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CONTENTS

S. No.	Particulars	Page No.
1	**Viral Crusaders and Vaccine for HIV** P. Rajendran …………………………………………………………..	3
2	**Plant Growth Promoting Rhizobacteria for Biocontrol of phytopathogens and Yield Enhancement of *Phaseolus vulgaris* L.** Pankay Kumar and R.C. Dubey…………..………………………….....	8
3	**Microbial Diversity in Coastal Mangrove Ecosystems** K. Kathiresan and R. Balagurunathan ………………………………....	40
4	**Studies on Cadmium Biosorption by Cadmium Resistant Bacterial Strains from Uppanar estuary, Cuddalore, Southeast Coast of India** K. Mathivanan and R. Rajaram ……………………………………	48
5	**Isolation and Characterization of Plant Growth Promoting Rhizobacteria (PGPR) from Vermistabilized Organic Substrates and their Effect on Plant Growth** M. Prakash, V. Sathishkumar, T. Sivakami and N. Karmegam ………	54
6	**Isolation of Thermo Tolerant *Escherichia coli* from Drinking Water of Namakkal District and Their Multiple Drug Resistance** K. Srinivasan, C. Mohanasundari and K. Rasna	63
7	**Isolation and Screening of Extremophilic Actinomycetes from Alkaline Soil for the Biosynthesis of Silver Nanoparticles** Vidhya and R. Balagurunathan ………………………………………	68
8	**BCL-2 Expression in Systemic *Lupus Erythematosus* Cases in Chennai, Tamil Nadu** M. Karthikeyan, P. Rajendran, S. Anandan, G. Ashok, S. Anbalagan, R. Nivedha,S. Melani Rajendran and Porkodi ………………………...	71

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Viral Crusaders and Vaccine for HIV

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INTRODUCTION

Evolution of HIV virus is a 'Concept of Silence'. There is no clear cut record of its origin unlike many other viruses whose emergence is well documented with details of their origin. According to some of the scientists the virus must have probably evolved way back in 1930s. However the known HIV sero-positive case could be traced to a preserved serum sample collected from Congo some time in 1959, giving us a hint that the virus evolved, simmered, primed in jungle of Congo in Chimpanzees and then jumped on to human who hunted Chimpanzees for meat purpose. From Congo the virus traveled to Haiti and from Haiti the virus reached the already immune-deficient gay population in New York and San Francisco. The earlier cases were initially identified as *Pneumocystis carinii* pneumonia or Kaposi sarcoma in 1981. The causative retrovirus called *Lymphadenopathy Associated Virus* (LAV) was identified first by Dr. Luc Montagnier in 1983 at Pasteur Institute, France and later in 1984 Dr. Robert Gallo reported isolation of a virus *Human T-cell Lymphotropic virus* type III (HTLV-III) at National cancer Institute, Bethesda, USA and both of which were renamed as Human Immunodeficiency virus type 1 (HIV-1). Ironically only Dr.LucMontagnier and Dr.Francoise sinoussi were given Nobel Prize for the discovery of HIV virus causing AIDS in human and not Dr.Robert Galo.

Subsequently the virus traveled to the rest of the world, culminating in a dreaded pandemic on par with the pandemic of Small pox and Plague. Today millions of people are living with HIV / AIDS and millions were already dead. Adding to the misery, many innocent children born to the AIDS parents are left as orphan. Although scientist and pharmaceutical companies have discovered many ARV drugs and HAART therapy, none of them could eliminate the virus, rather they either reduce the viral load or reduce the morbidity considerably.

For many the psychological feeling is that the HIV (AIDS) is a curse. The scientific fraternity has failed to stop, eliminate and eradicate the HIV from earth, despite the high scientific and technological knowledge. On the other hand Dr. Edward Jenner's vaccine was successful in eradicating small pox (more dangerous than HIV) with a simple principle of "similar things (virus) will cure similar diseases". The discovery of small pox vaccine in 1798 opened the Pandora box of vaccinology to the world.

Why do we need (HIV) AIDS vaccine? Is it that existing antiviral agents are not enough to eliminate HIV? A combination of awareness programs, promoting preventive measures, use of microbicides and use of antiviral agents should have facilitated the sharp decline of HIV around the world. On the other hand even today about 6800 are newly infected with HIV every day and 5700 die of AIDS (Global summary of AIDS epidemic, 2007) and in this rate HIV circulation will never halt. Secondly cost effectiveness of anti HIV drugs and their side effects and development of drug resistance, failure to implement safe sex, failure to protect children born to HIV infected mothers and failure to provide post exposure prophylaxis are the facts which pose great challenge to the viral crusaders and that is why we need a protective vaccine for HIV.

Apart from the above said hurdles there are other inborn difficulties in preparing vaccines due to structural configuration of the virus *per se*. For example in GP 120 antigenic structure, the CD4 epitome binding site is protected by variable loop and sugar bond and therefore any vaccine targeted for GP 120

binding to CD4 may not prevent the binding at all (Burton et al., 2004). Similarly the other targets for HIV such as Reverse Transcriptase, Protease and Integrase have their own integral protective components which are responsible for the ineffectiveness of various vaccine approaches.

