the present work a fuzzy logic based temperature controller is designed which will sense the inside and outside temperature of greenhouse, displays it on the screen, allows user to set inside temperature as per the requirement and activate the relays accordingly so as to maintain temperature.



System Block Diagram2.1 Hardware

Atmel's 89C52 microcontroller was used for system design, which after initialization, reads the sensors, displays the inside and outside temperature values of on LCD and act accordingly as per the algorithm. National Semiconductor's LM 35 ICs were used as temperature

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Design and Development of Microcontroller Based Low Cost System for CO_2 Trapping In Greenhouse

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Abstract –

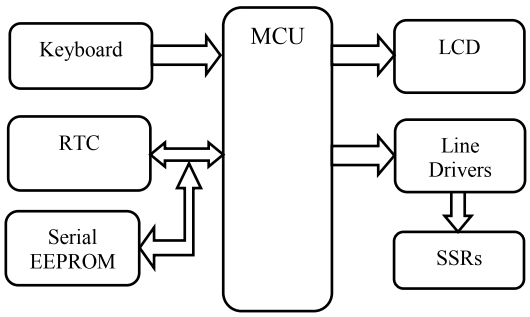
The scope of this paper includes the design and development of a low cost system for CO_2 trapping in greenhouse using microcontroller. The hardware of the system consists of interactive user interface, which allows the user to enable or disable the trapping system. The system also supports digital clock designed using IC PCF8583 clock/calendar catering the need of a clock for the system. The software was developed for the system for user interface which includes the keyboard, display and processing the settings made by the gardener and generating the output accordingly at different port lines.

Keywords: - Greenhouse, Microcontroller, RTC, SSR.

Introduction:

The classical definition of greenhouse is a structure that is covered with a material that is transparent to the visible portion of the electromagnetic spectrum, which is utilized in the growth of plant Automation in greenhouse is very important for successful management of the greenhouse crops [1]-[3]. Carbon Dioxide (CO_2) is essential for photosynthesis. The CO_2 released by the plants and soil during night could be trapped and hence CO_2 level could be increased by 6-7 times and photosynthetic activity by 3-4 times [4]. Normally air contains about 300ppm of CO_2. Studies have shown that levels of 1000 to 1500 ppm are very beneficial for good yields [5]. Therefore, the economical method to achieve this is designed to close all openings of the greenhouse in the late afternoon and open them in the morning automatically.

1. **System Block Diagram**



2. **Hardware**

3.1. The MCU

Atmel's 89C52 IC was used to design the system hardware to which 12MHz crystal was connected for generation of system clock. The port 2 pins were connected to the line driver IC ULN2803, which was used to drive the Solid State Relays (SSRs) to which mechanical system may be connected to drive doors and vents of greenhouse. The port1 pins P1.0, P1.1 and P1.2 were used for the keyboard design. Audible alerts were generated using buzzer connected to port 1 pin P1.3. Port pins P1.4 and P1.5 were used to interface the serial devices. P1.4 was defined as the serial Data line and P1.5 was used as the clock signal to the serial devices.

3.2. Keyboard and Display

A simple keyboard consisting of three keys was designed for the system which could be used to set clock and to start or stop trapping mechanism. The limited number of keys reduces the complexity in the operation. A 20x4 LCD display module was used to display the current time and also to show interactive messages for the user during the settings. This module has four rows of twenty characters in each row.

3.3. Real Time Clock and EEPROM

The system uses I^2C bus for integrated clock/calendar IC PCF 8583P by Philips semiconductor and serial EEPROM. The clock/calendar IC was very helpful in the current system which was used to control the mechanical system at proper time for trapping CO_2. The minutes and hours were used in the system, which may be modified as per the local time. The EEPROM IC24C02 was used in the design to store the settings made by the user. This facility avoids the need of making frequent settings in case of power failure and system resumes its functioning as per the previous settings when power is turned on. Both, RTC and EEPROM are serial devices which reduces the hardware and ultimately the power consumption [6].

3.4. Line drivers and SSRs

To control the mechanical system used for doors and vents of greenhouse as per the software, The single phase solid state relays (SSR) 006 JDA 330705 by ERI having rms on state current of 7 Amperes were used. The output port lines were first connected to the line driver IC ULN2803, which is designed to be compatible with standard TTL families, which drives the solid state relays (SSRs).

4. **Software**

Three main software modules were developed for the system. The modular programming approach was used so that individual modules could be upgraded as and when needed [6]. These modules are:

i. The initialization Module,
ii. The Keyboard and Display Module, and
iii. CO_2 Trapping Module

The initialization module, prepares the system for the normal operation. This module first defines the variables and initiates them to their default values. Reads the settings from the serial EEPROM and stores them to corresponding location. It also initiates the LCD display by sending the commands and displays the welcome message on it then displays the current time. The keyboard and display module scans the keyboard and detects the key pressed by the user and the display the related message on the LCD. If user wishes to change the current time, it could be done with the help of proper keys on the keyboard. Intelligence was developed in the system

such that if user does not press any key, the system remains unaffected and the current information from the RTC was used. But, in case the user modifies the minutes or the hour values, it will be immediately loaded to the corresponding locations in the RTC, which proceeds with the new values. The CO_2 module, if activated, generates the signal at 5 p.m., which may be used to drive a mechanical system which close all the doors and vents of the greenhouse so that CO_2 can be trapped and accumulated and in the morning, the system deactivates the signal at 8 a.m. so that the mechanical system can now open all the doors and vents of the greenhouse. This will enhance the CO_2 level. The signal generated by the controller remains activated between 5 p.m. to 8 a.m. and during this period complete vents of greenhouse will be closed. Here, the signal was kept continuously alive for the required duration so that in case of power failure, if trapping mechanism was activated, then system could take the action when the power resumes. The Control signals generated by microcontroller were used to drive the solid state relays through line drives. The mechanical system, to open and close greenhouse vents, needs to be designed and would be driven by these solid state relays.

Result And Discussion:

Respiration by the plant at night causes the Carbon dioxide levels to increase to around 500 ppm by early morning. The plant uses this high level in the first two or three hours after sunrise causing the levels to drop to around 150 ppm. At this stage the doors and vents could be opened to allow outside fresh air and the levels then rise back to around 300 ppm [4]. The actual test of this system was conducted by simulation, where the system clock time was changed and system actions were verified for the signal generation for the mechanical system. The controller generated the signals only when the CO_2 trapping was started. If user stops the CO_2 trapping, the system immediately deactivates the signal generated for the mechanical system. The functioning of this mechanism was tested by having different setting pertaining to clock and CO_2 trapping mode.

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Diversity of Aeromycoflora from Indoor Environment of Hostel

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Abstract:

An aeromycological study on indoor environment of hostel area was conducted during the month of dec-jan2018-19 which shows the variation in numbers as well as percentage of fungal species in these area. The study shows highest percentage contribution of Cladosporium spp(53%)on PDA and Czapeck-dox medium. The rest of the fungi like Aspergillus, Fusarium and Helminthosporium spp shows minimum percentage contribution.

Keywords: Aeromycoflora, PDA, CZA, Hostel.

Introduction :

Fungus is a member of the group of eukaryotic organisms that includes micro – organism such as yeast and molds as well as mushrooms. Fungi is a achlorophyllous organisms and they do not take part in photosynthesis hence it is called heterotroph. Fungi play and important role to decomposition of organic matter. Some fungi is mycotoxin and some are used for the production of antibiotic.

Aerobiology is deals with the studies of microrganisms present in the air. Meir (1930) was the first aerobiologists who used the term aerobiology for the studies of airborne fungal spores and their – organisms. The fungal species such as Aspergillus, Fusarium and Cladosporium is abundantly found in air .Fungi are common in indoor and outdoor environment,and nearly 10% of people worldwide have fungal allergy(Burge H.A2001). Epidemiological studies showed that to high concentration of micro – organism in the air can be allergic however even very low concentration of some particular micro – organism can cause serious disease. Singh and his coworkers are actively engaged in the field of aerobiology using various technique with reference to airspora inside hostel kitchen, cowshed etc.Climate play an important role in growth of fungi. The aim of the study is totally based on to isolate the fungal spores from indoor environment of hostel area, their prevalence and their impact on human being.

Material and methods:-

The present study deals with the Aeromycoflora isolated from the indoor environment of hostel area from J. B. College of science, Wardha i.e. ground floar, first floar and terrace.The fungi isolated on two different types of media i.e PDA and CZA media. 2 Petriplates of freshly prepared potato dextrose agar media and czapeck-dox media were exposed at each site for 10 min in the morning and evening. Then temperature is recorded. After exposure, the plates were incubated at 27◦C + upto 3-4 days in incubator. The slides were prepared with the help of Lactophenol cotton blue stain and identified using certain literatures.(Bernett and hunter,1972; cooke1963;Tilak 1989;Kalbende etal2012)

Result and Discussion:

The present work based on collection and identification of different fungi from three sites of hostel i.e. ground floar, first floar and Terrace.

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Outdoor Aerospora Study from Play Ground of Jbcs College, Wardha

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Abstract:

Air does not act as a natural environment for the growth and multiplication of Aero mycoflora, but it act as a very good medium for their dispersal from one place to another. Aeromycoflora of playground constitute fungal spores abundance and frequency in the environment. Aeromycoflora simply refers to the airborne fungal contributors of the environment. In air, all types of microorganisms are present like bacteria and fungi,spores are disperse in air from different sources and causes many diseases to human being and animals. Environmental factors like temp,and humidity play and important role for distribution of fungal spores. Air sampling was done at monthly intervals by using petriplate method for isolation of airborne fungi. Aspergillus niger, A. flavus, A. fumigatus & Cladosporium cladosporoides were more dominant fungi than other rest form of fungi. The present investigation shows that 27 fungal spores were recorded during the study period. Cladosporium cladosporoides was the highest percentage (24.16%) followed by Aspergillus and Fusarium spp.

Keywords: Aeromycoflora, Playground, PDA, Seasonal variations, Fungal spores.

Introduction:

Air borne fungi which are found in all types of environment. They are found largely in Indoor and outdoor environment. Earth atmosphere contain propagules of diverse group of microbes and other particle of biological origin (Rajendra et al 2017). The abundance of fungal spores are dependent upon many biotic and abiotic factors including temperature, humidity and sudden environmental changes, thus the airborne microorganism of any environment is specific in nature. Airborne microbe is a component of our environment and it is a potential economic and health implications (Gregory, 1961; Hashimoto, 1986). The aim of this study was to determine the Aeromycoflora and their identification, seasonal distribution of the airborne cultivable fungi in the air of a playground associated environments at outdoor in order to evaluate .

Materials And Methods :

For isolation of Aeromycoflora, Potato Dextrose Agar culture medium was used to isolate the variety of fungal spores from the environment. Aeromycoflora were studied from the play ground of JBCS Wardha. Culture plate exposure method containing PDA media were used for isolation of mycoflora present in the outdoor environment. This method also used by Tiwari P. (2008) for survey of aeromycoflora. The 10 cm diameter size petriplate were exposed twice in a day . The exposed petriplate were brought into the laboratory and incubated at 27 ± 1◦C for 4-5 days. At the end of incubation period the fungal colonies were counted and fungal spores identification were made by using lacto-phenol and cotton blue stain. Identification were done on the basis of colourization of colonies and their morphological characterization and also using available literature (Barnett, 1969; Nigmani et al. 2006). Temperature and Humidity were recorded in the college campus during the sampling period using a Hygrometer.

Result And Discussion:

The study deals with the exploration of Aeromycoflora from Playground of JBCS college which are situated at centre of the college. During this study a no of fungal propagules are encountered through a PDA media using gravity plate exposure methods. It shows that the variation in number of fungal species in this area. The Identified fungal spores belonging to

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Antifungal Activity of Two Medicinal Plants Against Some Selected Fungi

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Absract:

The aim of the study was totally based on evaluation of antifungal activity using plant extract against some selected fungi. The plants were selected on the basis of their reported ethnobotanical uses. Ethanolic extraction were done with the help of Soxhlet appratus by using dried leaves powdered of two medicinally important plants i.e Vitex negundo and Holarrhena antidysentrica .Well diffusion method were used to inhibit the growth of fungi on PDA media. Two plants extract shows the minimum inhibitory growth of fungi on PDA media.

Keywords: Antifungal, Fungi, Medicinal Plants, Solvent extract.

Introduction:

Pathogenic fungi are the main allergic agents in plants causing alterations during developmental stage including post-harvest to mankind. It will not be an exaggeration to say that use for herbal drug for human healthcare is probably as ancient as mankind. Medicinal plant represent rich source of antimicrobial agents (Mahesh and Satish, 2008). Plant extracts have been developed and proposed for use as antimicrobial substance (Del et al., 2000) Vitex negundo :(family- Verbenaceae). The genus consist of 250 species of which about 14 species are found in India and have some commercial and medicinal importance . Vitex negundo Linn; commonly known as five-leaved choste tree are mank's pepper is used as medicine fairly throughout the greater part of the India (Chopra et al., 1956). The Vitex negundo plant is a large aromatic shrub or sometimes a smaller slender tree and quadrangular, densely whitish branchlets up to 4.5 -5.5m in height. Plants is bitter, antiseptic, asthma and enlargement of spleen. Leaves contain an alkaloids nishindine and other constituents like vitamin C, carotene, benzoic acid (Husain, 1992).

Holarrhena antidysentrica: (family-Apocynaceae) commonly known as bitter oleander and locally as "inderjotulkh" (Baquar,1989). Seeds are 1-2cm long, linear or oblong concave with a coma shaped. The plant is use traditionally for a health disorders including Diarrhea ,dysentery (Jain, 1991). Alkaloids have been reported in the leaf of plant.

Material and Method:

Plant Collection: The plant were collected from the Dhaga forest, Wardha . Sterilization of plant material: The disease free and fresh plants were selected. The plant parts were washed with distilled water for three times. Then surface sterilized with 0.1%Mercuric chloride for 20 second. Again the leaves were washed thoroughly with distilled water (three time).

