Mohammad Saghir Khan · Almas Zaidi
Javed Musarrat *Editors*

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## Principles and Application of Microphos Technology

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# Preface

The rapidly increasing human population has placed tremendous pressure on agriculture sector to provide sufficient quantity and better quality foods in a more sustainable manner. In order to achieve food security, artificially developed chemicals (fertilizers/pesticides) have been used over the years in energy-intensive agricultural practices to overcome the nutrient deficiencies of the soils and hence to optimize the food production. Even though the synthetic fertilizers among agrochemicals, for example, single super phosphate, when applied in different production systems, have shown promising results, when used excessively and inadvertently, they cause a profound diminishing impact on soil health (soil fertility) and concurrently diminish the viability and productivity of crops. Phosphorus among soil constituents is one of the most important plant nutrients next to nitrogen. Even though there is no deficiency of phosphorus reserves in agronomic soils worldwide, the availability of soluble phosphorus to plants is a major global problem due largely to its rapid fixation and precipitation ability. This, in effect, leads to severe phosphorus deficit in soils. To mitigate such acute phosphatic problems, especially in resource crunch agricultural sector, chemically synthesized fertilizers are applied on a regular basis and at larger scale. Even though the use of artificial fertilizers in agricultural practices has resulted in some better results, their use and misuse has been questioned due in part to its cost and hazardous impact onto natural environment including soil ecosystems. Considering these challenging threats, the interest and awareness have been generated among scientists to uncover some easy-to-operate options. In this regard, in order to accomplish lab-to-land concepts, the naturally abundant yet functionally divergent phosphate-solubilizing microorganisms (PSM) have attracted greater attention of the farm practitioners due to its low cost and easy-to-apply approach. Indeed, PSM offer a practicable alternative to hugely expensive chemical P fertilizers. Application of PSM involving bacteria, fungi, and actinomycetes in agricultural practices has shown some overwhelming results with different crops like legumes, vegetables, and cereal, etc. Apart from providing phosphorus to plants, these organisms also profoundly increase the plant growth by supplying other major plant nutrients like nitrogen via $N_2$ fixation, increasing the availability of plant hormones, absolving the lethal impact of pathogenic microorganisms, and secreting

a few enzymes, etc. Thus, PSM possessing numerous multifunctional plant growth-promoting abilities could be of great practical help to both farmers and students/teachers/scientists across different ecological regions of the world.

**Phosphate-Solubilizing Microorganisms: Principles and Application of Microphos Technology** is an inclusive source of information on numerous useful aspects of phosphate-solubilizing microorganisms which could be applied and practiced for enhancing crop production in distinctly variable agro-ecosystems. This book highlights both fundamental information on the subject and strategies as to how the PSM could be raised to the level of microbial inoculants (microphos), mechanisms, and physiological functions of PSM and factors affecting the growth and phosphate-solubilizing potentials of such microbes. Furthermore, there are separate chapters on the role of phosphate-solubilizing fungi and actinomycetes in the survivability and development of some economically important plants. Discussion on cold-tolerant PSM as elaborated in this book may upgrade and popularize the use of such microbes in enriching the soil P pool and hence increasing the agricultural produce in temperate climatic zones of the world. The ecological diversity and biotechnological implications of PSM and their consequent impact on crops are discussed separately. Special attention is given on to assess the sole/synergistic/additive effects of PSM on some important legumes and cereal crops grown distinctively in different production systems. This book further describes the role of PSM in improving the nutrient uptake and consequently the yield of aerobic rice. The book also highlights a broad and updated view of the management of plant diseases using phosphate-solubilizing microbes. Moreover, the book describes as to how the consortia of plant growth-promoting rhizobacteria other than phosphate solubilizers facilitate the plant growth under stressed environment. The impact of PSM on the growth and development of some notable vegetable crops is also considered and effectively discussed.

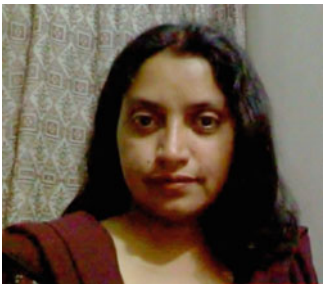
The major aim of **Phosphate-Solubilizing Microorganisms: Principles and Application of Microphos Technology** is to compose scientific information available so far in this area and to make this information available to readers and practitioners in a more meaningful and practical way so that maximum benefits of this technology could be achieved. The book gives an extensive and well-organized scientific coverage in the area of microphos and how the use of microphos technology could be exploited and extended to larger section of the agronomic society in an inexpensive and easy way. This book is likely to be of special interest to the postgraduate students, research scholars, teachers, scientists, and professionals working in the field of microbiology, soil microbiology, biotechnology, agronomy, plant sciences, plant physiology, and plant protection sciences. In addition to gratifying the desires of the academicians/professionals, **Phosphate-Solubilizing Microorganisms: Principles and Application of Microphos Technology** also provides information to the policy makers, inoculant making industries and the people practicing agriculture, and microbial biotechnology across the globe. Each chapter presented herein is contributed by highly experienced academicians/professionals, and attempts have been made to emancipate the quality information and updated knowledge on the subject for ultimate use in academics and/or agriculture practices.

We are very much grateful to our experienced and highly professional scientific colleagues who participated in this endeavor and contributed the state-of-the-art information and balanced scientific knowledge to make this book a reality. Chapters contributed by each scientist/teacher are well structured and involve suitable tables and well-formatted figures. The cooperation extended by our research scholars in designing and weaving the manuscripts presented in this book is deeply acknowledged. We are undeniably very appreciative of our family members who provided their full support and affection during the entire period of this book preparation. Above all, AZ and MSK are extremely thankful to their adorable children, Zainab and Butool, for their patient and helpful attitude all through the book project. Further, we appreciate the great efforts of book publishing team at Springer-Verlag, Switzerland, in responding to all our queries very promptly and earnestly. Finally, if someone finds any typographical mistakes or otherwise in this book, they are requested to inform us so that the mistakes can be corrected and improved in subsequent print/edition. We also invite suggestions and healthy criticism from the readers of this book in order to improve the scientific contents in future print/edition.