Adding to the misery speculations include whether to prepare a vaccine for HIV-1(Originated from 'SIVcpz' Chimpanzee) or HIV-2 (Originated from "SIV sm' Soothy Mangabey monkey) or the latest addition 'SIVg (Originated from Gorilla in Cameroon). Evidently the most ideal one is the vaccine from HIV-1 than others. More over the HIV-1 clusters are variable for Africa, Asia, America and Europe. The prevalence of sub types among various groups is different and there is no uniform distribution of subtype 'M' around the world. For example in Asia HIV-1 Group M subtype 'C' is most common in whereas subtype 'B' is common in North America. In addition to the common subtype existing in a particular country various other subtypes may also be prevalent in a small percentage. Eventually it is not possible to prepare a Universal vaccine with specific subtype of HIV-1. Also we need two types of vaccines, one for therapeutic and another one for protective. We have the following types of vaccines tested either in monkeys or in human volunteers.

VACCINES

Sub Unit Vaccine: Sub unit vaccines are prepared against individual peptide of gp160, gp 120 and gp 41 which can stimulate neutralizing antibody but may not stimulate CMI response. Although this type of vaccine is safe this is not successful because it is poorly antigenic and it induces only one type of antibody against a viral peptide.

Whole Cell Killed Vaccine: Killed vaccines prepared from SIV (Simian Immunodeficiency Virus) were tested against SIV negative monkeys which did not induce antibody, mucosal immunity and T cell response (Learmont et al., 1992; Learmont et al., 1995). More over there may be residual infectivity due to incomplete inactivation in some batches of vaccine as it happened in Salk vaccine before.

Live Attenuated Vaccine: Live attenuated virus vaccines initially appeared to be successful in preventing experimental challenge in non-human primates. However mutants arising out of these vaccines are pathogenic to humans. If the virus carries deletion in both 'nef' and 'long terminal repeat' then late immune suppression will occur (Learmont et al., 1992; Learmont et al., 1995). Any living attenuated vaccine is always looked upon suspiciously for possible mutation from a non-virulent to virulent form as it happened in live attenuated polio vaccine.

Vector Vaccine: The viruses which can resemble HIV but not harmful (Adeno Associated Virus, Bacteriophage Lambda, Canary pox, Herpes, Vaccinia etc., collectively called as 'Trojan horse viruses") can carry certain genes of HIV which when introduced to the host can stimulate anti-HIV immune response. This type of vaccines induced moderate protection but not completely reliable. Most studied of these are Vaccinia Ankara and the other one is Vaccinia New York strains. Canary pox vector is better than Vaccinia vector. But Adenovirus is the most preferred vector.

DNA Vaccine: These vaccines contain most of HIV protein and copies of small numbers of HIV genes. This type of vaccine induce wide spectrum of activation of Langerhan's cells, activation of near by Lymph nodes and activation of HIV specific killer cells. However there are speculations that the DNA may integrate to the host cell genome and might produce future complications.

Combined Vaccines: In this type of vaccine, scientists use any two vaccines, one for priming and another one for booster. For example in India, Tuberculosis Research Centre-Chennai (ICMR) tried on Phase I Prime Booster combined vaccine trial. In this vaccine the prime dose was Adeno Associated Virus with gag, protease and Reverse Transcriptase genes and the booster dose contained Modified Vaccinia virus Ankara with gag, protease, RT, ref, tat and pol genes. This trial result showed safety and moderate immune response. However the MVA is not a stable one.

Pseudo-Virion Vaccine: In this vaccine, a non infectious HIV virus was used to carry few important genes of Infectious HIV-1 which gave a moderate protection in infected monkeys.

Emery Vaccine: This is a three dose vaccine where in Professor D. Mrs. Harrier L. Robinson of Emery University experimented in 2000 monkeys with I & II doses of DNA priming vaccine and 3rd dose of recombinant vaccine (r MVA-Modified Ankara) after specific time gap and finally challenged with virulent (SHIV : combined SIV+HIV) in rectal mucosa. Although none of the monkeys developed AIDS like disease it is yet to be tried in human volunteers.

STEP: Merck Vaccine: Among all the vaccine trials, the much talked about vaccine is the STEP vaccine. In this vaccine they used Adenovirus type 5 (Ad5 is a mixture of 4 Adenoviruses) a defective virus to carry gag, pol or nef genes of HIV-1 (MRK Ad5 HIV-1). The first STEP trial was conducted in North and South America, Caribbean and Australia followed by 2nd trial in Phambili in South Africa by Merk and HIV Vaccine trial Network, USA. Unfortunately, the much anticipated STEP trial was abruptly stopped way ahead, as it does not protect all those who have been vaccinated. The probable reason for the failure of STEP trial is that many American and African people had natural antibody to Ad5 strain itself. Therefore the next step is to introduce some unusual Adenovirus types than the common one circulating in America and Africa.

BPL Inactivated Vaccine: United Kingdom Microbiological Research Center prepared a vaccine with Tissue culture adapted SIV treated with BPL and vaccinated the monkeys. This prevented integration of virus to host cell genome but it is a preliminary study and needs extensive research.

Dendrite cell Vaccine: Dr.Wei Lu from Reme Descartes University combined dendrite skin cells with killed HIV and injected in to monkeys. Subsequently they were challenged with live SIV. The result showed that the monkeys did not become sick and none died. Again it is premature to arrive at any conclusion and needs further research.