Preparation of plant extract: The collected plant material was air dried at room temperature for 10 to 15 days. The dried plants material was crushed by mortar and pestle without adding any solvent into it . the powder material was kept in air tight glass bottles. This stock powder was added in 150 ml solvent (Ethanol) by using a soxhlet extract for minimum 8-9

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Taxonomic Study on Plants of Malvaceae

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Abstract:

The present work deals with the identification of some weed plants belonging to family Malvaceae. In this study we select Dhaga forest area situated near Wardha city. The study is totally based on the identification of herb and shrub plants belonging to Malvaceae family. The study were carried on the month of September to January within 1 to 3 km area around the dhaga temple or forest. Near about 11 plants were identified on the basis of their morphological characterization. A total about 11 species belonging to 6genera of family Malvaceae.

Introduction :

The present study based on Angiospermic plants belonging to family Malvaceae also known as mallow family. The Malvaceae family comprises 88 genera and 2300species distributed across the world (Heywood 1978; Burkil 1997) also mentioned by Pradeepkumar 1996.The main characters of these family shows a large showy variously coloured flowers.One of the distinguishing character's include presence of various types of trichomes. In the Malvaceae family there are variations with herb, shrub and tree with mucilaginous sap in the all parts. Different taxonomist classified the family into tribe from time to time(bentham and Hooker in 1862). Plants of Malvaceae shows the diverse life forms. Habit and habitat of these malvaceae plants varies from plant to plant. It includes tufted or stellate hairs mucilage is found in root, stem , leaves and flowers of some species which gives it a slimy texture . The leaves are simple , alternate ,lobed free lateral stipule present in flowers, hermaphrodite, polypetalous, actinomorphic, Solitary inflorescence, sepal 5, basely connate calyx, stamens numerous monothecous , carpels as many as sepals long slender style, carpels5; axile placentation .

Study area:

Dhaga forest is situated near Wardha city. They are about 40 km away from Wardha. It is a very thick forest having the variations with Herb Shrub and Tree plant. We are selected about 1-3 km area around the temple of Shiva which are situated in Dhaga forest. It is much frequented tourist place in Wardha district .Best season to visit on September to January.

Climate:

Wardha city experienced humid and dry climate. Sometimes in the summer season the temperature rises above 46-48°C.and another time its stay average. Sufficient rain falls at rainy seasons from July to early October. In winter temperature falls upto 8-10 ◦C.
Soil is a fertile and vegetation of plants belonging to monocot and dicot plants including herb, shrub, tree and climbers.

Material and Methods:

The present study in based on the field of the area during the period of September 2018 to January 2019. Field visit and data collection were done once in every month. A total 11 species under 6 genera belonging to the family Malvaceae were collected and identified. The collected

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In-Vitro Binding Interaction of 2-(4-Hydrophenyl)-1H-Benzimidazole With BSA: Equilibrium Dialysis and FT-IR Spectroscopic Study

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Abstract:

This paper presented binding interaction of 2-(4-hydrophenyl)-1H-benzimidazole (4HPHBI) with bovine serum albumin (BSA) equilibrium dialysis and FT-IR study at physiological pH in various solvents. Findings were interpreted by scatchard plot in terms of association constants. This showed the increased in association constants with increase in concentrations of the 4HPHBI. It is seen that, the binding interaction supposed to be more in 1, 4-dioxane than DMSO and DMF. FT-IR study explained the binding interaction through shift in peak positions of amide I and II. FT-IR study concluded the changes in secondary structure of BSA on binding with 4HPHBI. In the present study, we have also reported the effect of foreign particles viz. arsenic and mercury on the binding interaction of 4HPHBI with BSA. Reactivity and association of 4HPHBI with BSA in presence of foreign moiety was determined and compared.

Keywords: BSA, Equilibrium dialysis, FT-IR study, Scatchard plot, association constant, foreign particles.

Introduction:

N-heterocyclic compounds are important class of compounds which are widely used in materials science, agro-chemistry, biomedical research and medicinal chemistry. Amongst the N-heterocyclic compounds, benzimidazole has a prominent place in organocatalysis, organometallic and material chemistry. 2-(4-Hydrophenyl)-1H-benzimidazole (4HPHBI) shows various biological properties especially, antimicrobial, antiviral, anticancer and antitumor[1-6]. Serum albumins are the most abundant proteins in the circulatory system of wide variety of organisms. The structure of HSA explains numerous physiological phenomena and provides further insight in pharmacokinetics. Variation in the temperature is found to be a key factor in binding affinities of HSA[7-8] as evident from various drugs viz. Ligustrazine, Ciprofloxacin[9], methotrexate[10] and cisplatin[11]. It is difficult to obtain HSA for experimental purposes. HSA and BSA exhibit similar chemical properties due to high percentage of sequence identities. BSA in lieu of HSA is used in this study because of low cost and easy availability. Various techniques are available to monitor the binding interactions of ligands to protein like NMR[12], isothermal titration calorimetry[13], U.V. visible absorbance[14], fluorescence[15], equilibrium & FT-IR[16], fluorescence and CD spectroscopy[17]. Equilibrium dialysis and FT-IR study also focused on the effect of foreign particles such as arsenic on protein-drug binding[18-19].

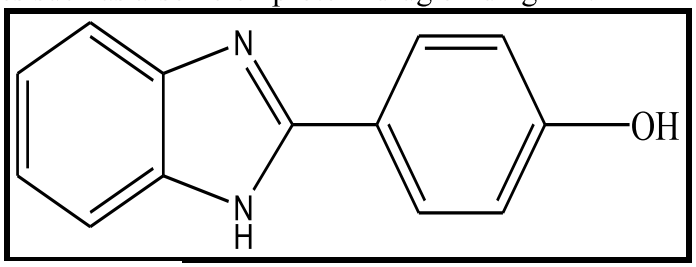

Figure 1: Structure of 2-(4-hydrophenyl)-1H-benzimidazole (4HPHBI)

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INDEX- Abstract

Production, Extraction, Quantification of Biopigment "Prodigiosin" from *Serratia Marcescens* and Its Antibacterial activity.

A.P. Sakharkar[1], S. S. Khandare[2]*, M. G. Ingale[3]

[1] P.G. Student,Post graduate Dept. of Microbiology, J. B. College of Science, Wardha,
[2] Asso. Professor and HOD, Dept. of Microbiology, J. B. College of science, Wardha,
[3]Asstt. Professor, Post graduate Dept. of Microbiology, J. B. College of Science, Wardha,

Prodigiosin is a biopigment and secondary metabolite produced by many strains of *Serratia marcescens* and other gram negative bacteria. Prodigiosin is a valuable molecule due to its reported antifungal, immunosuppressive and anti-proliferative activities.In the present study, total 17 natural samples ie.10 soil samples, 3 water samples and 4 sewage effluent samples were collected from wardha region and screened for prodigiosin production, Total five isolate were found to produce the red pigment viz: AK1, AK2, AK3, AK4, AK5. Isolate AK5 was found to be most efficient prodigiosin producer and was selected for further study. AK5 was identified on the basis of cultural, morphological and biochemical characterization as *Serratia marcescens.*Production of pigment was carried out on nutrient broth. Pigmentextraction was carried out by using methanol and petroleum ether as solvent and subjected to spectrum scanning in range between 300-700 nm followed by presumptive test to confirm pigment as prodigiosin. Qualitative analysis of pigment was also carried out bypaper chromatographyand Rf value of 0.42 was obtained. Further, pigment was also quantified by using standard formula and prodigiosin was estimated to be 4020 unit/cell by methanol extraction method and 1580unit/cell by petroleum ether extraction method. Antibacterial activity of prodigiosin was also tested against *E. coli*, *P.aeruginosa*, *S.aureus* and *S. typhi* and zone of inhibitions were found to be 12 mm, 11 mm, 13 mm & 12 mm respectively. Antibiotic susceptibility pattern of isolate was also studied against 7 different antibiotics which are routinely used clinically. .

Key Words: Progidiosin, *Serratiamarcescens*, Antibacterial

****

PRODUCTION, EXTRACTION AND QUANTIFICATION OF PRODIGIOSIN, A BIOPIGMENT PRODUCED FROM *SERRATIA MARCESCENS* AND ITS ANTIBACTERIAL ACTIVITY AGAINST PATHOGENIC ORGANISMS

A.P. Sakharkar[1], S. S. Khandare[2]*, M. G. Ingale[3]
Post graduate Dept. of Microbiology, J. B. College of Science, Wardha.
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Abstract

Prodigiosin is a biopigment and secondary metabolite produced by many strains of *Serratia marcescens*. Prodigiosin is a valuable molecule due to its immunosuppressive, anti-proliferative and antifungal activities. In the present study, total 17 natural samples ie.10 soil samples, 3 water samples and 4 sewage samples were collected from wardha region and screened for prodigiosin production, Total five isolate were found to produce the red coloured pigment viz: AK1, AK2, AK3, AK4, AK5. Isolate AK5 was found to be most efficient prodigiosin producer and was selected for further study. AK5 was identified on the basis of cultural, morphology and biochemical characteristics and comparing with Bergeys Manual of Determinative Bacteriology (9th edition) as *Serratia marcescens.* Production of pigment was carried out on nutrient broth. Pigment extraction was done by using petroleum ether and methanol as solvent and subjected to spectrum scanning in range between 400-700 nm followed by presumptive test to confirm pigment as prodigiosin. Qualitative analysis of pigment was also carried out by paper chromatography and Rf value of 0.42 was obtained. Further, pigment was also quantified by using standard formula and prodigiosin was estimated to be 4020 unit/cell by methanol extraction method and 1580 unit/cell by petroleum ether extraction method. Prodigiosin was evaluated for its antibacterial activity against *E. coli*, *P. aeruginosa, S. aureus, S. typhi* and zone of inhibitions of 12 mm, 12 mm, 13 mm and 11 mm respectively were recorded. The isolate was found to be completely susceptible to Chloramphenicol, Ciprofloxacin, Cloxacillin, Erythromycin, Gentamicin and resistant towards Ampicillin and Streptomycin.

Key Words: Prodigiosin, *Serratia marcescens,* Antibacterial activity

Introduction

The red pigment prodigiosin is having unique tripyrrole structure and belongs to the alkaloid class of secondary metabolites. This pigment can be obtained from a few members of *Pseudomonas, Streptomyces* and *Serratia* species [1]. It is a light sensitive pigment, insoluble in water and moderately soluble in ether and alcohol, and soluble in methanol, chloroform, acetonitrile and DMSO [2]. There are several organisms which can produce pigments and two major sources are plants (3) and microorganisms (4). There are various sources of pigments and plants occupies the main place among them. But problem with plant source is the attack of many pathogens on plants leads to the loss of the plants. [5]. There are variety of natural pigments

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Effect of Ultrasound on Particle Size and Gas Sensing Properties of Graphene/TiO_2Composite

Pradip Tekade[a], Kailash Nemade[b], Gauri Bhoyar[a]
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[b]Department of Physics, Indira Mahavidyalaya, Kalamb 445 401, India.
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Increased consciousness about the environmental issues, especially regarding health and safety, has focused on the need of air-quality monitoring.[1] Gas sensors have been playing crucial role in environmental monitoring, industrial safety, diagnosis of diseases, and traffic safety.[2-4]This paper reported the effect of particle size on gas sensing properties. Graphene/TiO_2 composite system were used to analyze the effect of particle size. The particle size of TiO_2 nanoparticles reduced using probe sonication technique. Four different time dose of probe sonication given to TiO_2, in order to reduce the particle size. The particle size of TiO_2 nanoparticles after probe sonication successfully estimated using X-Ray Diffraction analysis. As-obtained TiO_2 nanoparticles of reduced particle size used for the preparation of graphene/TiO_2 composites. Ultraviolet-visible spectroscopy also shows that particle size can reduced by probe sonication and it is also useful for band gap engineering. The doctor blade technique was employed to fabrication of sensors. As-fabricated sensors shows good dependence on the particle size. The sensors were tested towards CO_2 and LPG. In both cases, sensing response increases with reduction in particle size.

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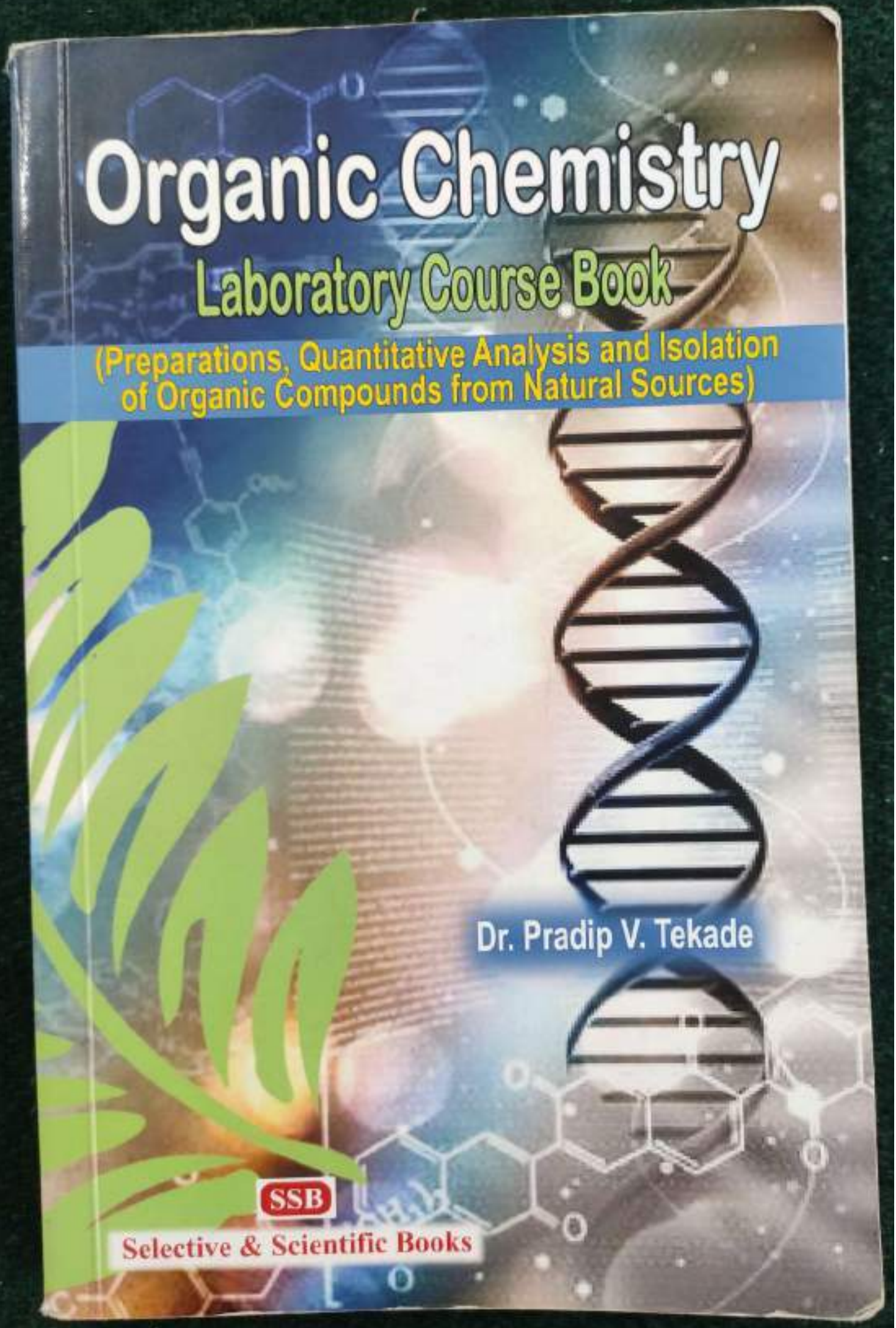
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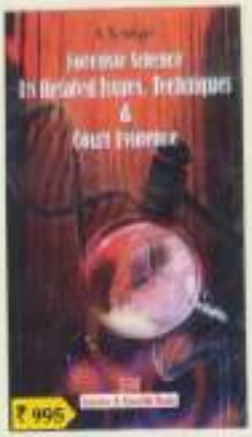