Aligarh, India — Mohammad Saghir Khan
Aligarh, India — Almas Zaidi
Riyadh, Saudi Arabia — Javed Musarrat

# The Editors

*Mohammad Saghir Khan*, *Ph.D.*, is an Associate Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. Dr. Khan received his M.Sc. from Aligarh Muslim University, Aligarh, India, and Ph.D. (microbiology) from one of the premier universities, "Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, India." He has been teaching microbiology to postgraduate students for the last 18 years and has research experience of 20 years. In addition to his teaching, Dr. Khan is engaged in guiding students for doctoral degree in microbiology. He has published over 100 scientific papers including original research articles, review articles, and book chapters in various national and international publication media. Dr. Khan has also edited eight books published by the leading publishers. Dr. Khan is deeply involved in research activities focusing mainly on rhizobiology, microbiology, environmental microbiology especially heavy metals–microbes–legume interaction, bioremediation, pesticide–PGPR–plant interaction, biofertilizers, and rhizo-immunology.



*Almas Zaidi* received her M.Sc., M.Phil., and Ph.D. (Agricultural Microbiology) degree from Aligarh Muslim University, Aligarh, India, and is currently serving as Assistant Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. Dr. Zaidi has been teaching microbiology (Ag.) at postgraduate level for the last 10 years and has research experience of 14 years. She has published over 50 research papers, book chapters, and review articles in journals of national and international repute. Dr. Zaidi has edited six books published by the leading publishers. Her main focus of research

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# Chapter 1
# Microphos: Principles, Production and Application Strategies

**Almas Zaidi, Md. Saghir Khan, and Ees Ahmad**

**Abstract** The increasing demand for inexpensive, ecologically sound and environmentally friendly agricultural practices has warranted the use of microbial fertilizers. The preparation from microbial inoculants (biofertilizers) especially the organisms capable of transforming insoluble phosphorus (P) to soluble and available forms is one of the better choices for enhancing crop production by supplying essential nutrients and other growth regulators in different production systems. Furthermore, the critical interactions between microbial communities with soil constituents and plants have provided some novel clues to better exploit them in agricultural practices. Even though the use of microbial preparation in agriculture is an old practice, the production of efficient inoculants expressing consistent performance under field soil is a major obstacle in their extensive and practical application. Therefore, the variations in the performance of microbial inoculants including microphos have greatly hampered their large-scale application. On the other hand, the selection of the technology for inoculant production and modes of their application are key to their success. We highlight here the various strategies employed to produce the phosphatic microbial inoculants (microphos), and how this inoculants can be applied under different agro-ecological niches is discussed and considered.

**Keywords** Phosphate-solubilizing microorganisms • Microphos • Rhizosphere • Phylogenetic tree • Plant growth regulators

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## 1.1 Introduction

The major and most essential macronutrient, phosphorus (P), is required by the plants for vital functions such as cell division, energy transfer, signal transduction, macromolecular formation, nucleic acid synthesis, photosynthesis and respiration, nitrogen fixation and production of oil, sugars and starches (Saber et al. 2005; Zaidi et al. 2009; Eftekhari et al. 2010; Elser 2012). Consequently, acquisition of sufficient concentration of P enhances the growth and development of plants in different production systems (Hayat et al. 2010; Ahemad et al. 2009; Vikram and Hamzehzarghani 2008). However, of the total soil P pool (0.5 %), only 0.1 % is plant available (Scheffer and Schachtschabel 1988) and the remaining soil P is inaccessible to plants (Rodríguez and Fraga 1999). Therefore, the deficiency of P impedes the growth and yields of plants heavily. Such P scarcity in agronomic practices is, however, corrected through the application of synthetic phosphatic fertilizers which indeed is expensive and hazardous. Moreover, greater portion of P applied exogenously to soils is rapidly fixed into soil constituents (Norrish and Rosser 1983; Borling et al. 2001; Hao et al. 2002) and, hence, becomes unavailable to plants. Even though the organic P constitutes a large fraction of P (as much as 50 % in soils), yet it is not directly used up as nutrient unless degraded by soil enzymes. Considering the high cost of chemical phosphatic fertilizers and ability of P to form a complex with soil constituents, it has become imperative to find an inexpensive and viable alternative to chemical P fertilizers. In this regard, the bio-preparation containing viable and sufficient number of efficient phosphate-solubilizing microorganisms (PSM) quite often called as "microphos" has provided some solution to the P problems (Ahemad and Khan 2010; Hui et al. 2011; Xiang et al. 2011; Khan et al. 2013). When applied to seed, plant surfaces or soil, PSM colonize the rhizosphere or the interior of the plant (endophytes) and facilitate growth by providing P to growing plants (Khan et al. 2006). Several PSM inhabiting the soils (Behbahani 2010; Ahemad and Khan 2011a; Marra et al. 2011; Sanjotha et al. 2011; Yadav et al. 2011; Abd El-Fattah et al. 2013; Saxena and Sharma 2007) include bacteria (Khan et al. 2010; Yasmin and Bano 2011; Oves et al. 2013), fungi (Khan et al. 2010) and actinomycetes (Franco-Correa et al. 2010; Kaviyarasi et al. 2011; Balakrishna et al. 2012; Hamdali et al. 2012). Several authors attribute the solubilization of inorganic insoluble P by PSM to the production of organic acids and chelating oxo acids from sugars (Gulati et al. 2009; Khan et al. 2010). Mechanistically, when applied to seeds and soils, PSM facilitates plant development by (i) supplying hugely important nutrients to plants (Sashidhar and Podile 2010); (ii) releasing phytohormones, for example, IAA (Naz et al. 2009; Kavamura et al. 2013), gibberellins (Dey et al. 2004; Cassan et al. 2009) and cytokinin and ABA (Zahir et al. 2004; Cassan et al. 2013); (iii) alleviating the stress induced by ethylene on plants by synthesizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce ethylene level (Ahmad et al. 2012); (iv) producing siderophores for iron sequestration (Roca et al. 2013) and cyanogenic compounds (Ghyselinck et al. 2013); (v) releasing antimicrobial compounds

capable of inhibiting the growth of phytopathogens (Khan et al. 2002; Guo et al. 2004; Saravanakumar et al. 2007; Khan et al. 2009; Sambanthamoorthy et al. 2012); and (vi) providing resistance to drought, salinity, waterlogging and oxidative stress (Alvarez et al. 1996; Stajner et al. 1997; Saleem et al. 2007). Therefore, the use of microphos in crop production is considered as an environment-friendly alternative to further applications of mineral P fertilizers. However, in order to produce microphos, the organisms with P-solubilizing ability must be isolated and characterized. Subsequently, the microphos are tested both under pot soil or field environment prior to their transfer to the practitioner/farmers for application in agricultural practices.