VLP Vaccine: Virus like proteins (VLP) are not true viruses. The scientists from Oxford University and British Biotechnology combined a virus like protein with P24 antigen of HIV and experimented a vaccine. But their CTL (Cytotoxic 'T' Lymphocytes) stimulation was less.

SANOFI PASTEUR Vaccine: In this vaccine, primary dose of VAX genes Recombination of gp120 was followed by VCP booster (Viral vector with GP 160) which resulted in development of both B & T cell response in Thailand (VCP 1521 : Canary pox + gp 160). The final verdict of this vaccine trial in a large scale clinical trial is awaited.

RV 144: During the latest AIDS vaccine conference held at Bangkok (Thailand) on 12-15th September 2011, 400 studies on HIV vaccine were presented and discussed. There was one vaccine termed RV 144 a Thailand trial showed higher protection. RV 144 contains gp120, env (B, E) which is primed with VAXGene gp 120 B/E (AIDSVAX B/E) boosting. This vaccine has a better scope and further studies are in progress.

HIV VACCINE TRIALS IN INDIA

In India the vaccine trials done are HIV-1 Clad C either in animals or in human.

1) Dr. D. N. Rao and colleagues from All India Institute of Medical Sciences, New Delhi used envelope (env) derived peptide immunogen along with adjuvant to immunize mice (Ahluwalia et al., 1997; Pun et al., 2009).
2) Dr. Pradeep Seth and his colleagues from AIIMS , New Delhi prepared HIV-1,gp 160, DNA Vaccine which induced antigen specific immune response in mice (Arora et al., 2001).
3) Dr. Pradeep Seth team had also used a modified Vaccinia Ankara (MVA) vaccine where in the envelope of subtype 'C' (env, gag & Protease genes) and core protein were incorporated. This vaccine induced high level of humoral and cell mediated response in mice (Kumar et al., 2004).

4) Dr.Varadarajan's group from Indian Institute of Science, Bangalore used gp 120 as a noval immunogen entity and induced Neutralizing antibody (Varadarajan et al., 2005; Chakraborty et al., 2006).
5) Dr. Ranga's group from Jawaharlal Nehru Centre for Advanced Science Research, Bangalore developed 'Tat' based DNA vaccine to elicit CMI in mice (Ramakrishna et al., 2006; Kashi et al., 2009).
6) NARI-Pune, Dr. Kulkarni and an USA based Institution (Targeted Genetics Corporation) used a vaccine (tg AACO9) which is a recombinant AAV-Serotype 2 Protein capsid and HIV-1 subtype 'C' gag +Protease + Part of RT . Human Clinical Trial was done in Pune (2005-2007). It was found to be safe, well tolerated and moderate T cell response was found for 'gag' gene (Kulkarni et al., 2008).
7) NICED-Kolkata: Dr. Sekhar Chakrabarti and his Colleagues from National Institute of Cholera and Enteric Diseases (ICMR) laboratory, Kolkata constructed a Recombinant MVA, TBC-M4 containing HIV-1,Clad 'C' with env, gag, tat, rev, nef & RT genes and was evaluated in 2 Yr Phase I clinical trial and found to be safe and elicited virus specific immune response in man and women.
8) TRC-Chennai: Dr.Ramanathan of Tuberculosis Research Centre (ICMR) in collaboration with NARI-Pune and YRG CARE, Chennai and International AIDS Vaccine Initiative (IAVI) and NACO first used TBC-M4 in clinical trials and later AD VAX (Adenovirus vector incorporated with HIV-1 Clad 'c' env, gag, Pol, nef & Tat genes (derived by Aaron Diamond AIDS research Centre, New York). The TBC M4 / ADVAX are being now evaluated in London. This combined vaccine gave a moderate protection in volunteers.

CONCLUSION

While killed vaccine did not elicit 'T' cell immunity, live attenuated vaccine is unacceptable, subunit vaccine induces only one type of antibody against any one antigen, the focus is now on viral vectors mounted with multiple genes of HIV (pol, env, gag and other minor genes) to induce both B and T cell activation to target the virus in all stages of replication. There are two basic problems in HIV vaccine development. The first one is the complexity of viral antigens, polymorphism and mutation. The second aspect is that the immune response of the human host. Till today scientist are bewildered why some people are HIV positive but AIDS negative. Why some high risk population after many exposures to HIV did not test positive for viral antibody. More over many times the antibody alone is not sufficient to kill HIV because HIV can make a cell to cell transfer by fusion of cells and therefore the virus is not exposed to antibody. The goals of an anti-HIV vaccine is that it must provide sterilizing immunity and it must act against both Free and Cell fixed viruses or otherwise it should depress the initial rate of replication by a low viral load maintenance of blood and body fluids including semen and a state of long term, non progression of disease. Evidently a successful vaccine is the one which can induce both humoral broadly neutralizing antibody and cellular immunity against HIV.

Eventually emphasis must be made on the fundamental questions of HIV vaccine discovery and discovery related research (Fauci et al., 2008). In future HIV vaccine alone may not be able to eliminate AIDS from earth. However HIV vaccine will definitely be an adjunct to the other facilitating intervention strategies such as, adapting to specific prevention procedure, AIDS screening mandatory, use of ARTs and plant based medicine, awareness programs on safe sex etc., by medical, paramedical and non-government organizations. Although search for an effective vaccine has been continuing for more than 25 years, it has remained elusive (Harindra et al., 2008). We can be optimistic that HIV vaccine will be available within a decade and we will see the light of the day very soon. At this juncture we are missing the father of vaccinology Dr. Edward Jenner who might have produced a simple vaccine for HIV instead of the present day viral crusaders who depend mainly on molecular biology.