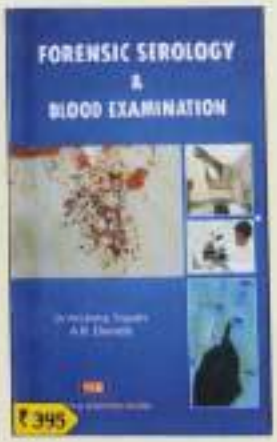

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STOCHASTIC COMPLEMENTATION OF ANALYZING THE RELATIONSHIP BETWEEN TWO SET OF NODES WITH RESPECT TO NETWORKING

Chandrasekaran K Arun [1], Dr. Vilas M Ghodki [2] , Dr. S.B. Kishor [3]

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[2] Associate Professor, Dept. of Computer Science , J.B. College, Wardha, Maharashtra, India

[3]Head, Dept. of Computer Studies and Research, S.P College, Chandrapur, Maharsashtra, India

ABSTRACT:

A stochastic complementation concept is an idea which occurs naturally, although not always explicitly, in the theory and application of finite Markov chains. This paper brings stochastic complementation to the forefront with an explicit definition and a development of some of its properties and its applications with respect to networking. Furthermore, stochastic complementation are explored with respect to problems involving uncoupling procedures in the theory of Markov chains. Also, concept of stochastic complementation is used for analyzing the relationship between two set of nodes with respect to networking.

Keywords: Markov chains, stationary distributions, stochastic matrix, stochastic compelementation , nearly reducible systems, Simon–Ando theory

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STOCHASTIC COMPLEMENTATION OF ANALYZING THE RELATIONSHIP BETWEEN TWO SET OF NODES WITH RESPECT TO NETWORKING

Chandrasekaran K Arun [1], Dr. Vilas M Ghodki [2] , Dr. S.B. Kishor [3]

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Keywords: Markov chains, stationary distributions, stochastic matrix, stochastic compelementation , nearly reducible systems, Simon–Ando theory

[1] INTRODUCTION

Traditional techniques like machine learning, statistical, pattern recognition, and data mining approaches (see, for example, [12]) usually assume a random sample of independent objects from a single relation. All these techniques have gone through the extraction of knowledge from data, almost always leading, in the end, to the classical double-entry tabular

format, containing features for a sample of the population. These features are therefore used in order to learn from the sample, provided that it is representative of the population as a whole. However, real-world data coming from many fields (such as World Wide Web, marketing, social networks, or biology; see [7]) are often multi relational and interrelated. The work recently performed in statistical relational learning [8], aiming at working with such data sets, incorporates research topics, such as link analysis [14], [19], web mining [1], [3], social network analysis [2], [20], or graph mining [5]. All these research fields intend to find and exploit links between objects (in addition to features—as is also the case in the field of spatial statistics [6], [17]), which could be of various types and involved in different kinds of relation-ships. The focus of the techniques has moved over from the analysis of the features describing each instance belonging to the population of interest (attribute value analysis) to the analysis of the links existing between these instances (relational analysis), in addition to the features. This paper is proposing to analyze the links exist between set of nodes in networking.

[2] SIMPLE CORRESPONDENCE ANALYSIS

As stated before, simple correspondence analysis (see, for instance, [9], [10], [12], [18]) aims to study the relation-ships between two random variables x_1 and x_2 (the features) having each mutually exclusive, categorical, outcomes, denoted as attributes. Suppose the variable $x_1$has n_1 observed attributes and the variable x_2 has n_2 observed attributes, each attribute being a possible out- come value for the feature. An experimenter makes a series of measurements of the features x_1; x_2 on a sample of vg individuals and records the outcomes in a frequency (also called contingency) table, fij, containing the number of individuals having both attribute x_1 = i and attribute x_2 = j. In our relational database, this corresponds to two tables, each table corresponding to one variable, and containing the set of observed attributes (outcomes) of the variable. The two tables are linked by a single relation (see Fig. 1 for a simple example). This situation can be modeled as a bipartite graph, where each node corresponds to an attribute and links are only defined between attributes of x_1 and attributes of x_2. The weight associated to each link is set to $w_{ij} = f_{ij}$, quantifying the strength of the relationship between i and j. The

$$A = \begin{bmatrix} O & A_{12} \\ A_{21} & O \end{bmatrix}, \quad P = \begin{bmatrix} O & P_{12} \\ P_{21} & O \end{bmatrix},$$

associated n * n adjacency matrix and the corresponding transition matrix can be factorized as where O is a matrix full of zeroes. Suppose we are interested in studying the relationships between the attributes of the first variable x1, which corresponds to the n1 first elements. By stochastic complementation (see (10)), $P_c=P_{12}P_{21}=D_1^{-1}A_{12}D_2^{-1}A_{21}$ .Computing the diffusion map for t = 1aims to extract the subdominant right-hand eigenvectors of P_c, which exactly corresponds to correspondence analysis (see, for instance, [24], (4.3.5)). Moreover, it can easily be shown that P_c has only real nonnegative eigenvalues, and thus, ordering the eigenvalues by modulus is equivalent to ordering them by value. In correspondence analysis, eigenvalues reflect the relative importance of the dimensions: each eigenvalue is the amount of inertia a given dimension exhibits in the frequency table [12]. The basic diffusion map after stochastic complementation on this bipartite graph therefore leads to the same results as simple correspondence analysis. Relationships between simple correspondence analysis and link analysis techniques have already been highlighted. For instance, Zha et al. [24] showed the

equivalence of a normalized cut performed on a bipartite graph and simple correspondence analysis. On the other hand, Saerens et al. investigated the relationships between Kleinberg's HITS algorithm [25], and correspondence analysis [18] or princi-pal component analysis [16].

[3] MULTIPLE CORRESPONDENCE ANALYSIS

Multiple correspondence analysis assigns a numerical score to each attribute of a set of p > 2 categorical variables [23], [18]. Suppose the data are available in the form of a star-schema: the individuals are contained in a main table and the categorical features of these individuals, such as education level, gender, etc., are contained in p auxiliary, satellite, tables. The corresponding graph is built naturally by defining one node for each individual and for each attribute while a link between an individual and an attribute is defined when the individual possesses this attribute. This configuration is known as a star-schema [13] in the data warehouse or relational database fields (see Fig. 2 for a trivial example).Let us first renumber the nodes in such a way that the attribute nodes appear first and the individuals nodes last. Thus, the attributes-to-individuals matrix will be denoted by A_{12}, it contains a 1 on the (i, j) entry when the individual j has attribute i, and 0 otherwise. The individuals-to-attributes matrix, the transpose of the attributes-to-individuals matrix, is A_{21}. Thus, the adjacency matrix of the graph is

$$\mathbf{A} = \begin{bmatrix} \mathbf{O} & \mathbf{A}_{12} \\ \mathbf{A}_{21} & \mathbf{O} \end{bmatrix}.$$

Now, the individuals-to-attributes matrix exactly corresponds to the data matrix $A_{21} = X$ containing, as rows, the individuals and, as columns, the attributes. Since the different features are coded as indicator (dummy) variables a row of the X matrix contains a 1 if the individual has the corresponding attribute and 0 otherwise. We thus have $A_{21} = X$ and $A_{12} = X^T$. Assuming binary weights, the matrix D_1 contains on its diagonal the frequencies of each attribute, that is, the number of individuals having this attribute. On the other hand, D_2 contains p on each element of its diagonal, since each individual has exactly one attribute for each of the p features (attributes corresponding to a feature are mutually exclusive). Thus,

$$\mathbf{D}_2 = p\,\mathbf{I} \text{ and } \mathbf{P}_{12} = \mathbf{D}_1^{-1}\mathbf{A}_{12}, \mathbf{P}_{21} = \mathbf{D}_2^{-1}\mathbf{A}_{21}.$$

Suppose we are first interested in the relationships between attribute nodes, thereby hiding the individual nodes contained in the main table. By stochastic complementation (4), the corresponding attribute-attribute transition matrix is

$$\begin{aligned} \mathbf{P}_c &= \mathbf{D}_1^{-1}\mathbf{A}_{12}\mathbf{D}_2^{-1}\mathbf{A}_{21} = \frac{1}{p}\mathbf{D}_1^{-1}\mathbf{A}_{12}\mathbf{A}_{21} \\ &= \frac{1}{p}\mathbf{D}_1^{-1}\mathbf{X}^T\mathbf{X} = \frac{1}{p}\mathbf{D}_1^{-1}\mathbf{F}, \end{aligned}$$

where the element f_{ij} of the frequency matrix $F = X^TX$, also called the Burt matrix, contains the number of co-occurrences of the two attributes i and j, that is, the number of individuals having both attribute i and attribute j. The largest nontrivial right eigenvector of the matrix P_c represents the scores of the attributes in a multiple correspondence analysis. Thus, computing the eigenvalues and eigenvectors of Pc and displaying the nodes with coordinates proportional to the eigenvectors, weighted by the corresponding eigenvalue, exactly corresponds to multiple

correspondence analysis. This is precisely what we obtain when computing the basic diffusion map on P_c with t = 1. Indeed, as for simple correspondence analysis, it can easily be shown that Pc has real nonnegative eigenvalues, and thus, ordering the eigenvalues by modulus is equivalent to ordering by value.

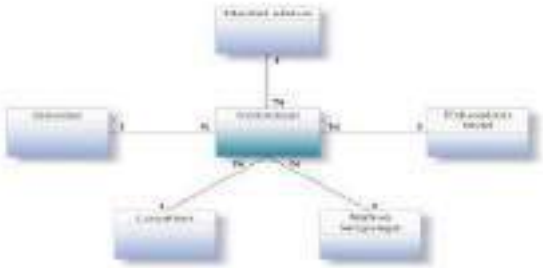

Fig. 1. Trivial example of a star-schema relation between a main variable, individual and auxiliary variables, Gender, Education level, etc. Each table contains outcomes of the corresponding random variable.

If we are interested in the relationships between elements of the main table (the individuals) instead of the attributes, we obtain,

$$\mathbf{P}_c = \frac{1}{\mu}\mathbf{A}_{21}\mathbf{D}_1^{-1}\mathbf{A}_{12} = \frac{1}{\mu}\mathbf{X}\mathbf{D}_1^{-1}\mathbf{X}^T,$$

which, once again, is exactly the result obtained by multiple correspondence.

[4] ANALYZING RELATIONS BY STOCHASTIC COMPLEMENTATION

In the previous section, the concept of stochastic complementation is briefly reviewed and applied to the analysis of a graph through the random-walk-on-a-graph model. From the initial graph, a reduced graph containing only the nodes of interest, and which is much easier to analyze, is built.

4.1 COMPUTING A REDUCED MARKOV CHAIN BY STOCHASSTIC COMPLEMENTATION

Suppose we are interested in analyzing the relationship between two sets of nodes of interest. A reduced Markov chain can be computed from the original chain, in the following manner: First, the set of states is partitioned into two subsets, S_1—corresponding to the nodes of interest to be analyzed—and S_2—corresponding to the remaining nodes, to be hidden. We further denote by n1 and n2 (with n1 + n2 = n) the number of states in S_1 and S_2, respectively; usually n2 > = n1. Thus, the transition matrix is repartitioned as

$$\mathbf{P} = \begin{array}{c} \\ S_1 \\ S_2 \end{array} \begin{array}{c} \begin{array}{cc} S_1 & S_2 \end{array} \\ \begin{bmatrix} \mathbf{P}_{11} & \mathbf{P}_{12} \\ \mathbf{P}_{21} & \mathbf{P}_{22} \end{bmatrix} \end{array}.$$

The idea is to censor the useless elements by masking them during the random walk. That is, during any random walk on the original chain, only the states belonging to S_1 are recorded; all the other reached states belonging to subset S_2 being censored, and therefore, not recorded. One can show that the resulting reduced Markov chain obtained by censoring the states S_2 is the stochastic complement of the original chain [15]. Thus, performing a stochastic complementation allows to focus the analysis on the tables and elements representing the factors/features of interest. The reduced chain inherits all the characteristics from the original chain; it simply censors the useless states. The stochastic complement Pc of the chain, partitioned as in (9),is defined as (see, for instance, [15])

$$\mathbf{P}_c = \mathbf{P}_{11} + \mathbf{P}_{12}(\mathbf{I} - \mathbf{P}_{22})^{-1}\mathbf{P}_{21}$$