## 1.2 Rationale for Using Microphos in Sustainable Agriculture

In contemporary agricultural practices, millions of tons of agrochemicals including P fertilizers are frequently but indiscriminately used to achieve optimum crop yields. Such synthetic chemicals are, however, not completely used up by plants and, hence, persist in different forms in soil. From here, they leach deep into the grounds and disrupt the composition and functions of beneficial rhizosphere microorganism (Ai et al. 2012), soil matrix (Ai et al. 2013; Lemanski and Scheu 2014) and via food chain, the human health (Ayala and Rao 2002). Furthermore, the chemical fertilizers are used either alone, for example, single super phosphate (Maheshwari et al. 2011), or as mixture (Malhi et al. 2007), for example, diammonium phosphate (DAP), for enhancing crop production in different soil ecosystems. The excessive use of agrochemicals is, however, posing some serious threats to the very sustainability of the environments and is being considered as one of the major problems around the world. So, due to the alarmingly very high costs of fertilizers and some acute environmental hazards associated with the use of synthetic fertilizers (López-Bellido et al. 2013), it has become increasingly important to find some low-cost alternative like the use of renewable resources which could both be inexpensive and could minimize the environmental threats (Bashan 1998; Vessey 2003; Adesemoye and Kloepper 2009). In this context, the discovery of plant growth-promoting rhizobacteria (Kloepper et al. 1986; Ahemad and Khan 2011b; Ahmad et al. 2013; Oves et al. 2013) and the preparation from PSM (microphos) have provided some relief to the poor agronomic practitioners largely due to: (i) low-cost technology with a high cost-benefit ratio, (ii) easy and abundant availability of PSM, (iii) enhances plant growth and crop yields through increased P supply and other growth regulators, (iv) reduces the environmental pollution caused from the manufacturing of the fertilizers and chemicals used, (v) improves soil health and conditioning, (vii) protects plants from pathogens damage and (viii) helps plant to grow under stressed conditions. Therefore, the discovery of PSM and, hence, the production of microphos have attracted greater attention of agronomists

than microbiologists in recent times because they can reduce/minimize the dependence on synthetic P fertilizers and, hence, can protect soil from chemical toxicity. During the last couple of decades, there has been some practical progress in this direction where some new and functionally exciting/novel PS microbes have been identified and used for enhancing agriculture productivity in a more sustainable manner (Khan et al. 2007, 2010).

## 1.3 Rhizosphere and PSM Colonization

Heterogeneously distributed microbial communities play an important role in the acquisition and transfer of various nutrients in soil. For P, soil microorganisms are involved in a range of processes that affect P transformation and thus influence the subsequent availability of P (as phosphate) to plant roots. The rhizosphere indeed is the narrow region of soil that is directly influenced by root secretions (Sørensen 1997) and associated soil microorganisms (Fig. 1.1) and plays some critical roles in plant growth and consequently in soil fertility (Avis et al. 2008). According to Bringhurst et al. (2001), the rhizosphere includes the region of soil bound by plant roots, often extending a few mm from the root surface. This region of soil is much richer in bacteria than the surrounding bulk soil (Hiltner 1904). In soil, microbes are often limited by energy, and hence, root exudates such as organic acids, sugars and amino acids provide energy to them and stimulate their growth and metabolic activities which in turn influence biogeochemical cycling of nutrients in soils (Cardoso and Freitas 1992; Stevenson and Cole 1999; Fontaine and Barot 2005). Studies based on molecular techniques have estimated more than 4,000 microbial species per gram of soil (Montesinos 2003). Of these, about $10^7$–$10^9$ colony-forming units of culturable bacteria have been found in per gram of rhizosphere soil (Benizri et al. 2001), whereas the population densities in the rhizoplane have been reported to range from $10^5$ to $10^7$ colony-forming units per gram of fresh weight (Benizri et al. 2001; Bais et al. 2006). Furthermore, the microbial populations first colonize the rhizosphere following soil inoculation (Gamalero et al. 2003) as shown by many techniques like microscopic tools, immuno-markers or by fluorescence in situ hybridization (FISH) and by using gnotobiotic conditions. Following colonization, bacterial cells are visualized as single cells attached to the root surfaces and subsequently as doublets on the rhizodermis, forming a string of bacteria (Hanson et al. 2000). From here onwards, the whole surface of some rhizodermal cells are colonized, and bacteria can establish even as microcolonies or biofilms (Benizri et al. 2001). In a similar manner, rhizoplane colonization has been studied using both in vitro-grown plants and plants grown in natural soil inhabiting a high microbial diversity. In order to provide benefits to plants, such microorganisms (inoculated one/natural inhabitants of soils) thus must be rhizosphere and/or rhizoplane competent (Elliot and Lynch 1984; Compant et al. 2005) for an extended period of times (Whipps 2001). Many factors can be involved in rhizosphere and rhizoplane competence by PGPB (Albareda et al. 2006). However,

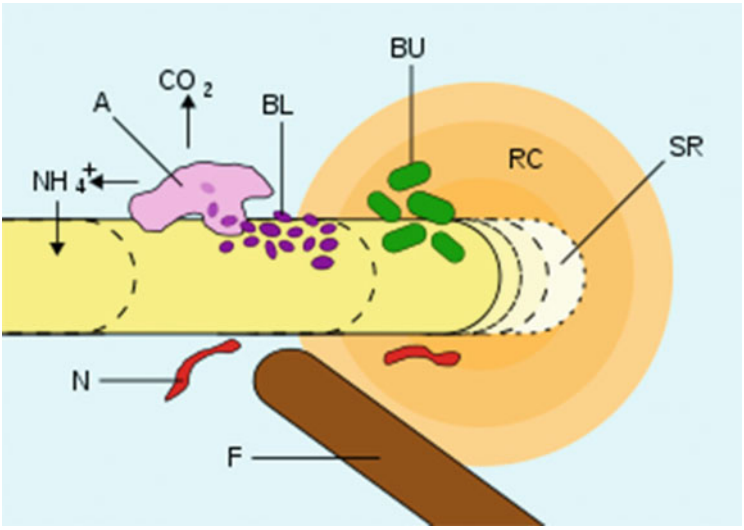


**Fig. 1.1** An illustration of the rhizosphere. *A* amoeba-consuming bacteria, *BL* energy-limited bacteria, *BU* non-energy-limited bacteria, *RC* root-derived carbon, *SR* sloughed root hair cells, *F* fungal hyphae, *N* nematode worm (adapted from http://en.wikipedia.org./wiki/rhizosphere)