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Plant Growth Promoting Rhizobacteria for Biocontrol of Phytopathogens and Yield Enhancement of *Phaseolus vulgaris* L.

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ABSTRACT

Phaseolus vulgaris is an important legume which originated in Latin America (4,000 BC) and spread the other parts of world, and now being widely cultivated in the tropics and subtropics as well as in temperate regions. In India it is cultivated in sub-Himalayan and higher Himalayan ranges at 1200-1800 m mainly in Maharashtra, Himachal Pradesh, Jammu & Kashmir and Uttarakhand. Due to its nutritive components, it is one of the 10 most important crops of the world. *P. vulgaris* was the first legume in which symbiosis with microsymbiont *Rhizobium etli* was identified. Plant growth promoting rhizobacteria associated with common bean such as *Rhizobium leguminosarum*, *Bacillus subtilis*, *Pseudomonas* sp. enhance its growth and yield in field condition affecting by directly and/or indirectly through production of IAA, siderophore, HCN, solubilization of phosphate, zinc and potassium, and control of several phytopathgens by producing lytic enzymes, antibiotics, oxalate oxidase.

INTRODUCTION

Rhizosphere is the immediate vicinity of plant roots with increased microorganisms where the biology and chemistry of the soil are influenced by the root. In the rhizosphere, very important and intensive interactions take place between the plant, soil, microorganisms and soil microfauna, influenced by compounds exuded by the root and by microorganisms feeding on these compounds (Antoun and Prévost, 2006). All this activity makes the rhizosphere the most dynamic environment in the soil. Gobat *et al*. (2004) have distinguished three rhizosphere fractions into three sub-regions: *(i)* endorhizosphere (interior of the root); *(ii)* rhizoplane (surface of the root); and *(iii)* rhizospheric soil that adheres to the root when the root system is shaken manually. The volume of the soil that is not influenced by the root is defined as non-rhizospheric soil or bulk soil. The rhizosphere is the front-line between plant roots and soil-borne pests. Therefore, it seems logical that microorganisms that colonize the same niche could be the ideal candidates for sustainable agriculture (Weller, 1988). Bacteria are the most abundant microorganisms in the rhizosphere (Antoun and Prévost, 2006).

Bacteria which colonize the rhizosphere of many plant species and confer beneficial effects, such as increased plant growth and reduced susceptibility to diseases, are known as 'plant-growth-promoting rhizobacteria' (PGPR) (Kloepper and Schroth, 1978). PGPR are also termed as 'biocontrol-plant growth promoting bacteria' (Bashan and Holguin, 1998), 'yield increasing bacteria' (Piao *et al*., 1992), 'plant health promoting rhizobacteria' (PHPR) or 'nodule promoting rhizobacteria' (NPR), and are associated with the rhizosphere which is an important zone for plant–microbe interactions (Burr and Caesar, 1984). On the basis of relationship with plants, PGPR can be divided into two groups: symbiotic bacteria and free living rhizobacteria (Khan, 2005). Moreover, PGPR can also be divided into two groups based on their residing sites: intracellular PGPR (iPGPR or symbiotic bacteria) which live inside the plant cells, produce nodules, and localized inside the specialized structures (Vessey, 2003) and extracellular PGPR (ePGPR or free-living rhizobacteria) which live outside the plant cells and do not produce nodules,

but still promote plant growth (Gray and Smith, 2005). The best-known iPGPR are rhizobia which produce nodules in leguminous plants.

A variety of bacteria have been used as soil inoculants intended to improve the supply of nutrients to crop plants. Species of *Rhizobium* (*Rhizobium*, *Mesorhizobium*, *Ensifer* (*Sinorhizobium*), *Bradyrhizobium*, *Azorhizobium*, *Methylobacterium*, *Burkholderia*, *Cupriavidas* (*Ralstonia*), *Devosia*, *Ochrobacturm*, *Phyllobacterium*, *Herbspirillum* and *Shinella*) have been successfully used worldwide to permit an effective establishment of the nitrogen-fixing symbiosis with leguminous crop plants (Bottomley and Maggard, 1990). On the other hand, non-symbiotic nitrogen fixing bacteria such as *Alcaligenes, Azospirillum, Azotobacter*, *Bacillus*, *Clostridium, Enterobacter* and *Klebsiella* are also used to inoculate a large area of arable land in the world with the aim of enhancing plant productivity (Lynch, 1983). Bean rust is a common and potentially serious disease of dry and snap beans with a worldwide distribution, but is most prevalent in tropical and sub-tropical areas. It causes 25 - 100% losses depending on stage of infection and the prevailing weather conditions (Schwartz *et al*., 2004). Severe rust infection results in defoliation, stunted growth and subsequent reduced yields while infected pods may be rejected in the market due to the development of disfiguring lesions. Bean production is also affected by other soil-borne pathogens world-wide such as wilt caused by *Fusarium oxysporum* f.sp. *phaseoli* (Fop). The disease has been reported in many beans producing region. Besides these two so many other fungal phytopathogens adversely affect the common beans growth and yield. Although fungicides have been effective in the control of fungal pathogens but various problems have arose including residues on produce, environmental pollution, development of new physiological races and the prohibitive cost (Gerhardson, 2002). Additionally, they have negative effects on human health and kill possible antagonists. PGPR have attracted much attention in their role in reducing plant diseases. Although their full potential has not yet been reached, the work to date is very promising. Some PGPR, if inoculated on seeds before planting, are able to establish themselves on crop roots. They use scarce resources, and thereby prevent or limit the growth of pathogenic microorganisms. Seed size and weight of common bean depend on genetic variations, cultivar and environmental conditions (Gonzalez de Mejia *et al*., 2005). The seed color of beans is determined by the presence and concentration of flavonol glycosides, anthocyanins and tannins (Aparicio-Fernandez *et al*., 2005). Beans are consumed as mature grain, as immature seed and also as a vegetable. Their pods can be obtained in as little as two-three month depending on varieties, and rotations are possible with other species until the main crop is harvested.