It can be shown that the matrix P_c is stochastic, that is, the sum of the elements of each row is equal to 1 [15]; it therefore corresponds to a valid transition matrix between states of interest. We will assume that this resulting stochastic matrix is aperiodic and irreducible, that is, primitive [26]. Indeed, Meyer showed in [15] that if the initial chain is irreducible or aperiodic, so is the reduced chain. Moreover, even if the initial chain is periodic, the reduced chain frequently becomes aperiodic by stochastic complementation [15]. One way to ensure the aperiodicity of the reduced chain is to introduce a small positive quantity on the diagonal of the adjacency matrix A, which does not fundamentally change the model. Then, P has nonzero diagonal entries and the stochastic complement, P_c, is primitive. Let us show that the reduced chain also represents a random walk on a reduced graph G_c containing only the nodes of interest. We therefore partition the matrices

$$\mathbf{A} = \begin{bmatrix} \mathbf{A}_{11} & \mathbf{A}_{12} \\ \mathbf{A}_{21} & \mathbf{A}_{22} \end{bmatrix}; \mathbf{D} = \begin{bmatrix} \mathbf{D}_1 & \mathbf{O} \\ \mathbf{O} & \mathbf{D}_2 \end{bmatrix}; \mathbf{L} = \begin{bmatrix} \mathbf{L}_{11} & \mathbf{L}_{12} \\ \mathbf{L}_{21} & \mathbf{L}_{22} \end{bmatrix}$$

from which we easily find $P_c = D_1$. Notice that if A is symmetric (the graph G_c is undirected), Ac is symmetric as well. Since P_c is stochastic, we deduce that the diagonal matrix D_1 contains the row sums of Ac and that the entries of A_c are positive. The reduced chain thus corresponds to a random walk on the graph G_c whose adjacency matrix is A_c. Moreover, the corresponding Laplacian matrix of the graph G_c can be obtained by

$$\begin{aligned} \mathbf{L}_c &= \mathbf{D}_1 - \mathbf{A}_c = (\mathbf{D}_1 - \mathbf{A}_{11}) - \mathbf{A}_{12}(\mathbf{D}_2 - \mathbf{A}_{22})^{-1}\mathbf{A}_{21} \\ &= \mathbf{L}_{11} - \mathbf{L}_{12}\mathbf{L}_{22}^{-1}\mathbf{L}_{21} \end{aligned}$$

since $L_{12} = A_{12}$ and $L_{21} = A_{21}$. If the adjacency matrix A is symmetric, L_{11} (L_{22}) is positive definite, since it is obtained from the positive semi definite matrix L by deleting the rows associated to S2 (S1) and the corresponding columns, thereby eliminating the linear relation-ship. Notice that L_c is simply the Schur complement of Thus, for an undirected graph G, instead of directly computing P_c, it is more interesting to compute L_c, which is symmetric positive definite, from which Pc can easily be deduced.

[4] EXPERIMENTS

This experimental section aims to answer four research question. Does the proposed stochastic complementation applied to the analysis of a graph through the random-walk-on-a-graph model provide realistic subgraph drawings?

4.1 Analyzing the Effect of Stochastic Complementation on a Newsgroups Data Set

The real-world data set studied in this section is the newsgroups data set. It is composed of about 20,000 un-structured documents, taken from 20 discussion groups (newsgroups) of the Usernet diffusion list. For the ease of interpretation, we decided to limit the data set size by randomly sampling 150 documents out of three slightly correlated topics ("sport/baseball," "politics/mideast," and "space/general"; 50 documents from each topic). Those 150 documents are preprocessed as described in [21]. The resulting graph is composed of 150 document nodes, 564 term nodes, and three topic nodes representing the topics of the documents. Each document is connected to its corresponding topic node with a weight fixed to 1. Thus, the class (or topics) nodes are connected to document nodes of the corresponding topics, and each document is also connected to terms contained in the document. Drawing a parallel with our illustrative example (see Fig. 5), topic nodes correspond to c-nodes, document nodes to e-nodes, and terms to a-nodes. The goal of this experiment is to study the similarity between the terms and the topics through their connections with the document nodes. The reduced Markov chain is computed by setting S_1 to the nodes of the graph corresponding to the terms and the topics.

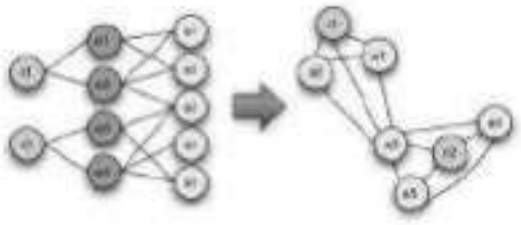

Fig. 2. Example illustrating stochastic implementation

The remaining nodes (documents) are rejected in the subgroup S_2. The KDM PCA quickly provides the same mapping as the basic diffusion map when increasing the value oft. However, it can be observed on the embedding with t = 1 that several nodes are rejected outside the "triangle," far away from the other nodes of the graph. A new mapping, where the terms corresponding to each node are also displayed (for the visualization convenience, only terms cited by 25 documents or more are shown) for KDM PCA with t = 1.It can be observed that a group of terms are stuck near each topic nodes, denoting their relevance to this topic (i.e., "player," "win," "hit," and "team" for the sport topic). We also observe that terms lying in-between two topics are also commonly used by both topics ("human," "nation," and "European" seem to be words used in discussions on politics as well as on space),or centered in the middle of the three topics (common terms without any specificity, such as "work," "Usa," or "make"). Globally, the projection provides a good representation of the use of the terms in the different discussion groups for both the basic diffusion map and the KDM PCA. Terms rejected outside the "triangle" are often only

cited by few documents and seem to have few links with other terms. They are probably out of topic as, for instance, the series of terms on printer in the outlier group on the left of the sport topic (“printer,” “Packard,” or “Hewlett”).

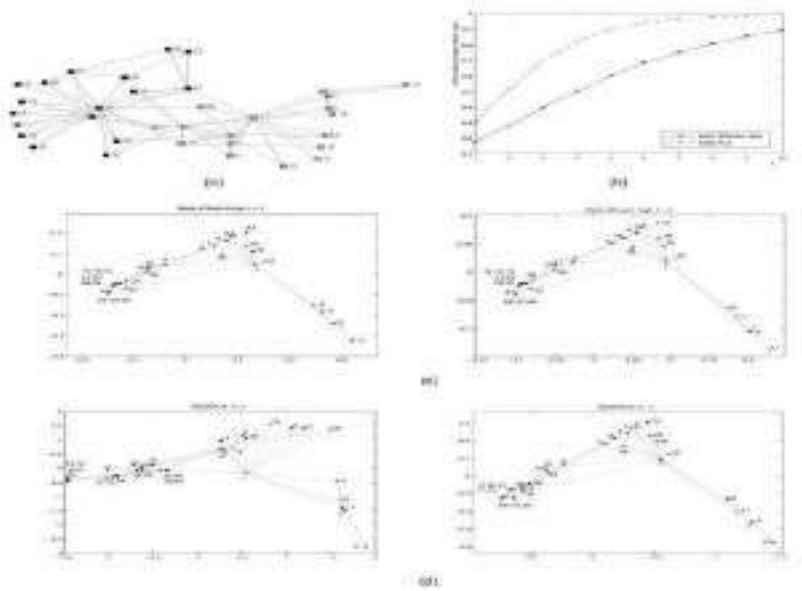

Fig. 2. Zachary Karate Social Network

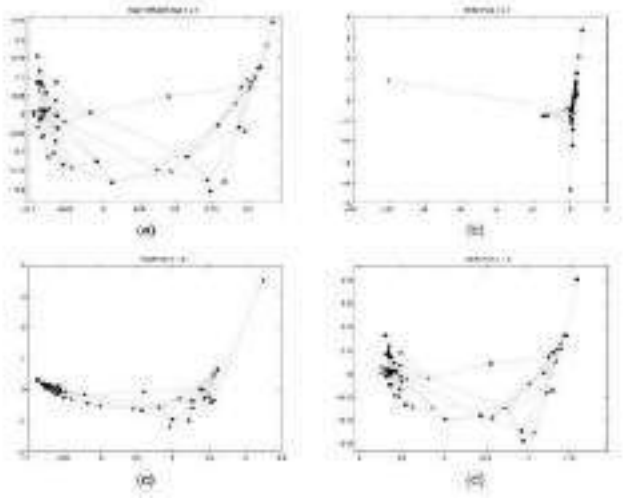

Fig 3. The graph mapping obtained by the diffusion map

4.2 DISCUSSION OF THE RESULTS

Let us now come back to our research questions. As a first observation, we can say that the two-step procedure (stochastic complementation followed by a diffusion map projection) provides an embedding in a low-dimensional subspace from which useful information can be extracted. Indeed, the experiments show that highly related elements are displayed close together while poorly related elements tend to be drawn far apart. This is quite similar to correspondence analysis to which the procedure is closely related. Second, it seems that stochastic complementation reasonably preserves proximity information, when combined with a diffusion map (KDM PCA) or an ISOMAP projection (MDS). For the diffusion map, this is normal, since both stochastic complementation and the diffusion map distance are based on a Markov chain

model—stochastic complementation is the natural technique allowing to censor states of a Markov chain. On the contrary, stochastic complementation should not be combined with a Laplacian Eigenmap, a curvilinear component analysis, or a Sammon nonlinear mapping—the resulting mapping is not accurate. Finally, the KDM PCA provides exactly the same results as the basic diffusion map when t is large. However, when the parameter t is low, the resulting projection tends to highlight the outlier nodes and to magnify the relative differences between nodes. It is therefore recommended to display a whole range of mappings for several different values of t.

[5] CONCLUSIONS AND FURTHER WORK

This work proposed to apply stochastic complementation in the analysis of a graph through the random-walk-on-a-graph model and provides realistic subgraph drawings. Here, the network is viewed as a graph, where the nodes correspond to the elements contained in the sets and the links correspond to the relations between the sets. It seems that stochastic complementation reasonably preserves proximity information, On the contrary, stochastic complementation should not be combined with a Laplacian Eigenmap, a curvilinear component analysis, or a Sammon nonlinear mapping—the resulting mapping is not accurate. Further work will be devoted to the combining stochastic implementation with a Laplacian Eigenmap, a curvilinear component analysis, or a Sammon nonlinear mapping—and produce the resulting mapping is accurate.

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FOR **PUBLISHING / PRESENTING / PARTICIPATION**

PAPER ENTITLED

A Study Of Least Square-Support Vector Machine Based Intrusion Detection System To Defect Denial-of Service Attacks.

AT National Conference on Computer Technology , Management and it's Applications (NC2TMA-2018) March 27th - 28th, 2018

Dr. Santosh Deshpande
Head of Department

Dr. Vikas Inamdar
Director

A STUDY OF LEAST SQUARE-SUPPORT VECTOR MACHINE BASED INTRUSION DETECTION SYSTEM TO DETECT DENIAL-OF SERVICE ATTACKS

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Keywords: Feature Selection , Intrusion Detection , DoS Attacks

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[1] INTRODUCTION

Intrusion detection system (IDS) is a complement of traditional networks protection techniques namely user authentication, data encryption, and firewall as the first line of defense for computer and networks security. IDS have been recognized as intense research area in the past decade owing to the rapid increase of sophisticated attacks on computer networks [1]. The objective of IDS is to detect any anonymous or unusual activity as an attempt of breaking the security policy of computer networks. There are three broad categories of detection approaches: 1) classification; 2) data clustering; and 3) anomaly-based approach. In the classification approach, we classify the given data set into different types of attacks. Data classification is a supervise machine learning technique. In the second data clustering approach, we categorize the given data set into different categories on the basis of similarity and dissimilarity. Data clustering is an unsupervised machine learning technique. The third anomaly-based approach identifies deviations from the normal usage behavior patterns to identify the intrusion. In general, anomaly-based approach is semi supervised machine learning technique. Each approach has own advantages and disadvantages over the other approaches [2]. This paper address the first approach i.e. data classification for building intrusion detection model. There are many issues and challenges in the existing data classification approaches. The first is called imbalance class problem, where the number of examples of attack class is very rare. That is the data set distribution reflects a significant majority of normal class and a minority of attack class [3]. The second is to identify the appropriate classifier for intrusion detection from a large number of existing classifiers [4]. The third is pre-processing the raw data so that processed data can be used as input for a classifier. Accuracy of a classifier depends upon the quality of input data set. The quality of data depends upon the quality feature vector of data set.

[2] FEATURE SELECTION

Feature selection is a technique for eliminating irrelevant and redundant features and selecting the most optimal subset of features that produce a better characterization of patterns belonging to different classes. Methods for feature selection are generally classified into filter and wrapper methods [12]. Filter algorithms utilize an independent measure (such as, information measures, distance measures, or consistency measures) as a criterion for estimating the relation of a set of features, while wrapper algorithms make use of particular learning algorithms to evaluate the value of features. In comparison with filter methods, wrapper methods are often much more computationally expensive when dealing with high-dimensional data or large-scale data. In this study hence, we focus on filter methods for IDS. Due to the continuous growth of data dimensionality, feature selection as a pre-processing step is becoming an essential part in building intrusion detection systems [13]. Mukkamala and Sung [14] proposed a novel feature selection algorithm to reduce the feature space of KDD Cup 99 dataset from 41 dimensions to 6 dimensions and evaluated the 6 selected features using an IDS based on SVM. The results show that the classification accuracy increases by 1 percent when using the selected features. Chebrolu et al. [15] investigated the performance in the use of a Markov blanket model and decision tree analysis for feature selection, which showed its capability of reducing the number of features in KDD Cup 99 from 41 to 12 features. Chen et al. [16] proposed an IDS based on Flexible Neural Tree

(FNT). The model applied a pre-processing feature selection phase to improve the detection performance. Using the KDD Cup 99, FNT model achieved 99.19 percent detection accuracy with only 4 features. Recently, Amiri et al. [12] proposed a forward feature selection algorithm using the mutual information method to measure the relation among features. The optimal feature set was then used to train the LS-SVM classifier and to build the IDS. Horng et al. [17] proposed an SVM-based IDS, which combines a hierarchical clustering and the SVM. The hierarchical clustering algorithm was used to provide the classifier with fewer and higher quality training data to reduce the average training and test time and to improve the classification performance of the classifier. Experimented on the corrected labels KDD Cup 99 dataset, which includes some new attacks, the SVM-based IDS scored an overall accuracy of 95.75 percent with a false positive rate of 0.7 percent.

[3] INTRUSION DETECTION FRAMEWORK BASED ON LEAST SQUARE SUPPORT VECTOR MACHINE

The framework of the proposed intrusion detection system is depicted in Fig. 1. The detection framework is comprised of four main phases: (1) data collection, where sequences of network packets are collected, (2) data preprocessing, where training and test data are preprocessed and important features that can distinguish one class from the others are selected, (3) classifier training, where the model for classification is trained using LS-SVM, and (4) attack recognition, where the trained classifier is used to detect intrusions on the test data. Support Vector Machine is a supervised learning method. It studies a given labeled dataset and constructs an optimal hyperplane in the corresponding data space to separate the data into different classes. They named this new formulation the Least Squares SVM (LS-SVM). LS-SVM is a generalized scheme for classification and also incurs low computation complexity in comparison with the ordinary SVM scheme [4].