the competence of bacteria varies among different rhizospheres/rhizoplane (Gamalero et al. 2003) which has been described to be linked to root exudation (Lugtenberg et al. 2001). For instance, carbon fixed by plant photosynthesis is known to be partly translocated into the root zone and released as root exudates (Bais et al. 2006). Moreover, various carbohydrates, amino acids, organic acids and other compounds, which provide a source of nutrients for root-associated bacteria, are released in the rhizosphere (Walker et al. 2003). Such exudates act as chemoattractants towards which the bacterial population moves and in effect allow them to colonize and multiply in both the rhizosphere and the rhizoplane (Lugtenberg and Kamilova 2009). Plant exudates thus provide a rich source of energy and nutrients for the bacteria in rhizosphere, resulting in more microbial populations in the region than outside the region (Haas and Defago 2005). The colonization of plant rhizosphere by *Bacillus subtilis* sp. and *Pseudomonas* sp. has been well studied (Trivedi et al. 2005; Steenhoudt and Vanderleyden 2000). Rhizobacteria may depend on other microbes for nutrient sources as one microbe may convert plant exudates into a form that can be used by another microbe. Thus, rhizosphere has appeared as a versatile and dynamic ecological environment of intense plant–microbe interactions (Mayak et al. 2004) harnessing essential micro- and macronutrients affecting plant growth, although the process of root colonization is under the influence of various parameters such as bacterial traits, root exudates and several other biotic and abiotic factors (Benizri et al. 2002). Broadly, chemotaxis is generally considered to play an important role for successful rhizosphere/rhizoplane colonization (Andrews and Harris 2000; Walsh

et al. 2001). Recently, it has been reported that soil microorganisms, including free-living as well as associative and symbiotic rhizobacteria belonging to the genera *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Proteus*, *Pseudomonas*, *Rhizobium*, *Serratia* and *Xanthomonas* in particular, are the integral parts of rhizosphere biota (Glick 1995; Kaymak 2011) and have shown successful rhizosphere colonization.

## 1.4 Occurrence of Phosphate-Solubilizing Microbes

Soil is a dynamic system that harbours numerous microbial communities, and it is reported that one gram of fertile soil contains $10^1$ to $10^{10}$ bacteria (Hayat et al. 2010). In soil ecosystems, bacteria are found in different forms such as bacilli, spiral and cocci. Of these, the rod-shaped bacilli are common in soil and have been found as prominent P solubilizers among various bacteria (Khan et al. 2010). However, the composition and structure of PSM within soil varies greatly and are influenced largely by the physico-chemical characteristics of soil (Kim et al. 1997; Khan et al. 2007). Besides soils, PSM have also been reported in various rhizosphere soils of different crops, for example, wheat [(*Triticum aestivum*) Rawat et al. 2011; Babana et al. 2013], maize [(*Zea mays*) Ranjan et al. 2013], rice [(*Oryza sativa*) Panhwar et al. 2012], sugar cane (*Saccharum officinarum*), onion [(*Allium cepa*) Ranjan et al. 2013], garlic (*Allium sativum*), betel vine plant [(*Piper betel* L.) Tallapragada and Seshachala 2012], chickpea (*Cicer arietinum* L.), pea (*Pisum sativum*), green gram [*Vigna radiata* (L.) Wilczek)], lentil (*Lens esculentus*), mentha (*Mentha arvensis*), potato (*Solanum tuberosum*), tomato [(*Lycopersicon lycopersicum)* Ranjan et al. 2013], chilli (*Capsicum annuum*), cabbage (*Brassica oleracea* var. *capitata*), mustard (*Brassica campestris*), jasmine (Ranjan et al. 2013), rhizoplane (Compant et al. 2013), phyllosphere (Ryan et al. 2008; Vorholt 2012), rock phosphate deposit area soil (Richardson et al. 2009), marine environment (Zhu et al. 2011) and polluted soils (Luo et al. 2011). Due to variation in PSM populations in different agro-ecological habitat and considering their functional diversity, it has become extremely important to search PSM with varied biological and chemical properties so that the soil microbial diversity, mechanistic basis of nutrient transformation and plant growth promotion by PSM could be revealed.

## 1.5 Production Strategies of Microphos (PSM Inoculants)

The production of efficient microbial inoculants involving P-solubilizing activity (microphos) broadly includes (a) collection of samples and determination of microbial diversity; (b) isolation, screening and selection of PSM from heterogeneous

microbial populations; (c) bioassay of P-solubilizing activity of the microbial strains; (d) characterization and identification of PSM; (e) bioassay of plant growth-promoting activities; (f) selection of suitable carriers, mixing of inocula with selected carriers and development of microbial inoculants; and (g) pot/field trials of prepared microphos before commercial recommendation for agricultural practices.

### *1.5.1 Collection of Samples and Assessment of Microbial Diversity*

The soil samples are collected generally in sterile polythene bags from a depth of 15–12 $cm^2$ from conventional/polluted non-rhizosphere and rhizosphere soils, mixed thoroughly and are used for determining microbial diversity. The total bacterial, fungal, actinomycetal populations, phosphate-solubilizing micro-organisms (PSM) and asymbiotic nitrogen fixers, for example, *Azotobacter*, can be isolated using standard media and microbiological methods (Holt et al. 1994). For this, soil samples are serially diluted in sterile normal saline solutions (NSS), and 100 µl of diluted suspension is spread plated (Buck and Cleverdon 1960) on nutrient agar [g/l:beef extract 3; peptone 5; agar 15; pH 7], Martin's medium [g/l: dextrose 5; potassium dihydrogen orthophosphate 1; magnesium sulphate 0.5; streptomycin 0.006; Rose Bengal 2 part in 3,000 part of medium; 1 g of chloramphenicol/nalidixic acid can be dissolved in 100 ml of sterile water and 0.3 ml of this solution is added to 100 ml of Rose Bengal medium after it is cooled to 45 °C], Kenknight's medium [g/l: dextrose 1; potassium dihydrogen phosphate 0.1; sodium nitrate 0.1; potassium chloride 0.1; magnesium sulphate 1.50] or starch casein agar (SCA) medium [g/l: starch 10; casein 0.3; $KNO_3$ 2; NaCl 2; $K_2HPO_4$ 2; $MgSO_4 \cdot 7H_2O$ 0.05; $CaCO_3$ 0.02; $FeSO_4 \cdot 7H_2O$ 0.01 agar 18; pH 7.2; tetracycline (100 µg/ml) and amphotericin B (50 µg/ml) are added to medium after autoclaving to prevent bacterial growth and fungal growth, respectively (Williams and Davies 1965; Porter and Tresner 1960)], Pikovskaya (Table 1.1) medium, Ashby's medium (Table 1.1) and yeast extract mannitol (YEM) agar medium (Table 1.1) for total bacterial counts, fungal populations, actinomycetes, phosphate solubilizers, *Azotobacter* and rhizobia, respectively.