COMMON BEAN (*PHASEOLUS VULGARIS*)

Leguminosae is the second largest family of flowering plants comprisimg of about 750 genera and more than 20,000 species; among them only 15% have been explored for rhizobial diversity. Common bean is important legume for human nutrition and a mojor protein and calorie source in the world (Sharon, 2003); it belongs to subfamily *Papilionaceae* of *Fabaceae* (*Leguminosae*). The word *Phaseolus* comes from the Greek *phaselua*, which refers to a canoe-like boat reminiscent of a bean pod (Albala, 2007). It is dominant in the staple diets of lower income people in Americas, Africa and Asia, together with maize. Beans are extensively diverse crops in terms of their uses, cultivation methods and their morphological variability. Their value was realized as early as 2300 years ago, when Theophrastus (370-285 B.C.) wrote the bean best reinvigorates the soil. Beans are not a burdensome crop to the ground: they even seem to manure it, because the plant is of loose growth and rots easily (Van Kessel and Reeves, 2000). The diversity of beans is also displayed in the range of environments to which they have adapted. They are found from sea level up to 3,000 meter above sea level and are cultivated in monoculture, in associations, or in rotations.The beans are smooth, plump, kidney-shaped, up to 1.5 cm long, range widely in color, and are often mottled in two or more colors. Common bean is known by different name in different country such as rajma, cheema, kidney beans, pinto bean, caparrones bean, lima bean, tepary bean and navy beans.

It is grown extensively in five major continental areas: Eastern Africa, North and Central America, South America, Eastern Asia and Western and Southern Eastern Europe (Adams *et al*., 1985). Archeological investigations showed that common beans originated on the American Continent, specifically in southern United States, Mexico, Central America, and the northern part of South America (Gepts and Dpbouk, 1991). In particular, the species *P. vulgaris* was introduced into Europe in the 16th century; since then it has become a very important crop in many regions of the world such as Africa and India.

In India agriculture is the main occupation of the people and about 70% of the population is engaged in this activity. Livestock rearing is complementary to agriculture. In India, the total area under cultivation is about 169.7 M ha; an additional area of 0.4 M ha is under plantation crops (FAO). In our country, the area under which common bean are cultivated is 9700 million ha (only 36% area) as compared to 27,086 million ha all over the world. While its production in India is 4,340 million tones (only 23%) as compared to 18,943 million tonnes in the world (FAO, Anonymous, 2003). Although it is most important pulse crops of India. However, it is less preferred in comparison to other pulses like *Vigna radiata*, *V. mungo*, etc., which consequently resulted in its limited cultivation restricted to certain parts of the country. Even then this pulse crop has gained popularity within Indian farmers due to its high lucrative features like short growth cycle, good adaptability, high market price and the most important for poor farmers, particularly women, hence it is also known as woman's crop (Spence, 2006; Kumar *et al*., 2008).