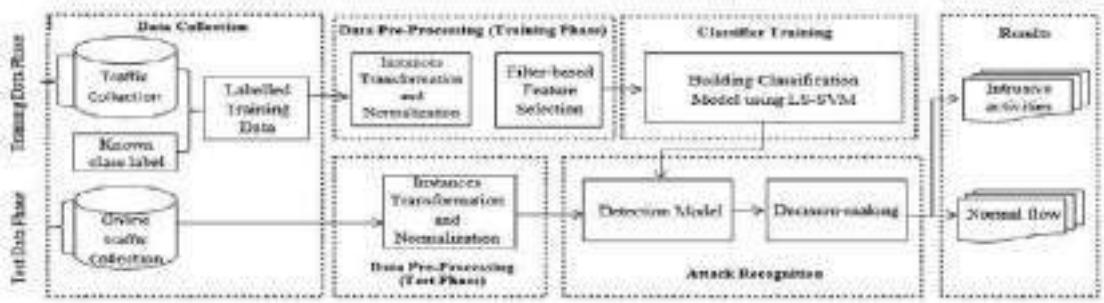

Figure 1: The framework for the LS-SVM based IDS

A. DATA COLLECTION

Data collection is the first and a critical step to intrusion detection. The type of data source and the location where data is collected from are two determinate factors in the design and the effectiveness of an IDS. To provide the best suited protection for the targeted host or networks, this study proposes a network-based IDS to test our proposed approaches. The proposed IDS runs on the nearest router to the victim(s) and monitors the inbound network traffic. During the training stage, the collected data samples are categorised with respect to the transport/Internet layer protocols and are labeled against the domain knowledge. However, the data collected in the test stage are categorised according to the protocol types only.

B. DATA PROCESSING

The data obtained during the phase of data collection are first processed to generate the basic features such as the ones in KDD Cup 99 dataset . This phase contains three main stages shown as follows.

C. Data Transferring

The trained classifier requires each record in the input data to be represented as a vector of real number. Thus, every symbolic feature in a dataset is first converted into a numerical value. For example, the KDD CUP 99 dataset contains

D. NSL-KDD Cup Dataset

The KDD Cup 1999 dataset is used as a benchmark for evaluating of IDS techniques [5]. The majority of examples in this data set have been extracted from the DARPA 1998 IDS evaluation [13]. KDD data set has a huge number of redundant examples. Duplicate examples may have a negative effect on the training process of machine learning classifiers. Through out in this empirical study, we used NSL-KDD Cup 99 dataset [7]. This data set is an improved version of KDD data set and publically available [7]. In this paper, KDD Train+ and KDD Test+ data sets that have 20% of the records of the entire NSL-KDD data set are used . The data set has 41 features. These features can be classified into four groups, namely basic features, content features, time-based features and host-based features. The details of these 41 features are presented in table I. Training data set consist of 29 different attacks. These attacks are further categorized into four different types and presented in Table 1. The brief introduction of four different types of attacks is as follows: 1) Denial of Service Attack (DoS): It is a class of attacks in which an attacker makes some computing or memory resource too busy or to handle legitimate requests, or denies legitimate users access to a machine. 2) Probing Attacks (Probe): It is a class of attacks in which an attacker scans a network of computers to gather information or find known vulnerabilities. An attacker with a map of machines and services that are available on a network can use this information to look for exploits. 3) Remote to Local Attacks (R2L): It is a class of attacks in which an attacker sends packets to a machine over a network but who does not have an account on that machine; exploits some vulnerability to gain local access as a user of that machine.

DoS	Probe	R2L	U2R
Neptune	Satan	Guess_Password	Buffer_overflow
Teardrop	Nmap	Warezmaster	Loadmodule
Land	Portsweep	Warezclient	rootkit
Smurf	IPSweep	Sendmail	
Pod		Multihop	
		ftpwrite	
		Imap	
		Spy	
		Phf	

Table 1: List of Attacks

E. Performance Evaluation

Several experiments have been conducted to evaluate the performance and effectiveness of the proposed LSSVM-IDS. For this purpose, the accuracy rate, detection rate, false positive rate and F-measure metrics are applied. The accuracy metric, detection rate and false positive rate are defined by

$$Accuracy = \frac{TP + TN}{TP + TN + FN + FP},$$

$$Detection\ Rate = \frac{TP}{TP + FN},$$

$$False\ Positive\ Rate = \frac{FP}{FP + TN},$$

where True Positive (TP) is the number of actual attacks classified as attacks, True Negative (TN) is the number of actual normal records classified as normal ones, False Positive (FP) is the number of actual normal records classified as attacks, and False Negative (FN) is the number of actual attacks classified as normal records. The F-measure is a harmonic mean between precision p and recall r [44]. In other words, it is a statistical technique for examining the accuracy of a system by considering both precision and recall of the system. F-measure used in this paper assigns the same weights to both Precision Rate (PR) and Recall Rate (RR), and is given by

$$F\text{-}measure = \frac{2(Precision * Recall)}{Precision + Recall}.$$

The precision (PR) is the proportion of predicted positives values which are actually positive. The precision value directly affects the performance of the system. A higher value of precision means a lower false positive rate and vice versa. The precision is given by

$$Precision = \frac{TP}{TP + FP}.$$

Table 2 summarises the classification results of the different selection methods in regard to detection rates, false positive rates and accuracy rates. It shows clearly that the detection model combined with the FMIFS has achieved an accuracy rate of 99.79, 99.91 and 99.77 percent for KDD Cup 99, NSL-KDD and Kyoto 2006+, respectively, and significantly outperforms all other methods. In addition, the proposed detection model combined with FMIFS enjoys the highest detection rate and the lowest false positive rate in comparison with other combined detection models. The proposed feature selection algorithm is computationally efficient when it is applied to the LSSVM-IDS. Fig. 2 shows the building (training) and test times consumed by the detection model using FMIFS compared with the detection model using all features. The figure shows that the LSSVM-IDS + FMIFS performs better than LSSVM-IDS with all 41 features on all datasets. There are significant differences when performing experiments on KDD Cup 99 and NSL-KDD and a slight difference on Kyoto 2006+ dataset by comparison with the two aforementioned models.

	KDD Cup 99			NSL-KDD			Kyoto 2006+		
	DR	FPR	Accuracy	DR	FPR	Accuracy	DR	FPR	Accuracy
LSSVMIDS + FMIFS	99.06	0.13	99.79	98.76	1.28	99.01	99.66	0.13	99.77
LSSVMIDS + MMIFS ($\beta = 0.1$)	99.36	0.23	99.71	95.46	1.53	97.96	98.59	0.16	99.32
LSSVMIDS + MMIFS ($\beta = 1$)	99.26	0.24	97.63	93.26	1.47	96.75	98.03	0.58	99.12
LSSVMIDS + FLCFS	98.47	0.61	98.03	93.29	1.41	96.65	98.07	0.82	98.99
LSSVMIDS + All features	99.16	0.57	99.19	91.32	1.38	95.96	98.29	0.33	97.42

Table 2 : Performance Classification for All Attacks Based on the Three Datasets

[4] CONCLUSION

Recent studies have shown that two main components are essential to build an IDS. They are a robust classification method and an efficient feature selection algorithm. In this paper, a supervised filter-based feature selection algorithm has been proposed, namely Flexible Mutual Information Feature Selection (FMIFS). FMIFS is an improvement over MIFS and MMIFS. FMIFS suggests a modification to Battiti's algorithm to reduce the redundancy among features. FMIFS eliminates the redundancy parameter b required in MIFS and MMIFS. This is desirable in practice since there is no specific procedure or guideline to select the best value for this parameter. FMIFS is then combined with the LSSVM method to build an IDS. LSSVM is a least square version of SVM that works with equality constraints instead of inequality constraints in the formulation designed to solve a set of linear equations for classification problems rather than a quadratic programming problem. The proposed LSSVM-IDS + FMIFS has been evaluated using three well known intrusion detection datasets: KDD Cup 99, NSL-KDD and Kyoto 2006+ datasets. The performance of LSSVMIDS + FMIFS on KDD Cup test data, KDDTest+ and the data, collected from Kyoto dataset has exhibited better classification performance in terms of classification accuracy, detection rate, false positive rate and Fmeasure than some of the existing detection approaches. In addition, the proposed LSSVM-IDS + FMIFS has shown comparable results with other state-of the-art approaches when using the Corrected Labels sub-dateset of the KDD Cup 99 dataset and tested on Normal, DoS, and Probe classes; it outperforms other detection models when tested on U2R and R2L classes. Furthermore, for the experiments on the KDD 21 dataset, LSSVM-IDS + FMIFS produces the best classification accuracy compared with other detection systems tested on the same dataset. Finally, based on the experimental results achieved on all datasets, it can be concluded that the proposed detection system has achieved promising performance in detecting intrusions over computer networks. Overall, LSSVM-IDS + FMIFS has performed the best when compared with the other state-of-theart models. Although the proposed feature selection algorithm FMIFS has shown encouraging performance, it could be further enhanced by optimising the search strategy

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STUDY OF FUNGAL SPORES FROM OUTDOOR ENVIRONMENT OF WARDHA CITY (M.S)

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ABSTRACT

Air is the main source of fungi and they are abundantly present in the outdoor environment. Airborne fungi are the most common organisms in nature. Many physical, Chemical and biological factors are responsible for the growth of fungi. Fluctuation of temperature which gives variations with number of fungal spores. The present study was carried out in the year 2014-15 using gravity petriplate exposure method containing PDA media for trapping the fungal spores.The present study showed that number of fungal spores present in the vegetable market and studying their percentage contribution.. The study shows a the percentage of Aspergillus spp and cladosporium spp was more dominant and other fungi like Penicillium ,Alternaria, fusarium Curvularia which are predominant fungi and Cunninghamella, Stachybotrytis spp was less no of counted.

Keywords: *Aeromycoflora, Vegetable market, PDA medium*

INTRODUCTION

Aeromycology is the study of dispersal and distribution of fungal spores and their mycelia in the air; and their dependence on meteorological conditions like temperature, rainfall, humidity, wind speed etc. The prevalence of airborne fungal spores are depended upon many biotic and abiotic factors, thus the airborne microorganism of any environment is specific in nature. Number and type of fungi vary with time of day, weather and seasonal fluctuations, condition of the surrounding areas, climatic conditions and the presence of a local source of spores (Pepeljnjak and Segvic Klaric , 2003). Airborne microbe is a component of our environment and is of potential economic and health implications (Gregory, 1961; Hashimoto, 1986). Molds are frequent contaminant of fresh vegetables. Airborne fungi are also considered as a key factor and as indicator of the level of air pollution. Occurance of Aeromycoflora in vegetable and fruit market were also studied. (Sharma and Bhattacharjee,2001;Medhi and Sharma 2010).The aim of this study was to monitor the occurrences and seasonal distribution of the airborne cultivable fungi in the air of a Vegetable market associated environments at outdoor in order to evaluate .

MATERIALS AND METHODS

The Vegetable market was situated in the centre of wardha city and surrounded by railway station and Bus stand. For isolation of aeromycoflora, PDA culture media were used. Aeromycoflora of the Vegetable market of Wardha city was observed by gravity petriplate exposure method containing PDA medium. This method also used by Tiwari P. (2008) for survey of aeromycoflora. The 10 cm diameter size Petriplate were exposed twice in a day . The exposed Petriplate were brought into the laboratory and incubated at 28 ± 1°C. for 4-5 days. At the end of incubation period the fungal colonies were counted, isolated and identified with the help of available literature (Barnett, 1969; Nigmani et al. 2006). For fungal spores identification, slides were prepared with the help of glycerine jelly as mounting media and lacto-phenol cotton blue as the standard stain. Temperature and Humidity were recorded in the Vegetable market during the sampling period using a Hygrometer. For ecological studies, percentage frequency and percentage contribution of individual species during the survey period was calculated using the standard formula (Tiwari, 1999)

$$\text{Percentage contribution} = \frac{\text{Total no of indivisual spp}}{\text{Total no of all spp}} \times 100$$

RESULT AND DISCUSSION

The present work deals with the Aeromycoflora of vegetable market from Wardha city. Kakde and Kakde (2012) was also shows air borne fungal spores in the vegetable market. In the present study total 831 fungal colonies belonging to 50 fungal forms, represent a group of a genus Ascomycotina ,Zygomycotina And Deuteromycotina. Most of the fungal colonies which represent the deuteromycotina. The Aspergillus and Cladosporium spp was more dominant fungi at the time of study period. The

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ANTIBACTERIAL ACTIVITY OF COPPER (II) OXIDE NANOSTRUCTURE SYNTHESIZED BY MICROWAVE IRRADIATION

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ABSTRACT

The multi-functionality of cupric oxide (CuO) nanostructure leads to fascinating applications in various fields. Herein, we report a simple, template-free, and surfactant-less wet-chemical microwave irradiated synthesis of CuO nanomaterials having ellipsoidal morphology. The morphology, structural, and optical properties of as-synthesized CuO nanostructures are investigated in detail. CuO nanoellipsoids are found to possess high surface area around 30m^2/g with the band gap of 1.85 eV and hence used to study the antibacterial activity. The antibacterial study was performed with gram positive and gram negative bacterial strains of Staphylococcus aureus and Escherichia coli, respectively.