Each sample should be replicated at least three times and incubated at $28 \pm 2$ °C for 2, 3, 5, 5 and 5 to 7 days for quantifying the populations of bacteria, fungi, actinomycetes, PSM and *Azotobacter*, respectively. Where microbiological assay is not done immediately, the samples are kept in sterile polythene bags and stored at 4 °C for a short period of time. Standard culture medium and growth conditions should be used for isolation and enumeration of microbial populations as given in Table 1.2.

**Table 1.1** Chemical composition of media used for assessment of microbial diversity in soil

| Media component | Amount (g/l) | | | |
|---|---|---|---|---|
| | Pikovskaya medium | NBRIP medium | Ashby's medium | Yeast extract mannitol agar |
| Dextrose | 10.0 | 10.0 | – | – |
| Mannitol | – | – | 20.0 | 10.0 |
| Yeast extract | – | – | – | 1.0 |
| $Ca_3(PO_4)_2$ | 5.0 | 5.0 | – | – |
| $CaCO_3$ | | – | 5.0 | 2.0 |
| $MgCl_2{\cdot}6H_2O$ | 5.0 | 5.0 | – | – |
| $MgSO_4{\cdot}7H_2O$ | 0.25 | 0.25 | 0.2 | 0.2 |
| KCl | 0.2 | 0.2 | – | – |
| $(NH_4)_2SO_4$ | 0.1 | 0.1 | – | – |
| $K_2HPO_4$ | – | – | 0.2 | 0.5 |
| $K_2SO_4$ | – | – | 0.1 | – |
| NaCl | – | – | – | – |
| Bromophenol blue (BPB) | – | 0.025 | – | – |

**Table 1.2** Culture medium and growth conditions used for isolation and enumeration of microbial populations

| Microbes | Medium | Incubation temperature (°C) | pH of medium | Incubation period (days) |
|---|---|---|---|---|
| Bacteria | Nutrient agar | $28 \pm 2$ | $7 \pm 0.2$ | 1–2 |
| Fungi | Martin's agar | $28 \pm 2$ | $7 \pm 0.2$ | 3–5 |
| Actinomycetes | Kenknight's agar | $28 \pm 2$ | $7 \pm 0.2$ | 5–7 |
| PSM | Pikovskaya agar | $28 \pm 2$ | $7 \pm 0.2$ | 5–7 |
| *Azotobacter* spp. | Ashby's agar | $28 \pm 2$ | $7 \pm 0.2$ | 5–7 |
| Rhizobia | YEM agar | $28 \pm 2$ | $7 \pm 0.2$ | 2–5 |

### *1.5.2 Isolation, Screening and Selection of PSM*

Gerretsen (1948) initially demonstrated that microbial activity in the rhizosphere could dissolve sparingly soluble inorganic P and increase plant growth. Subsequently, Pikovskaya (Pikovskaya 1948) devised a medium (Table 1.1) for the isolation and screening of PSM. Later on, a modified Pikovskaya medium using bromophenol blue dye as suggested by Gupta et al. (1994) and National Botanical Research Institute P [NBRIP] medium (Table 1.1) developed by Nautiyal (1999) are used for the isolation and selection of P solubilizers. However, there are conflicting reports on the performance of these media. For example, the bromophenol blue method used to improve the clarity and visibility of the yellow-coloured halo has not necessarily improved the plate assay (Nautiyal 1999). Moreover, the Pikovskaya medium contains yeast extract, and it is desirable

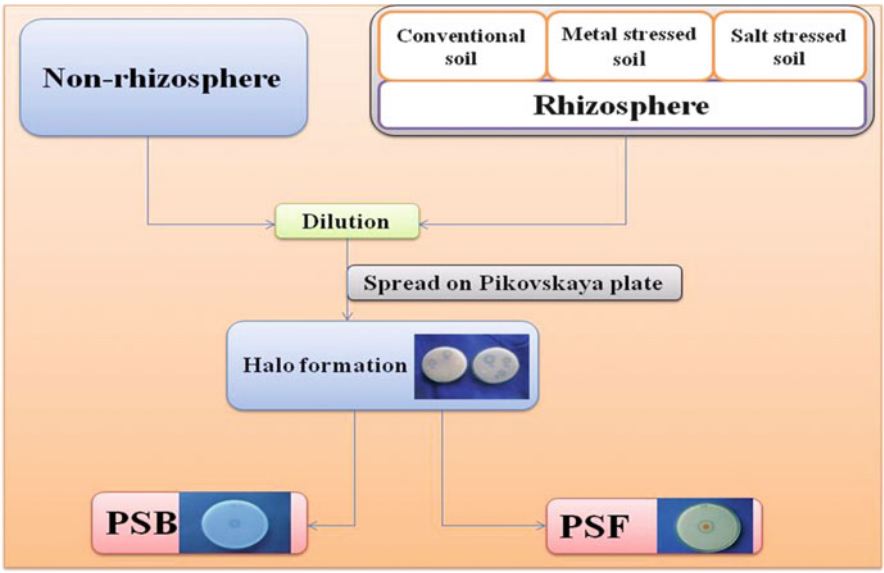


**Fig. 1.2** Isolation and selection of P-solubilizing microorganism from different sources

to formulate a defined medium to elucidate the role of microorganisms in P mineralization. On the contrary, the NBRIP medium has several advantages over other media (Nautiyal 1999). For instance, the NBRIP medium can be used as a defined medium because it excludes the use of yeast extract. Secondly, NBRIP is more efficient in a broth assay compared to the Pikovskaya medium.