Common bean and Rhizobia: For the first time, Beijerinck (1888) isolated a bacterium from root nodules of legumes and named it *Bacillus radicicola*; this was subsequently renamed *Rhizobium* (Frank, 1889). The earliest classification of rhizobia was based on specificity of symbiotic plant range of bacterial species. Jordan (1982) coined the new genus, *Bradyrhizobium japonicum* described for isolate of *Glycine max*. In the first edition of Bergey's Manual of Systematic Bacteriology (Kreig and Holt, 1984) two rhizobial genera (*Bradyrhizobium* and *Rhizobium*) and four species were described which were based solely on growth rate and symbiotic host ranges. To date root nodulating bacteria comprise of 13 genera and 98 species (http://www.rhizobia.co.nz). Recently, Ardley and co-workers reported a new genus (*Microvirga*) containig three species in rhizobia group (un- published). Common bean is a promiscuous host plant since the capacity to nodulate *P. vulgaris* effectively is present in a genetically heterogeneous group of bacteria originating from all over the world (Laguerre *et al.,* 1993). Root-nodulating bacteria are of extreme importance in legumes. They provide them with the advantageous factor in relation to nitrogen fixation besides scavenging of phytopathogens. Earlier, it was generally accepted that legumes were nodulated only by the members of α-proteobacteria. The legume nodulation by members of β-proteobacteria was first reported by Moulin *et al.* (2001) from African legume. On the basis of widespread occurrence of β-proteobacteria in nodulating legume, rhizobia are now divided as α-rhizobia and β-rhizobia (Verma *et al*., 2004). Another recently described β-proteobacterium is *Herbaspirillum lusitanum* which has been reported to nodulate *P. vulgaris* from Portugal (Valverde *et al*., 2003). Most recently, *Rhizobium lusitanum* was reported to nodulate *P. vulgaris* (Valverde *et al*., 2006). Besides, *R. leguminosarum* bv. *phaseoli* (Jordan, 1984), *R. tropici* (Martinez-Romero *et al.,* 1991), *Ensifer meliloti*, and *R. leguminosarum* bv. *trifolii* (Michiels *et al.,* 1998), two new species able to form nitrogen-fixing nodules on roots of *P. vulgaris* have been described as *R. gallicum* bv. *phaseoli* and *R. giardinii* bv. *phaseoli* (Amarger *et al.,*1997). In Kenya, the dominant types of *Phaseolus*-nodulating rhizobia of acidic soil differ from a high-pH soil, where *Rhizobium tropici* dominates in the acidic soil (Anyango *et al*., 1995). Because, *R. tropici* is the most acid-tolerant bacterium described to date (Graham *et al*., 1994). It is tempting to assume that *R. tropici* might generally be better adapted to acidic soils than the other species of *Phaseolus*-nodulating rhizobia. There are several workers who have reported different species of rhizobia to nodulate *P. vulgaris* such as *R. etli* (D'Haeze *et al*., 2007), *R. gallicum* (Shamseldin *et al*.,

2005), *R. giardinii* bv. *giardinii* (Mhamdi *et al*., 2002), *R. leguminosarum* (García-Fraile *et al*., 2010), *R. tropici* (Gressent *et al*., 2002), *E. meliloti* bv. *mediterranense* (Zurdo-Piñeiro *et al*., 2009) (Table 1). Besides the presence of *Rhizobium* and other genera in root nodules of *P. vugaris*, some other bacterial species such as *Bacillus, Pseudomonas, Enterobacter etc.* are also present in the rhizosphere of common bean. They are also responsible for enchancement of growth and yield of common bean.

Common bean and micorbial consortium: A microbial consortium is a group of different species of microorganisms that act together as community. For developing a consortium one can choose microorganisms that are resistant to environmental shock, fast acting, synergistically active, producing natural enzymatic activity, easy to handle, having long shelf-life, good sustainability, non-pathogenic, non-corrosive of consistent quality and economical. By combining several microorganisms, multiple antifungal activities is also got to be combined and one may assume that at least one biocontrol mechanism will be functional under the conditions faced by the released biocontrol agents. Moreover, combinations of biocontrol agents are expected to result in a higher level of protection which has reduced variability of biological control (Jatiyanon and Kloepper, 2002). In fact, several authors have demonstrated that combination of microorganisms can result in improved plant stand (Camprubi *et al.*, 1995). Mixtures of biocontrol agents also resulted in enhanced control of several fungal diseases (Raupach and Kloepper, 1998). Sung and Chung (1997) showed that a mixture of bacteria that produce chitinase and antibiotics could efficiently suppress rice sheath blight caused by *R. solani*. Essalmani and Lahlou (2003) found that culture filtrate of *R. leguminosarum* and heat-killed bacterial cells protected legume plants against infection by *F. oxysporum* MR 84 to a high degree and also proved that the culture filtrate and heat-killed bacterial cells of *R. leguminosarum* contain signals able to induce resistance in plant. Many workers study underlines the potential of bacterial antagonists in promoting plant disease suppression by inducing plant defence mechanism (Kloepper *et al*., 1992; Cherif *et al*., 2003) (Table 2).

PLANT GROWTH PROMOTING RHIZOBACTERIA

PGPR have the potential to contribute in the development of sustainable agricultural systems (Schippers *et al.,* 1995). Generally, PGPR function in three different ways: *(i)* synthesizing particular compounds for the plants (Zahir *et al.,* 2004), *(ii)* facilitating the uptake of certain nutrients from the soil (Çakmakçi *et al.,* 2006), and *(iii)* preventing the plants from diseases (Deshwal *et al*., 2003b; Singh *et al*., 2008, 2010). But the possible explanation is that the PGPR enhance plant growth by both direct and indirect method, specific mechanisms involved have not all been well-characterized (Dey *et al.,* 2004). Direct mechanisms of plant growth promotion by PGPR can be demonstrated in the absence of plant pathogens or other rhizosphere microorganism, while indirect mechanisms involve the ability of PGPR to reduce the deleterious effects of phytopathogens on crop yield. PGPR have been reported to directly enhacnc plant growth by a variety of mechanisms such as fixation of atomospheric nitrogen that is transferred to the plant (Kennedy *et al.,* 2004), production of siderophore that chelate iron and make it available to the plant root (Nelson *et al.,* 2004), solubilization of minerals such as phosphorus (Banerjee and Yasmin, 2002), zink (Iqbal *et al.,* 2010), potassium (Han and Lee, 2006) and synthesis of phytohormones such as indole acetic acid (Patten and Glick, 2002), abscisic acid (Dobbelaere *et al.,* 2003), gibberellic acid (Mahmoud *et al.,* 1984), cytokinins (Timmusk *et al.,* 1999) and ethylene (Zahir *et al.,* 2004). Direct enhancement of mineral uptade due to increase in specific ion fluxes at the root surface in the presensce of PGPR has also been reported (Bertrand *et al.,* 2000).