Keywords: *Nanoellipsoidal CuO, antibacterial activity, microwave synthesis, S. aureus, E. coli.*

1. INTRODUCTION

CuO is one of the simplest members of the Cu family which shows its multi-functionality as a p-type semiconductor with narrow band gap of 1.24 eV.[1] It is a well-known material for the properties proliferating its applications in various fields viz. photovoltaics,[2] superconductors,[3] lithium batteries,[4] gas sensors,[5] magnetic storage media,[6] field emission,[7] methanol synthesis,[8] electrochemical sensing,[9] water gas shift reaction,[10] spin dynamics,[11] supercapacitors,[12] and heterogeneous catalysis.[13] Besides the inherent multi-functional properties arising from hetero-nanomorphologies, CuO nanomaterials have also emerged out as a potential candidate for antibacterial and antimicrobial activity.[14] The properties of CuO nanomaterials are closely related to its morphologies and crystallite size.[15, 16] Various morphologies of CuO viz. micro-spheres,[17] nanoplatelets,[18] nanoparticles,[19] nanorods,[20] nanowires,[21] nanoneedles,[22] nanoribbons,[23] nanosheets,[24] nanoshuttles,[25] nanobundles[26] etc. have attracted considerable attention due to their fundamental importance in above mentioned effective applications. Till date, various methods have been adopted to synthesize CuO, including solid-state reaction, sol–gel, electrochemical, sono-chemical, hydrothermal/solvothermal, microwave assisted hydrothermal, precipitation-pyrolysis, metal organic decomposition, and thermal decomposition.[27-35] Moreover, some green biological routes have also been emerged.[36] All these techniques are found to be efficient in producing single crystalline materials of diverse morphologies. However, most of these techniques either take long duration to carry out the reactions or not cost effective.

In addition to the need of short reaction duration, microwave synthesis also evenly suppresses side reactions, and thus enhances the yield with best reproducibility.[37] Microwave assisted hydrothermal technique has produced several hierarchical nanostructures of CuO including nanoflowers and nanopetals.[32,38,39] However, there are not many reports on the microwave-irradiated synthesis of CuO using commercial microwave oven.[40-43] Herein, we have adopted the same simple microwave-irradiated route for the wet-chemical surfactant-less synthesis of copper oxide (CuO) nanostructures under ambient condition. The as-synthesized CuO nanostructures were characterized by X-ray diffraction (XRD), field emission scanning electron microscopy (FESEM), Brunauer-Emmett-Teller (BET) surface area analyzer, UV-vis. spectrophotometer for its structural, morphological, surface and properties. The as-synthesized nanostructure is found to possess good crystallinity, uniform morphology, and high purity and its antibacterial activity is tested against the gram positive bacterial strains of Staphylococcus aureus (NCIM 2127) and the gram negative bacterial strains of Escherichia coli (NCIM 2065) using the disc diffusion assay method.

2. EXPERIMENTAL

2.1 CHEMICALS

Copper sulphate ($CuSO_4 \cdot 5H_2O$, 99.95%), sodium hydroxide (NaOH), ethanol (C_2H_5OH),.Bacterial strains of Staphylococcus aureus (NCIM 2127) and Escherichia coli (NCIM 2065) were obtained from National Chemical Laboratory (NCL), Pune, India. Muller–Hinton agar medium was used for the growth of microorganisms. All the chemicals received were of analytical grade and were used without any further purification.

2.2 SYNTHESIS OF CUO NANOMATERIAL

In the present study, microwave irradiated synthesis of CuO nanomaterial was carried out using the microwave irradiated technique as reported elsewhere with some modifications [44]. The commercial microwave chamber was used with the reaction conditions of 500 W for 10 min. During the reaction, color of the solution changed initially from blue to colorless and then slowly turns black. The black colored colloidal solution was centrifuged to separate out the precipitates. These precipitates were then washed using double distilled water, absolute ethanol, and acetone in sequence. This procedure was repeated several times. Finally, black colored powder was dried at 60°C for 4 h and used for further characterization.

2.3 CHARACTERIZATION

The crystallographic properties of the as-synthesized samples were studied using PANalytical high resolution X-ray diffraction (PW 3040/60) operated at 40 kV and 30 mA using Cu Kα X-rays (1.54Å). The surface morphology of as-prepared copper oxide nanostructures were analyzed using Carl Zeiss SUPRA 40 field-emission scanning electron microscope (FESEM). The effective Brunauer-Emmett-Teller (BET) surface area of the as-synthesized nanomaterials was measured using a Quantachrome ChemBET TPR/TPD analyzer. Optical property of the CuO nanostructures was studied using UV-vis. absorption spectrophotometer (Schimadzu 1800).

2.5 ANTIBACTERIAL ACTIVITY

Antibacterial activity of CuO nanostructure was tested against gram positive bacterial strains of Staphylococcus aureus (NCIM 2127) and gram negative bacterial strains of Escherichia coli (NCIM 2065) using the disc diffusion assay method with impregnated disks of as-synthesized CuO nanopetals. Approximately, 25.0 mL of molten and cooled nutrient agar media was poured in the sterilized petri dishes and was kept overnight at room temperature to ensure any contamination. The bacterial test organism S. aureus and E. Coli was grown in nutrient broth for 24 h at 37 °C. Bacterial lawns were prepared by using a 100 µL nutrient broth culture of each bacterial organism. Four dilutions of as-synthesized CuO nanopetals was prepared for testing viz. 5 mg/mL, 2.5 mg/mL, 2 mg/mL and 1.5 mg/mL in ethanol. Nanostructured impregnated discs were then placed on the bacterial lawn. These plates were incubated at 37°C for 24 h.

3. RESULTS AND DISCUSSION

3.1 STRUCTURAL PROPERTY

X-ray diffraction (XRD) analysis was carried out to identify the crystal structure and phase purity of samples. Figure 1 illustrates the XRD pattern of as synthesized material. The material shows the diffraction peaks that match with JCPDS card no. 01-080-1916 indicating single monoclinic phase of CuO. The average crystallite size calculated by Scherrer formula is found to be ~12 nm.

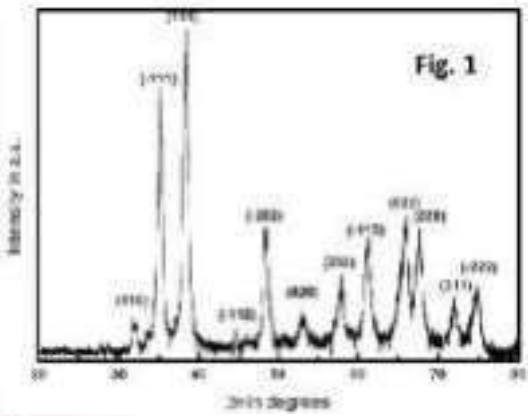

Figure 1. XRD pattern of as synthesized material

3.2 MORPHOLOGY

Figure 2 shows FESEM images of as synthesized CuO nanostructures which resemble ellipsoid-like morphology in nanodimension. There is a crucial role of concentration of NaOH to decide the preferential growth direction of nanostructure which supports the formation of ellipsoidal geometry of the nanostructure. [45] Figure 3 depicts the energy dispersive absorption spectrum which confirms the stoichiometry of Cu:O as 1:1.

Figure 2. FESEM images of CuO nanostructures

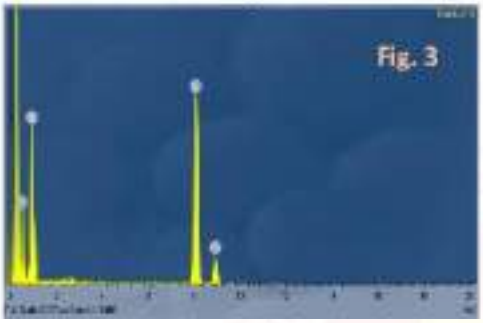

Figure 3. EDAX spectra of as synthesized nanostructure

3.3 SURFACE AREA

The Brunauer–Emmett–Teller (BET) technique was used to measure the surface area, pore radius, and pore volume of the as synthesized CuO nanostructure. Figure 3 depicts the nitrogen adsorption-desorption plots for the same. The effective specific surface area of the nanostructure was measured to be 30 m^2/g. This larger surface area in this system could be well utilized to study the multi-functionality of CuO in particular the surface phenomenon viz. catalysis, antibacterial properties.

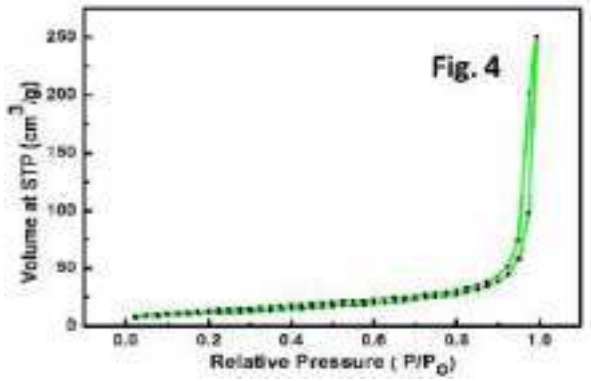

Figure 4. Nitrogen adsorption-desorption isotherm for CuO nanoellipsoids measured at 77 K

3.4 ANTIBACTERIAL ACTIVITY

Antibacterial activity of as synthesized CuO nanostructure has been studied by many groups relating to the role of different parameters viz. size effect, morphology, dissolution of copper ions in different medium etc.[14, 46-48]. Thus, as-synthesized nanostructure of CuO has been treated for its antibacterial activity against both gram-positive and gram-negative bacteria. Figure 5 shows the image of zone of inhibition with different concentrations of CuO nanoellipsoids as an antibacterial agent against E. Coli and S. aureus.

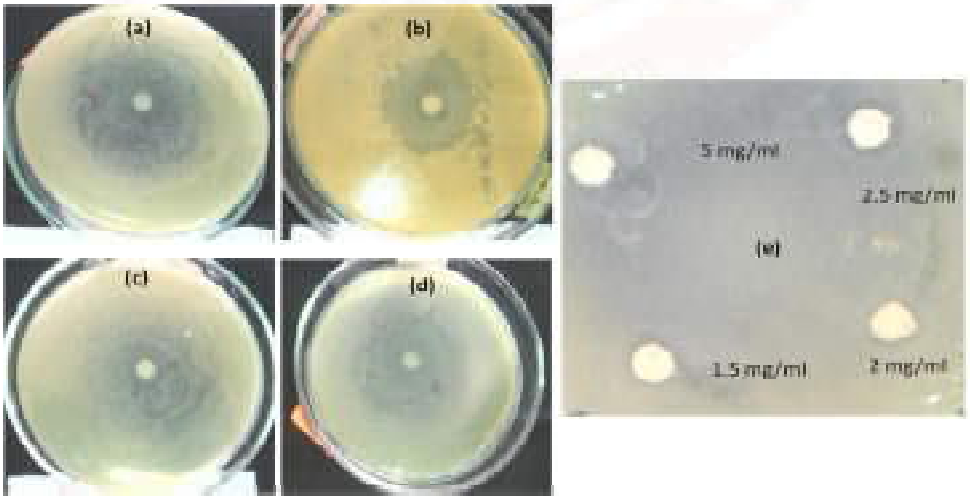

Figure 5. Zone of Inhibition of CuO nanopetals against E. Coli concentration **(a)** 1.5 mg/mL **(b)** 2 mg/mL **(c)** 2.5 mg/mL **(d)** 5 mg/mL **(e)** Zone of inhibition against S. aureus with different concentration of CuO nanostructure

Concentration of CuO nanoellipsoids (mg/mL)	Zone of inhibition (in mm) against	
	E. Coli	S. Aureus
5	25	18
2.5	23	15
2	20	12
1.5	18	06

Table 1. Zone of inhibition using CuO nanopetals against E.Coli and S.aureus

The diameter of inhibition zone reflects the magnitude of susceptibility of microbes (Table 1). E. Coli which exhibited a larger zone of inhibition than that of S. aureus is found to be more susceptible to CuO nanopetals. Table 1 also highlights the role of concentration of CuO nanopetals in the inhibition zone measurements and relates direct proportion between them. Our observations are in good agreement with Azam et al.[58] who also observed the same trend in case of CuO nanoparticles.

The metabolisms, differences in the cell structure, physiology or degree of contact of organisms with CuO nanomaterial are possible parameters that affect the antibacterial activity. The antibacterial activity of CuO nanoellipsoids can be attributed to the adhesion of bacterial cells over the surface of CuO nanoellipsoids, which releases the copper ions. These copper ions get attached with negatively charged bacterial cell wall leading to rupturing of the bacterial cell wall, thereby protein denaturation and ends with cell death. The effectiveness of CuO nanostructures in antibacterial activity is also known to be due to the indirect effects or changes in the surrounding charge environment of bacteria.[48]

4. CONCLUSIONS

In present work, we successfully synthesized CuO nanoellipsoids in absence of any surfactant and/or template using wet chemical microwave irradiation technique. The as-synthesized nanostructures were found to possess larger surface area. The as-synthesized CuO nanostructure is demonstrated for the antibacterial activity which gives excellent result against the bacteria E. coli and S. aureus.

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PARTIAL CHARACTERIZATION AND PURIFICATION OF A NOVEL CLASS II A PEDIOCIN PRODUCED BY *PEDIOCOCCUS PARVULUS* STRAIN MF233 ISOLATED FROM BATTER OF IDLI A TRADITIONAL FERMENTED FOOD OF SOUTH INDIA FOR MANAGEMENT OF FOOD BORNE PATHOGENS

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ABSTRACT

Pediococcus parvulus MF 233 isolated from batter of idli a traditional fermented food of south India, identified based on its 16S rRNA gene sequence, produced pediocin that had broad spectrum of inhibition against Gram positive and Gram negative antibiotic resistant pathogenic, food spoilage organisms, Salmonella typhi, Escherichia coli., Pseudomonas aeruginosa and Staphylococcus aureus. Pediocin (designated as pediocin MF233) was purified by ammonium sulphate precipitation, dialysis (MWCO 1000D) and ion exchange chromatography (DEAE Cellulose) showed increased specific activity from 3.66 to 9.52, 27.50 and 216, with 2.6, 2.88 and 7.87-fold increase in purification of protein respectively. The molecular weight of pediocin MF233 was 3000 Da. Pediocin MF233 found to be most heat stable and showed full activity at 121 ^{0}C for 10 minutes and maintained full stability after storage for 60 days at –20 ^{0}C and for 40 days at 4^{0}C, partial stability at 37^{0}C for 20 and 40 days while stability decreased slowly thereafter and lossed completely by 60 days. Pediocin MF233 showed stability at pH 2 to 6. Active principle of Pediocin MF233 was proteinaceous in nature since it was inactivated by proteolytic enzymes, but not by non–proteolytic enzymes. UV radiation did not affect the activity of Pediocin MF233. The studies concluded that the ability of Pediocin MF233 produced by Pediococcus parvulus MF233 in inhibiting a wide-range food pathogenic and spoilage bacterium, is of potential interest for food safety and may have future applications as food preservative.

KEY WORDS

Pediocin MF233, partial purification, characterization, foodborne pathogens, inhibitory activity.