Despite the variation in the effectiveness of different media, first of all, PSM is isolated from soils/any source using serial plate dilution method or by enrichment culture technique (Fig. 1.2). The serially diluted rhizospheric or non-rhizospheric soil samples are then spread (100 µl) or streaked or spot (10 µl) inoculated on solid Pikovskaya plates or any plates containing insoluble P (e.g. tricalcium phosphate) and incubated. After proper incubation of inoculated solid Pikovskaya plates for 5–7 days (bacteria) and 3–5 days (fungi and actinomycetes) at $28 \pm 2$ °C, the P-solubilizing microbes are detected by the formation of clear halo around their colonies (Plate 1.1). The development of a clear zone around the colony on the culture plates are taken as an index of P solubilization. However, the reliability of this halo-based technique is questioned as many isolates in other studies did not produce any visible halo/zone on agar plates but could solubilize insoluble inorganic P in the liquid medium (Gupta et al. 1994; Louw and Webley 1959). The phosphate solubilizers are then maintained on medium, for example, Pikovskaya, which is used for PSM isolation until use. Since P-solubilizing organisms exhibit many-fold variations in P-dissolving activity (Khan et al. 2007) and instability with regard to their P-solubilizing activity (Illmer and Schinner 1992), they are repeatedly subcultured to test the persistence of P-solubilizing potential. Once the efficient PSM are selected, they are tested for their ability to solubilize insoluble P

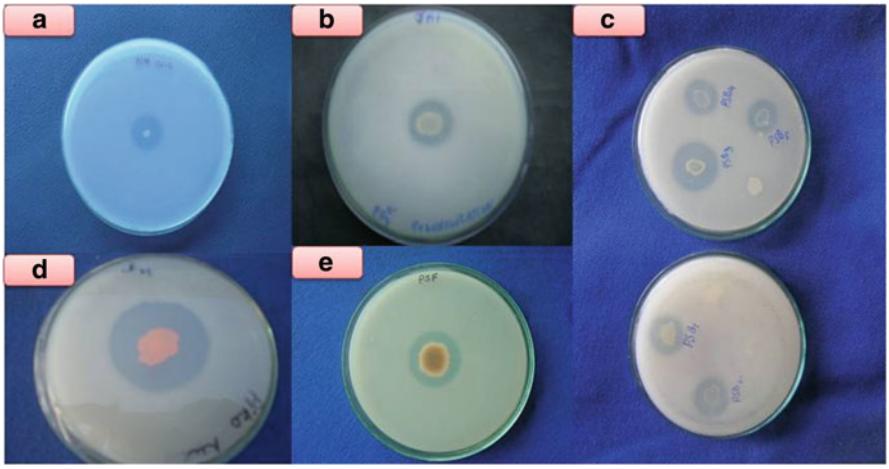


**Plate 1.1** Phosphate solubilization on Pikovskaya plate by some notable P solubilizers. (**a**) *Bacillus*, (**b**) *Azotobacter*, (**c**) *Serratia*, (**d**) Fungi, (**e**) *Pseudomonas* sp.

under liquid culture medium. Finally, the efficient P-solubilizing organisms are selected and used for the development of inoculants whose performance is tested under pot/field environments against various crops of economic importance.

### *1.5.3 Bioassay of P-Solubilizing Activity*

The microbial strains expressing PS activity during screening process are further enriched by inoculating into the Pikovskaya medium, incubated at $28 \pm 2$ °C for 7 days and then observed on solid plates for halo formation. The solubilization index (SI) and solubilizing efficiency (SE) of such microbes are calculated by the formula suggested by Premono et al. (1996) as

$$\text{Solubilization Index (SI)} = \left(\text{colony diameter} + \text{zone of halo}\right)/\text{colony diameter}$$
$$\text{Solubilizing Efficiency (SE)} = \left(\text{zone of halo}/\text{colony diameter}\right) \times 100$$

The colonies forming clear halo around microbial growth indicating P solubilization are counted and further used to determine the relative P-solubilizing efficiency [RPSE] in liquid Pikovskaya medium. The clear halo around bacterial growth is measured, and cultures are further used to determine the extent of P solubilization in liquid Pikovskaya medium. For quantitative measurement, 100 ml of Pikovskaya broth containing 5 g TCP is inoculated with 1 ml of $10^8$ cells/ml of each culture. The flasks are incubated for 5, 10 and 15 days with shaking at 120 rpm at $28 \pm 2$ °C. A 20 ml culture broth from each flask is removed and centrifuged ($9{,}000 \times g$) for 30 min, and the amount of water-soluble P released into the

supernatant is estimated by the chlorostannous-reduced molybdophosphoric acid blue method (King 1932; Jackson 1967). To 10 ml of supernatant, 10 ml chloromolybdic acid (ammonium molybdate 15 g; distilled water 400 ml and 10 N HCl 400 ml. These materials are mixed slowly with rapid stirring and cooled, and the volume is made to one litre with distilled water) and 5 drops of chlorostannous acid (stannous chloride 10 g; concentrated HCl 25 ml; the stock solution is kept in airtight bottle and one ml of stock solution is mixed in 132 ml of distilled water at the time of experiment) are added, and the volume is adjusted to 50 ml with distilled water. The absorbance of blue colour developed is read at 600 nm. The amount of P solubilizer is calculated using the calibration curve of $KH_2PO_4$. The change in pH following TCP solubilization is also recorded. Each independent experiment should be repeated three times after several subcultures to ensure the reproducibility of the results. Solubilization index and SE of the bacterial isolates showing greater solubilization on both solid and liquid media and persistence of PS activity after several subcultures are the criteria for the selection of efficient PS strains for further studies.

### *1.5.4 Microbiological and Biochemical Characterization of PSM*

The phosphate solubilizers are identified firstly by microbiological and biochemical tests. The microbiological tests may include the assessment of colonial morphology [shape, margin (serrated or smooth)], colour and characteristics such as the secretion of watery or mucoid/gummy substances from colonies, Gram reaction and shape of microbes. The biochemical reaction may involve indole reaction, citrate utilization, methyl red test, Voges–Proskauer, catalase, oxidase test, starch, gelatin, lipid hydrolysis, mannitol salt utilization test and sugar fermentation test, etc. The resulting characteristics are compared with those given in *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994), and strains are identified to generic level only.

#### 1.5.4.1 Antibiotic Sensitivity Behaviour of Isolated Cultures

Antibiotic sensitivity behaviour of the isolated P solubilizers is determined using the antibiotic discs of known potency by disc diffusion method of Bauer (1966) in order to find antibiotic markers for the PSM strains. For this, freshly prepared and autoclaved nutrient broth is inoculated by isolated bacterial cultures and incubated for 24 h at 28 ± 2 °C. 100 µl of overnight grown test culture is taken on nutrient agar plates and is evenly spread with sterile glass rod spreader. Plates are then mounted with individual antibiotic (e.g. amoxicillin, chloramphenicol, ciprofloxacin, cloxacillin, nalidixic acid, nitrofurantoin, norfloxacin, novobiocin, doxycycline

hydrochloride, erythromycin, etc.) disc using a sterile forceps. Each antibiotic-mounted plate is incubated at $28 \pm 2$ °C for 24–48 h. After incubation, the zone of inhibition is measured, and the strains are scored as resistant (R) and susceptible (S). Following the standard antibiotic disc sensitivity testing method (Margalejo et al. 1984), the plates are recorded for comparing the zone of inhibition (diameter in mm) with chart provided by the disc manufacturers.