Table 1: Rhizobia from *Phaseolus vulgaris.*

Class: α-Proteobacteria, Order: Hyphomicrobiales, Family: Rhizobiaceae

Genus	Species	References
Rhizobium	*R. etli* bv. *phaseoli*	Segovia *et al*., 1993
	R. etli	van Berkum *et al*., 1996
	R. etli CNPAF512	Michiels *et al*., 1998
	R. etli	Cermola *et al*., 2000
	R. etli	Diouf *et al*., 2000
	R. etli ISP42	Rodríguez-Navarro *et al*., 2000
	R. etli	Velazquez *et al*., 2001
	R. etli bv. *phaseoli*	Mhamdi *et al*., 2002
	R. etli	Mhamdi *et al*., 2002
	R. etli	Silva *et al*., 2003
	R. etli	Aguilar *et al*., 2004
	R. etli	Shamseldin *et al*., 2005
	R. etli CE3	D'Haeze *et al*., 2007
	R. etli	Gutierrez and Barraquio 2010
	R. gallicum	Amargar *et al*., 1997
	R. gallicum	Velazquez *et al*., 2001
	R. gallicum bv. *gallicum*	Mhamdi *et al*., 2002
	R. gallicum	Mhamdi *et al*., 2002
	R. gallicum	Silva *et al*., 2003
	R. gallicum	Shamseldin *et al*., 2005
	R. gallicum	Mnasri *et al*., 2007
	R. giardinii	Amargar *et al*., 1997
	R. giardinii	Velazquez *et al*., 2001
	R. giardinii bv. *giardinii*	Mhamdi *et al*., 2002
	R. leguminisorum bv. *phaseoli*	Jordan, 1984
	R. leguminisorum bv. *phaseoli*	Pimentel *et al*., 1990
	R. leguminosarum bv. *viciae* and bv. *trifolii*	Velazquez *et al*., 2001
	R. leguminisorum bv. *phaseoli*	Mhamdi *et al*., 2002
	R. leguminosarum bv. *viciae*	Mhamdi *et al*., 2002
	R. leguminosarum	Mhamdi *et al*., 2002
	R. leguminosarum bv. *trifolii*	Abril *et al*., 2007
	R. leguminisorum	García-Fraile *et al*., 2010
	R. leguminisorum RPN5	Kumar, 2012
	R. lusitanum sp. nov	Valverde *et al*., 2006
	R. phaseoli	Ramírez-Bahena *et al*., 2008
	R. tropici	Martinez-Romero *et al*., 1991
	R. tropici PRF 81	Hungria *et al*., 2000
	R. tropici type B	Diouf *et al*., 2000
	R. tropici	Gressent *et al*., 2002
Sinorhizobium (*Ensifer*)	*S. americanum*	Toledo *et al*., 2003
	S. fredii	Velazquez *et al*., 2001
	S. fredii-like (genotype *Phaseolus*)	Mhamdi *et al*., 2002
	S. fredii bv. mediterranense	Zurdo-Piñeiro *et al*., 2009
	S. medicae (genotype *S. medicae*)	Mhamdi *et al*., 2002
	S. meliloti (genotype *S. meliloti*)	Mhamdi *et al*., 2002
	S. meliloti (genotype 'Medicago)	Mhamdi *et al*., 2002
	S. meliloti bv. mediterranense	Zurdo-Piñeiro *et al*., 2009
β-Proteobacteria, Order: Burkholderiales, Family: Oxalobacteraceae		
Herbspirillum	*H. lusitanum*	Valverde *et al*., 2003
	H. seropedicae	Schmidt *et al*., 2011

Table 2: Co-inoluculation of PGPR*s* in *Phaseolus vulgaris*

Co-inoculation	References
Pseudomonas putida and *R. phaseoli*	Grimes *et al*., 1984
Bacillus polymyxa and *Rhizobium etli*	Petersen *et al*., 1996
Glomus mosseae and *R. leguminosarum*	Hassan *et al*., 1997
Rhizobium etli and *Bacillus* spp.	Srinivasan *et al*., 1997
Bacillus sp. CECT 450 and *R.tropici* CIAT 899	Camacho *et al*., 2001
Pseudomonas spp. and *R. leguminosaru*	Martins *et al*., 2004
Rhizobium spp. and *Azospirillum*	Remans *et al*., 2008
Rhizobium tropici (CIAT899) and *Paenibacillus polymyxa* (DSM 36)	Figueiredo *et al*., 2008
R. leguminoarum bv. phaseoli (ARC 301), *Azotobacter chroococcum* (AZ1) and *Bacillus megaterium var phosphaticium* (BM3)	Gharib *et al*., 2009
Chryseobacterium balustinum Aur9 and *R. tropici* CIAT899 or *R. etli* ISP42	Estévez *et al*., 2009
Azospirillum lipoferum S-21, *Pseudomonas fluorescens* P-93 and *Rhizobium* spp.	Yadegari and Rahmani, 2010
Rhizobium sp. BHURC01 and *P. fluorescens*	Verma *et al*., 2010
Pseudomonas sp. LG and *R. phaseoli*	Stajkovic *et al*., 2011
R. leguminisorum RPN5, *Bacillus subtilis* BPR7 and *Pseudomonas* sp. PPR8	Kumar, 2012