INTRODUCTION:

The empirical use of microorganisms and their natural products for the preservation of foods (biopreservation) have been a common practice in the history of mankind.[1] The lactic acid bacteria generally considered as food grade organisms since they are involved in numerous food fermentations, known to man for millennia, do not pose any health risk to man and are designated as generally regarded as safe (GRAS) status.[2] The inhibition of food spoilage microbes could be attributed to the production of antimicrobial compounds including organic acids, hydrogen peroxide, antibiotics and bacteriocins.[3] Many species of *Lactobacillus*, used in the manufacture of fermented dairy products, inhibit the growth of other bacteria

Substituted diethyl 4-(substituted-phenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5 dicarboxylate found to be effective as active biological compound.The physico-chemical properties of the transition metal complexes of these newly synthesized N-heterocyclics with certain metals are reported here. The proton-ligand and metal-ligand stability constants of complexes of diethyl 4-(substituted-phenyl)-2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylate with Ni(II), Cu(II), and Co(II) were determined in mixed solvents (70% dioxane). Presence of OH/NH group(s) in these compounds confirmed at 0.1 M ionic strength in 70% dioxane-water mixture pH-metrically. Ni(II), Cu(II), and Co(II) metal ions formed 1 : 1 and 1 : 2 complex with all the three ligands. The result shows that the ratio of logK1/logK2 is positive in all cases. This indicates that there is little or no steric hindrance to the addition of secondary molecules.

Sonal D. Bajaj
Pradip V. Tekade

Proton/Metal– Ligand Stability Constants of Complexes of N-Heterocycle

pH-Metric Study of Stability Constant of First Transition Metal Ions with Novel Bio-inspired 1,4-Dihydropyridine Ligands

Dr. PRADIP V. TEKADE , M.Sc , Ph.D , NET, SET, GATE, Associate Professor, Department of Chemistry, Jankidevi Bajaj College of Science, Wardha.He has published 50 research papers in Journals and 6 Books.Dr. SONAL D. BAJAJ , M.Sc , Ph.D., B.Ed.She has published 14 research papers in various national and international Journals and two books.

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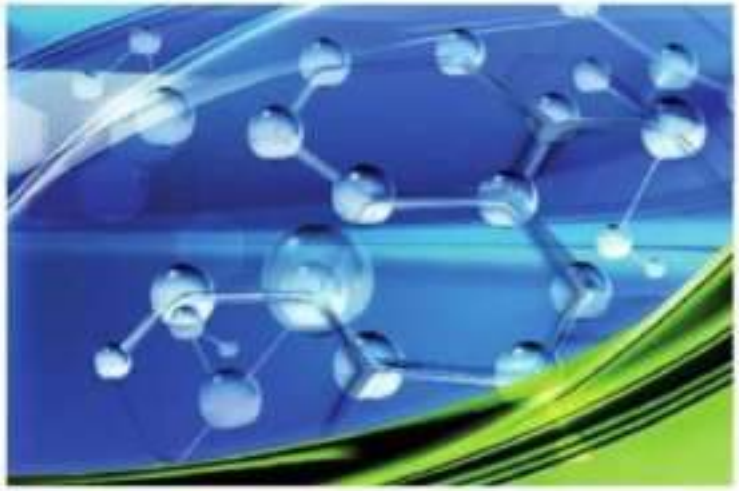
The ultrasound velocity measurements are helpful to study the intermolecular interactions and thermodynamical properties of pure components and their mixtures.Therefore, in the present book, ultrasonic studies of substituted propenamide studied in ethanolic solutions of various concentrations at different temperatures 303, 308 and 313 K with a view to understand molecular interactions in these solutions. Moreover , the complex formation in the solution of some N-heterocyclic compounds studied with some metal ions by determination of stability constant using spectrophotometry. The spectrophotometric study of chelating properties of some newly substituted heterocycles viz 2-(4,5-dihydro-1,2-oxazol-5-yl)phenol-N-methylaniline, 4-(1H-benzimidazole-2-yl)phenol and Ethyl 4-(4-hydroxyphenyl)-6-methyl-2-oxo-1,2,3,4-tetrahydropyrimidine-5-carboxylate with Ni (II) and Cu(II) metal ions by Job's variation method reported here.The study of thermo-acoustical parameters and study of stability constant of complexes by spectrophotometric study gives an important information regarding the presence of molecular interaction.It has an application in drug absorption and transmission.



Sonal Dilip Bajaj
Pradip V. Tekade

Acoustical and Spectrophotometric Study of Substituted N-Heterocyclics

Determination of Specific Molecular Interaction and Stability Constant of Substituted N-Heterocyclics

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Articles : Mechanical Engineering Section

DATAANALYSIS PROCEDURE FOR DISCOVERING RELATIONSHIPS IN A GRAPH WITH RESPECT TO NETWORKING

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Abstract - This paper precisely proposes a link-analysis based technique allowing to discover relationships existing between nodes in a computer network or, more generally, a graph. More specifically, this work is based on a random-walk through the database defining a Markov chain having as many states as nodes in the computer network. Suppose, for instance, we are interested in analyzing the relationships between nodes in a computer network, a two-step procedure is developed in analyzing the relationships. First, a much smaller, reduced, Markov chain, only containing the nodes but preserves the main characteristics of the initial chain, is extracted by stochastic complementation. For extracting the reduced Markov by stochastic complementation, an efficient algorithm is proposed. Secondly, the reduced chain is analyzed by, for instance, projecting the states in the subspace spanned by the right eigenvectors of the transition matrix called the basic diffusion map, or by computing a kernel principal-component analysis on a diffusion-map kernel computed from the reduced graph and visualizing the results. Indeed, a valid graph kernel based on the diffusion-map distance, extending the basic diffusion map to directed graphs, is introduced.

Keywords - *Diffusion Map , Stochastic complementation, Feature Redundancy*

I. INTRODUCTION

Wireless sensor networks (WSNs) are being used for diverse applications such as low cost area monitoring, environment monitoring, industrial and machine health monitoring, structural monitoring and military surveillance [1], [2]. In these applications, WSNs generate a large amount of data in the form of streams. In recent times, data mining techniques have been used to extract useful knowledge from WSN data [3], through discovering relationships among the sensor nodes which are known as behavioral patterns [4]. More recently, research has been focused to mine different types of behavioral patterns, e.g., sensor association rules [5], [6], [9] from stored (static) sensor data, context association rules [10] from sensor data stream, associated sensor patterns [7] and regularly frequent sensor patterns [8] from static as well as stream data. Traditional statistical, machine learning, pattern recognition, and data mining approaches [28] usually assume a random sample of independent objects from a single relation. Many of these techniques have gone through the extraction of knowledge from data, almost always leading, in the end, to the classical double-entry tabular format, containing features for a sample of the population. These features are therefore used in order to learn from the sample, provided that it is representative of the population as a whole. However, real-world data coming from many fields such as World Wide Web, marketing, social networks, or biology [16] are often multi relational and interrelated. The work recently performed in statistical relational learning [22], aiming at working with such data sets, incorporates research topics, such as link analysis [36] web mining [1],[9], social network analysis [8], or graph mining[11]. All these research fields intend to find and exploit links between objects which could be of various types and involved in different kinds of relationships. On the other hand, when dealing with a starschema database , this two-step procedure reduces to multiple correspondence analysis. The proposed methodology therefore extends correspondence analysis to the analysis of a relational database. In short, this paper has three main contributions:A two-step procedure for analyzing weighted graphs or relational databases is proposed. .

- It is shown that the suggested procedure extends correspondence analysis.

- A kernel version of the diffusion map distance, applicable to directed graphs, is introduced.

The paper is organized as follows: the basic diffusion map distance and its natural kernel on a graph in Section II. In Section IV we some experimental resultsinvolving several data sets.

II.THE DIFFUSION MAP DISTANCE AND ITSNATURAL KERNEL MATRIX

In this section, the basic diffusion map distance [24] is briefly reviewed and some of its theoreticaljustifications are detailed. Then, a natural kernel matrix isderived from the diffusion map distance, providing ameaningful similarity measure between nodes.

A. The Diffusion Map Distance

In our two-step procedure, a diffusion map projection,based on the so-called diffusion map distance, will beperformed after stochastic complementation. Now, sincethe original definition of the diffusion map distance dealsonly with undirected, aperiodic, Markov chains, it will firstbe assumed in Section 2 that the reduced Markov chain,obtained after stochastic complementation, is indeed undirected,aperiodic, and connected—in which case thecorresponding random walk defines an irreducible reversibleMarkov chain. Notice, that it is not required that the

original adjacency matrix is irreducible and reversible; theseassumptions are only required for the reduced adjacencymatrix obtained after stochastic complementation.The original derivation of the diffusion map, introducedindependently by Nadler et al., and Pons and Latapy [22],[13], is detailed in but other interpretationsof this mapping appeared in the literature . For an application of the basicdiffusion map to dimensionality reduction, see [35].Since P is aperiodic, irreducible, and reversible, it is wellknown that all the eigenvalues of P are real and theeigenvectors are also real [7]. Moreover, allits eigenvalues $\in [-1, +1]$, and the eigenvalue 1 has multiplicityone [7]. With these assumptions, Nadler et al. andPons and Latapy [42], [43], [46], [47] proposed to use asdistance between states i and j

$$d_{ij}^2(t) = \sum_{k=1}^{n} \frac{(x_{ik}(t) - x_{jk}(t))^2}{\pi_k} \qquad (1)$$

since, for a simple random walk on an undirected graph,the entries of the steady-state vector $\boldsymbol{\pi}$ are proportional (the $\propto$ sign) to the generalized degree of each node (the total ofthe elements of the corresponding row of the adjacencymatrix [28]). This distance, called the diffusion mapdistance, corresponds to the sum of the squared differencesbetween the probability distribution of being in any stateafter t transitions when starting (i.e., at time t = 0) from twodifferent states, state i and state j. In other words, twonodes are similar when they diffuse through the network—and thus influence the network—in a similar way. This is anatural definition which quantifies the similarity betweentwo states based on the evolution of the states' probabilitydistribution. Of course, when $i = j, d_{ij}(t) = 0$ .Nadler et al. [22] showed that this distance measurehas a simple expression in terms of the right eigenvectorsof P:

$$d_{ij}^2(t) = \sum_{k=1}^{n} \lambda_k^{2t}(u_{ki} - u_{kj})^2, \qquad (3)$$

where $u_{ki} = [\mathbf{u}_k]_i$ is component i of the kth right eigenvector, $\mathbf{u}_k$, of P and λ_k is its corresponding eigenvalue. Asusual, the λ_k are ordered by decreasing modulus, so that thecontributions to the sum in (3) are decreasing with k. Onthe other hand, $\mathbf{x}_i(t)$ can easily be expressed in thespace spanned by the left eigenvectors of $\mathbf{P}$, the $\mathbf{v}_k$,

$$\mathbf{x}_i(t) = (\mathbf{P}^{\mathrm{T}})^t \mathbf{e}_i = \sum_{k=1}^{n} \lambda_k^t \mathbf{v}_k \mathbf{u}_k^{\mathrm{T}} \mathbf{e}_i = \sum_{k=1}^{n} (\lambda_k^t u_{ki}) \mathbf{v}_k, \qquad (4)$$

where $\mathbf{e}_i$ is the ith column of $\mathbf{I}$,

$\mathbf{e}_i = [0, \ldots, 0, 1, 0, \ldots, 0]^{\mathrm{T}}$ with the single 1 in position i. The resulting mapping aims to represent each state i in an-dimensional euclidean space with coordinates $(|\lambda_2^t| u_{2i}, |\lambda_3^t| u_{3i}, \ldots, |\lambda_n^t| u_{ni})$, as in (4). Dimensions areordered by decreasing modulus, $|\lambda_k^t|$. This original mappingintroduced by Nadler and coauthors will be referred to asthe basic diffusion map in this paper, in contrast with thediffusion map kernel (KDM) that was introduced inSection II.The weighting factor, $\mathbf{D}^{-1}$, in (2) is necessary to obtain(3), since the $\mathbf{v}_k$ are not orthogonal. Instead, it can easily beshown that we have redefinethe inner

product as $(x, y) = \mathbf{x}^{\mathrm{T}}\mathbf{D}^{-1}\mathbf{y}$, where the metric ofthe space is $\mathbf{D}^{-1}$ [7].Notice also that there is a close relationship betweenspectral clustering (the mapping provided by the normalizedLaplacian matrix; see, for instance, [15], [35]) andthe basic diffusion map. Indeed, a common embedding ofthe nodes consists of representing each node by thecoordinates of the smallest nontrivial eigenvectors (correspondingto the smallest eigenvalues) of the normalizedLaplacian matrix, $\tilde{\mathbf{L}} = \mathbf{D}^{-1/2}\mathbf{L}\mathbf{D}^{-1/2}$. More precisely, if $\mathbf{u}_k$ isthe kth largest right eigenvector of the transition matrix P and λ_k is the kth smallest nontrivial eigenvector of thenormalized Laplacian matrix

A subtle, still important, difference between this mappingand the one provided by the basic diffusion map concerns theorder in which the dimensions are sorted. Indeed, for the basicdiffusion map, the eigenvalues of the transition matrix P areordered by decreasing modulus value. For this spectralclustering model, the eigenvalues are sorted by decreasingvalue (and not modulus), which can result in a differentrepresentation if P has large negative eigenvalues. Thisshows that the mappings provided by spectral clustering andby the basic diffusion map are closely related.Notice that at least three other justifications of thiseigenvector-based mapping appeared before in the literature,and are briefly reviewed here. It has been shownthat the entries of the subdominant right eigenvector of thetransition matrix P of an aperiodic, irreducible, reversible,Markov chain can be interpreted as a relative distance to its"stationary distribution" . This distance may be regarded as an indicatorof the number of iterations required to reach this equilibriumposition, if the system starts in the state from whichthe distance is being measured. These quantities are onlyrelative, but they serve as a means of comparison among thestates [30]. The same embedding can be obtained byminimizing the criterion

$$\sum_{i=1}^{n}\sum_{j=1}^{n} a_{ij}(z_i - z_j)^2 = \mathbf{z}^{\mathrm{T}}\mathbf{L}\mathbf{z}$$

Here, z_i is the coordinate ofnode i on the axis and the vector z contains the z_i. Theproblem sums up in finding the smallest nontrivialeigenvector of $(\mathbf{I} - \mathbf{P})$, which is the same as the secondlargest eigenvector of P, and this is once more similar to thebasic diffusion map. Notice that this mapping has beenrediscovered and reinterpreted by Belkin and Niroyi [2], [3]in the context of nonlinear dimensionality reduction. Thelast justification of the basic diffusion map, introduced in[15], is based on the concept of two-way partitioning of agraph. Minimizing a normalized cut criterion whileimposing that the membership vector is centered withrespect to the metric D leads to exactly the same embeddingas in the previous interpretation. Moreover, some authors

showed that applying a specific cut criteria to bipartitegraphs leads to simple correspondence analysis. More generally, these mappings are, of course, alsorelated to graph embedding and nonlinear dimensionalityreduction, which have been highly studied topics in recentyears, especially in the manifold learning community (see,i.e., [21], [30], [37] for recent surveys or developments).Experimental comparisons with popular nonlinear dimensionalityreduction techniques are presented in the following section.