#### 1.5.4.2 Identification of Phosphate-Solubilizing Organisms

Microbial cultures showing greater P-solubilizing activity in vitro, when grown on Pikovskaya medium, and exhibiting optimum solubilization of insoluble P in liquid culture medium are selected and presumptively identified to the genus level using morphological and biochemical test. Such organisms are then identified to the species level using whole-cell fatty acid methyl ester (FAME) profile and 16S rDNA sequence analysis (Chung et al. 2005; Chen et al. 2006). For 16S rDNA sequence analysis, partial 16S rRNA gene sequences of selected strains are done using universal primers, 518 F (5′CCAGCAGCCGCGGTAATACG3′) and 800R (5′TACCAGGGTATCTAATCC3′). All nucleotide sequence data should then be deposited in the public domain (e.g. GenBank sequence database). There are various agencies which are providing molecular sequencing for identifying bacterial cultures to species level, for example, Macrogen Inc., Seoul, South Korea. The online programme BLASTn is then used to find related sequences with known taxonomic information in the databank at the NCBI website (http://www.ncbi.nml.nih.gov/BLAST) to accurately identify and compare the isolates with nearest neighbour sequence available in the NCBI database.

#### 1.5.4.3 Construction of Phylogenetic Tree

The sequence obtained from nucleotide-sequencing agencies is initially estimated by the BLASTn online programme facility of NCBI (http://www.ncbi.nml.nih.gov/BLAST) and then aligned with all related sequences obtained from GenBank by ClustalW (Thompson 1994). Phylogenetic tree is then reconstructed by neighbour-joining method (Saitou and Nei 1987). Bootstrapped neighbour-joining relationships are estimated with MEGA4 software (Tamura et al. 2007).

### *1.5.5 Bioassay of Plant Growth-Promoting Activities of PS Bacteria*

#### 1.5.5.1 Screening for 1-Aminocyclopropane-1-Carboxylate (ACC) Deaminase Activity

Using the spot inoculation method, 5 µl of each isolated PS bacterium is placed on a section of plate (marked in 16 equal parts) containing DF (Dworkin and Foster 1958) salt minimal medium [g/l: $KH_2PO_4$ 4; $Na_2HPO_4$ 6, $MgSO_4{\cdot}7H_2O$ 0.2, glucose 2.0, gluconic acid 2.0; citric acid 2.0; trace elements, 1 mg $FeSO_4{\cdot}7H_2O$, 10 µg $H_3BO_3$, 11.19 µg $MnSO_4{\cdot}H_2O$, 124.6 µg $ZnSO_4{\cdot}7H_2O$, 78.22 µg $CuSO_4{\cdot}5H_2O$, 10 µg $MoO_3$, pH 7.2 and 2.0 g $(NH_4)_2SO_4$ as nitrogen source] supplemented with three mM ACC instead of [$(NH_4)_2SO_4$)] and incubated at $28 \pm 2$ °C for 72 h. The bacterial growth should be checked daily as suggested by Penrose and Glick (2003). At least one ACC deaminase-positive bacterial strain should be used as a control in this type of study (Nascimento et al. 2011), and all the samples should be tested in duplicate, and experiments must be repeated at least three times to ensure the reproducibility of the results.

Quantitative Assay of ACC Deaminase Activity

The ACC deaminase activity of P solubilizers (Ahmad et al. 2013) can be assayed following the method of Honma and Shimomura (1978) later modified by Penrose and Glick (2003). According to this method, the amount of α-ketobutyrate is measured which is produced by reaction of the enzyme ACC deaminase which cleaves ACC to α-ketobutyrate and $NH_3$. The number of mmol of α-ketobutyrate produced by this reaction is determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1 mmol. A stock solution of 100 mM α-ketobutyrate (Sigma-Aldrich) is prepared in 0.1 M Tris–HCl, pH 8.5, and stored at 4 °C. Just prior to use, the stock solution is diluted with the same buffer to make a 10 mM solution from which a standard concentration curve is generated. Each in a series of known α-ketobutyrate concentrations is prepared in a volume of 200 ml, 300 ml of the 2,4-dinitrophenylhydrazine reagent (0.2 % 2,4-dinitrophenylhydrazine in 2 M HCl) (Sigma-Aldrich) is added and the contents are vortexed and incubated at 30 °C for 30 min during which time the α-ketobutyrate is derivatized as a phenylhydrazone. The colour of the phenylhydrazone is developed by the addition of two ml 2 M NaOH; after mixing, the absorbance of the mixture is measured at 540 nm. Using this method, the ACC deaminase activity can be measured in bacterial extracts prepared in the following manner. The ACC deaminase-positive bacterial strains, for example, P solubilizers ($10^8$ cells/ml) are inoculated in Luria–Bertani broth (g/l: tryptone 10; yeast extract 5; NaCl 10; pH 7.5) and incubated in a shaking incubator at 200 rpm for 24–48 h at $28 \pm 2$ °C. Then, cultures are centrifuged at $8{,}000 \times g$ for 10 min at 4 °C, and the

biomass of P solubilizers is harvested. The supernatant is removed, and the cells are washed with 5 ml DF salts minimal medium. Following an additional centrifugation for 10 min at 8,000 × *g* at 4 °C, the cells are resuspended in 7.5 ml DF salts minimal medium in a fresh culture tube. Just prior to incubation, the frozen 0.5 M ACC solution is thawed, and an aliquot of 45 ml is added to the cell suspension to obtain a final ACC concentration of 3.0 mM. The bacterial cells are re-shaken in the incubator to induce the activity of ACC deaminase at 200 rpm for 24 h at the same temperature as is done for overnight-incubated cultures. The bacteria cultures are harvested by centrifugation at 8,000 × *g* for 10 min at 4 °C. The supernatant is removed, and the cells are washed by resuspending the cell pellets in 5 ml 0.1 M Tris–HCl at pH 7. Each bacterial cell pellet, prepared as described above are suspended in 1 ml of 0.1 M Tris–HCl, pH7.6 and transferred to a 1.5-ml micro-centrifuge tube. The contents of the 1.5-ml micro-centrifuge tube are spun at 16,000 × *g* for 5 min, and the supernatant is removed. The pellet is suspended in 600 ml of 0.1 M Tris–HCl, pH 8.5. A 30 µl of toluene is added to the cell suspension and vortexed at the highest setting for 30 s. At this point, a 100-ml aliquot of the "toluenized cells" is set aside and stored at 4 °C for protein assay by Lowery et al. (1951) method at a later time. The remaining toluenized cell suspension is immediately assayed for ACC deaminase activity. All sample measurements should be carried out in duplicate. 200 µl of the toluenized cells are placed in a fresh 1.5-ml micro-centrifuge tube; 20 ml of 0.5 M ACC is added to the suspension, briefly vortexed and then incubated at 30 °C for 15 min. Following the addition of 1 ml of 0.56 M HCl, the mixture is vortexed and centrifuged for 5 min at 16,000 × *g* at room temperature. One ml of the supernatant is vortexed together with 800 ml of 0.56 M HCl. Thereupon, 300 ml of the 2,4-dinitrophenylhydrazine reagent (0.2 % 2,4-dinitrophenylhydrazine in 2 M HCl) is added to the glass tube; the contents are vortexed and then incubated at 30 °C for 30 min. Following the addition and mixing of 2 ml of 2 N NaOH, the absorbance of the mixture is measured at 540 nm.

#### 1.5.5.2 Quantitative Assay of Indole Acetic Acid

Indole-3-acetic acid (IAA) synthesized by P solubilizers (Wani and Khan 2010; Ahemad and Khan 2012) is quantitatively evaluated by the method of Gordon and Weber (1951), later modified by Brick et al. (1991). For this, the PS bacterial strains are grown in Luria–Bertani (LB) broth. Luria–Bertani broth (100 ml) having 0, 50, 100, 200, 400 and 500 µg/ml tryptophan is then inoculated with 1 ml culture ($10^8$ cells/ml) of PS cultures and incubated for 3, 6, 9 and 12 days at 28 ± 2 °C with shaking at 125 rpm. After incubation, 5 ml of culture of each treatment is spun (9,000 × *g*) for 15 min, and an aliquot of 2-ml supernatant is mixed with 100 µl of orthophosphoric acid and 4 ml of Salkowski' reagent (2 % 0.5 M $FeCl_3$ in 35 % perchloric acid) and incubated at 28 ± 2 °C in darkness for 1 h. The absorbance of developed pink colour is read at 530 nm. The IAA concentration in the supernatant is determined using a calibration curve of pure IAA as a standard. The experiment should be repeated three times on different time intervals.

#### 1.5.5.3 Qualitative and Quantitative Estimation of Siderophores

The PS bacterial strains are further tested for siderophore production using Chrome Azurol S (CAS) agar medium following the method of Alexander and Zuberer (1991). Chrome Azurol S (CAS) agar medium is prepared from four solutions as (i) *Solution 1*, Fe-CAS indicator solution: A 10 ml of 1 mM $FeCl_3 \cdot 6H_2O$ [in 10 Mm HCl} is mixed with 50 ml of an aqueous solution of CAS (1.21 mg/ml). The above solution is then added to 40 ml of HDTMA (1.82 mg/ml) and cooled to 50 °C. (ii) *Solution 2*, buffer solution: A 30.24 g of PIPES is dissolved in 750 ml of a salt solution containing 0.3 g $KH_2PO_4$, 0.5 g NaCl and 1 g $NH_4Cl$, pH 6.8, with 50 % KOH, and water is added to bring the volume to 800 ml. (iii) *Solution 3*: (in 70 ml water) 2 g glucose, 2 g mannitol, 493 mg $MgSO_4$. $7H_2O$, 11 mg $CaCl_2$, 1.17 mg $MnSO_4$. $H_2O$, 1.4 mg $H_3BO_3$, 0.04 mg $CuSO_4 \cdot 5H_2O$, 1.2 mg $ZnSO_4$. $7H_2O$ and 1 mg $Na_2MoO_4 \cdot 2H_2O$. Autoclaved, cooled to 50 °C, then added to the buffer solution along with 30-ml filter-sterilized 10 % (W:V) casamino acids (Solution 4). The indicator solution is added last with sufficient stirring to mix the ingredients without forming bubbles. Chrome Azurol S agar plates are then prepared separately and divided into equal sectors and spot inoculated with 10 µl of $10^8$ cells/ml and incubated at $28 \pm 2$ °C for 5 days. Development of yellow-orange halo around the bacterial growth is considered as positive for siderophore synthesis. Each individual experiment should be repeated three times to ensure the reproducibility of results. The production of siderophore by the PSB strains are further detected quantitatively using Modi medium ($K_2$ $HPO_4$ 0.05 %; $MgSO_4$ 0.04 %; NaCl 0.01 %; mannitol 1 %; glutamine 0.1 %; $NH_4NO_3$ 0.1 %). Modi medium is inoculated with $10^8$ cells/ml of PSB and incubated at $28 \pm 2$ °C for 5 days. Catechol-type phenolates are measured on ethyl acetate extracts of the culture supernatant using a modification of the ferric chloride–ferrocyanide reagent of Hathway. Ethyl acetate extracts is prepared by extracting 20 ml of supernatant twice with an equal volume of solvent at pH 2. Hathway's reagent is prepared by adding 1 ml of 0.1 M ferric chloride in 0.1 N HCl to 100 ml of distilled water, and to this, 1 ml of 0.1 M potassium ferrocyanide is added (Reeves et al. 1983). For the assay, one volume of the reagent is added to one volume of sample, and absorbance is determined at 560 nm for salicylates with sodium salicylate as standard and at 700 nm for dihydroxy phenols with 2, 3-dihydroxy benzoic acid (DHBA) as standard.

#### 1.5.5.4 Assay of Hydrogen Cyanide and Ammonia

Hydrogen cyanide (HCN) production by PS cultures is detected by the method of Bakker and Schipper (1987). For HCN production, PS bacterial strains are grown on an HCN induction medium (g/l: tryptic soy broth 30; glycine 4.4; agar 15) for 3–4 days at $28 \pm 2$ °C. For each bacterial strain, a 100 µl of $10^8$ cells/ml is spread on the Petri plates. A disc of Whatman filter paper no. 1 dipped in 0.5 % picric acid and 2 % $Na_2CO_3$ is placed at the lid of the Petri plates. The plates are then sealed with