IV. EXPERIMENT AND ANALAYSIS

A. Graph Reduction Influence and Embedding Comparison

The objective of this experiment is twofold. The first aim isto study the influence of stochastic complementation ongraph mapping. The second one is to compare five populardimensionality reduction methods, namely, the diffusionmap kernel PCA (KDM PCA or simply KDM), the LaplacianEigenmap (LE) [3], the Curvilinear Component Analysis(CCA) [14], Sammon's nonlinear Mapping (SM) [25], andthe classical Multidimensional Scaling [6], [12], based ongeodesic distances (MDS). For CCA, SM, and MDS, thedistance matrix is given by the shortest path distancecomputed on the reduced graph whose weights are set tothe inverse of the entries of the adjacency matrix obtainedby stochastic complementation. Notice that the MDSmethod computed from the geodesic distance on a graphis also known as the ISOMAP method after [6]. Providedthat the resulting reduced Markov chain is usually dense,the time complexity of each algorithm is as follows: ForKDM PCA, LE, and MDS, the problem is to compute the dominant eigenvectors of a square matrix since the graphis mapped on a d-dimensional space, which is

$O(d\,\tau\,n_1^2)$,where$n1$ is the number of nodes of interest being displayedand τ is the number of iterations of the power method. ForSM and CCA, the complexity is about $O(\tau\,n_1^2)$, where τ isthe number of iterations (these

algorithms are iterative byrecorded. On the other hand, computing the shortest pathdistances matrix takes $O(n_1^2 \log(n_1))$. Thus, each algorithmhas a time complexity between $O(n_1^2)$ and $O(n_1^3)$.In this experiment, we address the task of classificationof unlabeled nodes in partially labeled graphs, that is,semisupervised classification on a graph . Notice thatthe goal of this experiment is not to design a state-of-theartsemisupervised classifier, rather it is to study theperformance of the proposed method, in comparison withother embedding methods.Three graphs are investigated. The first graph isconstructed from the well-known Iris data set [4]. Theweight (affinity) between nodes representing samples isprovided by

$w_{ij} = \exp[-d_{ij}^2/\sigma^2]$, where d_{ij} is the euclidean distance in the feature space and σ^2 is simply the samplevariance. The classes are the three iris species. The secondgraph is extracted from the IMDb movie database [37]. The last graph,extracted from the CORA data set , is composed ofscientific papers from three topics. A citation graph is builtupon the data set, where two papers are linked if the firstpaper cites the second one. The tested graph contains1,410 nodes divided into three classes representing machinelearning research topics.For each of these three graphs, extra nodes are added torepresent the class labels (called the class nodes). Each classnode is connected to the graph nodes of the correspondingclass. Moreover, in order to define cross-validation folds,these graph nodes are randomly split into training sets andtest sets (called the training nodes and the test nodes,respectively), the edges between the test nodes and the classnodes being removed. The graph is then reduced to the testnodes and to the class nodes by stochastic complementation(the training nodes are rejected in the S2 subset, and thus,censored), and projected into a 2D space by applying one ofthe projection algorithms described before. Terms and topic nodes are displayed jointly.

between the test nodes and the class nodes is accuratelyreconstructed in the reduced graph, these nodes from the testset should be projected close to the class node of theircorresponding class. Wereport the classification accuracy forseveral labeling rates, i.e., portions of unlabeled nodes whichconstitute the test set. The proportion of the test nodes variesbetween 50 percent of the graph nodes (twofold crossvalidation)to 10 percent (10-fold cross validation). Thismeans that the proportion of training nodes left apart(censored) by stochastic complementation increases withthe number of folds. The whole cross-validation procedure isrepeated 10 times (10 runs) and the classification accuracyaveraged on these 10 runs is reported, as well as the95 percent confidence interval.For classification, the assigned label of each test node issimply the label provided by the nearest class node, in termsof euclidean distance in the 2D embedding space. This willpermit to assess if the class information is correctly preservedduring stochastic complementation and 2D dimensionalityreduction. The parameter t of theKDM PCA is set to 5, in viewof our preliminary experiments.Figs. 1a, 1b, and 1c show the classification accuracy, aswell as the 95 percent confidence interval, obtained on thethree investigated graphs for different training-test setpartitioning (folds). The x-axis represents the number offolds, and thus, an increasing number of nodes left apart(censored) by stochastic complementation (from 0, 50, . . . , upto 90 percent). As a baseline, the whole original graph(corresponding to one single fold and referred to as 1-fold) isalso projected without removing any class link and withoutperforming a stochastic complementation; this situationrepresents the ideal case, since all the class information iskept. All the methods should obtain a good accuracy score inthis setting—this is indeed what is observed.First, we observe that, although obtaining very goodperformance when projecting the original graph (1-fold),CCA and SM perform poorly when the number of folds,and thus, the amount of censored nodes, increases. On theother hand, LE is quite unstable, performing poorly on theCORA data set. This means that stochastic complementationcombined with CCA, SM, or LE does not workproperly. On the contrary, the performance of KDM PCAand MDS remains fairly stable; for instance, the averagedecrease of performance of KDM PCA is around 10 percent,in comparison with the mapping of the original graph(from 1-fold to 2-fold—50 percent of the nodes arecensored), which remains reasonable. MDS offers a goodalternative to KDM PCA, showing competitive performance;however, it involves the computation of the all-pairsshortest path distance.These results are confirmed when displaying the mappings.Figs. 1a, 1b, and 1c show a mapping example ofthe test nodes, as well as the class nodes (the white markers)of the CORA graph, for the 10-fold cross-validation setting.Thus, only 10 percent of the graph nodes are unlabeled andprojected after stochastic complementation of the 90 percentremaining nodes. It can be observed that the LaplacianEigenmap managed toseparate the different

classes, but mostly in terms of angularsimilarity. On the KDM PCA mapping (Fig. 8d), the classnodes are well-located, at the center of the set of nodesbelonging to the class. On the other hand, the mappingsprovided by CCA and SM after stochastic complementationdo not accurately preserve the class information.

Figure 1(a): Classification accuracy obtained by the five compared projection methods for the Iris ((a), three classes), IMDb

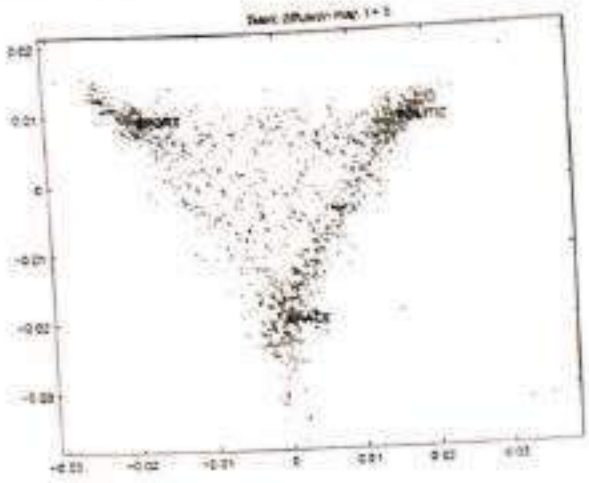


Figure 1 (b) : KDM PCA, or KDM), the LaplacianEigenmap ((e), LE), the Curvilinear Component Analysis

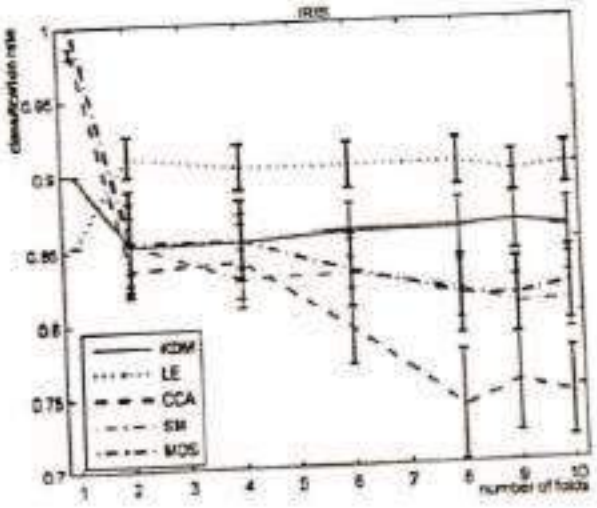
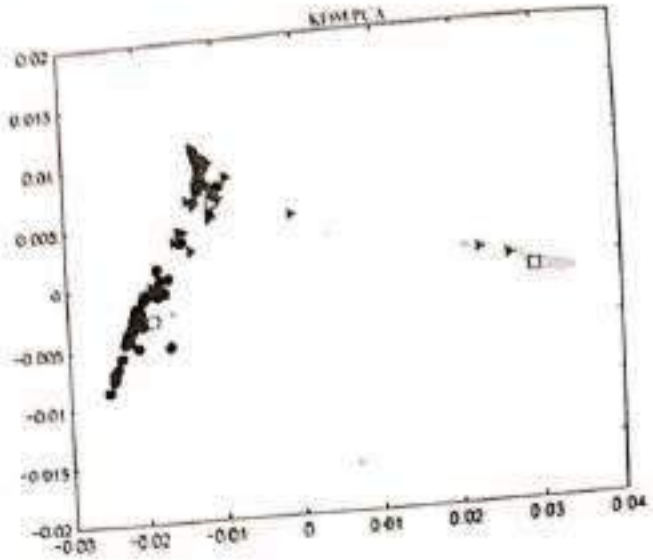


Figure 1(c) : The mapping of 10 percent of the Cora graph (10-folds setting) obtained by the five projection methods



V Conclusion

Let us now come back to our research questions. As a firstobservation, we can say that the two-step procedure(stochastic complementation followed by a diffusion mapprojection) provides an embedding in a low-dimensionalsubspace from which useful information can be extracted.Indeed, the experiments show that highly related elementsare displayed close together while poorly related elementstend to be drawn far apart. This is quite similar tocorrespondence analysis to which the procedure is closelyrelated. Second, it seems that stochastic complementationreasonably preserves proximity information, when combinedwith a diffusion map (KDM PCA) or an ISOMAPprojection (MDS). For the diffusion map, this is normal,since both stochastic complementation and the diffusionmap distance are based on a Markov chain model—stochasticcomplementation is the natural technique allowingto censor states of a Markov chain. On the contrary,stochastic complementation should not be combined witha LaplacianEigenmap, a curvilinear component analysis, ora Sammon nonlinear mapping—the resulting mapping isnot accurate. Finally, the KDM PCA provides exactly thesame results as the basic diffusion map when t is large.However, when the parameter t is low, the resultingprojection tends to highlight the outlier nodes and tomagnify the relative differences between nodes. It istherefore recommended to display a whole range ofmappings for several different values of t.

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DATAANALYSIS PROCEDURE FOR DISCOVERING RELATIONSHIPS IN A GRAPH WITH RESPECT TO NETWORKING

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Abstract - This paper precisely proposes a link-analysis based technique allowing to discover relationships existing between nodes in a computer network or, more generally, a graph. More specifically, this work is based on a random-walk through the database defining a Markov chain having as many states as nodes in the computer network. Suppose, for instance, we are interested in analyzing the relationships between nodes in a computer network, a two-step procedure is developed in analyzing the relationships. First, a much smaller, reduced, Markov chain, only containing the nodes but preserves the main characteristics of the initial chain, is extracted by stochastic complementation. For extracting the reduced Markov by stochastic complementation, an efficient algorithm is proposed. Secondly, the reduced chain is analyzed by, for instance, projecting the states in the subspace spanned by the right eigenvectors of the transition matrix called the basic diffusion map, or by computing a kernel principal-component analysis on a diffusion-map kernel computed from the reduced graph and visualizing the results. Indeed, a valid graph kernel based on the diffusion-map distance, extending the basic diffusion map to directed graphs, is introduced.

Keywords - *Diffusion Map , Stochastic complementation, Feature Redundancy*

I. INTRODUCTION

Wireless sensor networks (WSNs) are being used for diverse applications such as low cost area monitoring, environment monitoring, industrial and machine health monitoring, structural monitoring and military surveillance [1], [2]. In these applications, WSNs generate a large amount of data in the form of streams. In recent times, data mining techniques have been used to extract useful knowledge from WSN data [3], through discovering relationships among the sensor nodes which are known as behavioral patterns [4]. More recently, research has been focused to mine different types of behavioral patterns, e.g., sensor association rules [5], [6], [9] from stored (static) sensor data, context association rules [10] from sensor data stream, associated sensor patterns [7] and regularly frequent sensor patterns [8] from static as well as stream data. Traditional statistical, machine learning, pattern recognition, and data mining approaches [28] usually assume a random sample of independent objects from a single relation. Many of these techniques have gone through the extraction of knowledge from data, almost always leading, in the end, to the classical double-entry tabular format, containing features for a sample of the population. These features are therefore used in order to learn from the sample, provided that it is representative of the population as a whole. However, real-world data coming from many fields such as World Wide Web, marketing, social networks, or biology [16] are often multi relational and interrelated. The work recently performed in statistical relational learning [22], aiming at working with such data sets, incorporates research topics, such as link analysis [36] web mining [1],[9], social network analysis [8], or graph mining[11]. All these research fields intend to find and exploit links between objects which could be of various types and involved in different kinds of relationships. On the other hand, when dealing with a starschema database , this two-step procedure reduces to multiple correspondence analysis. The proposed methodology therefore extends correspondence analysis to the analysis of a relational database. In short, this paper has three main contributions:A two-step procedure for analyzing weighted graphs or relational databases is proposed. .

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