PGPR that indirectly enhance plant growth via suppression of phytopathogens do so by a variety of mechanisms, such as the ability to produce siderophores that chelate iron making it unavailable to pathogens (Pandey *et al.*, 2005b), the ability to synthesize anti-fungal metabolites like antibiotics (Kang *et al.*, 2004), expression of fungal cell wall-lysing enzymes *e.g.* β-1, 3-glucanases (Ruiz Duenas and Martinez, 1996), β-1, 4-glucanases (Diby *et al*., 2005), cellulases (Chatterjee *et al*., 1995), chitinases (Gupta *et al.,* 2006), proteases (Szekeres *et al.,* 2004) and hydrogen cynide (Senthilkumar *et al*., 2009), which suppress the growth of fungal pathogens. Thus, the PGPR successfully compete with pathogens for nutrients of specific niches on the roots and thereafter develop systemic resistance. Among these a very important bacterial enzyme like 1-aminocyclopropane-1-carboxylate (ACC) deaminase plays a significant role in the regulation of a plant hormone, ethylene and enhance the growth and development of plants (Glick, 2005; Saleem *et al*., 2007). Bacterial strains containing ACC deaminase can at least alleviate the stress-induced ethylene-mediated negative impact on plants (Glick, 2005). Remans *et al*. (2007) examined the potential of PGPR containing ACC-deaminase to enhance nodulation of common bean (*P. vulgaris*). It was observed that the effect on nodulation of PGPR was strongly dependent on P nutrition. Various functions of PGPR has been demonstrated in Figure 1.

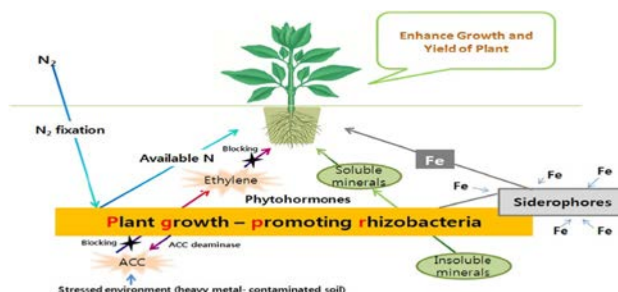

Figure 1: Functions of plant growth-promoting rhizobacteria (Sources: http://www.envitop.co.kr/ 10chumdan/07/sp1.htm)

Besides these PGP activities, PGPR also enhances resistance to drought, salinity, waterlogging and oxidative stress (Saleem *et al*., 2007) and production of water-soluble B group vitamins such as niacin, pantothenic acid, thiamine, riboflavine and biotin (Martinez-Toledo *et al*., 1996; Revillas *et al*., 2000). PGPRs are well known for their role in enhancing the soil fertility and promoting crop productivity by providing essential nutrients (Zaidi and Khan, 2006) and growth regulators (Wani *et al*., 2007). They also promote the growth of plants by alleviating the stress induced by ethylene-mediated impact of plant by synthesizing ACC deamiase (Belimov *et al*., 2005). The application of PGPR has also been extended to remediate contaminated soils in association with plants (Khan *et al*., 2009). The major applications of PGPR include agriculture, horticulture, forestry and environmental restoration. Thus, it is an important need to enhance the efficiency of insufficient amounts of external inputs by employing the best combinations of beneficial bacteria in sustainable agriculture production systems. PGPR may use one or more of above mentioned traits in the rhizosphere. Biochemical and molecular approaches are providing new insight into the genetic basis of these traits, the biosynthetic pathway involved, their regulation and importance for biological control in laboratory and field studies (Nelson, 2004).

Plant roots offer a niche for the proliferation of soil bacteria that thrive on root exudates and lysates. Root exudation is part of the rhizodeposition process, which is a major source of soil organic carbon released by plant roots (Nguyen, 2003).The quantity and quality of root exudates are determined by plant species, the age of an individual plant and external factors like biotic and abiotic stresses. Plant root exudates directly affect microbial populations within the rhizosphere (Morgan *et al*., 2005) by providing growth substrates such as sugars, amino acids, organic acids, fatty acids, nucleotides, sterols, vitamins and other compounds that influence the growth of bacteria and fungi (Broeckling *et al*., 2008). In turn, the plant is affected by the increased activity of the microorganisms in the rhizosphere. A young seedling typically exuding about 30-40 % of their fixed carbon as root exudates (Whipps, 1990). Root exudates mediate both positive and negative interactions in the rhizosphere.The positive interactions include symbiotic associations with beneficial microbes such as mycorrhizae, rhizobia and PGPR. Negative interactions include association with parasitic plants, pathogenic microbes and invertebrate herbivores.

The plant not only secretes components but also takes up the exudates components (Kamilova *et al*., 2006b). In the era of sustainable crop production, the plant–microbe interactions in the rhizosphere play a pivotal role in transformation, mobilization, solubilization, etc. of nutrients from a limited nutrient pool, and subsequently uptake of essential nutrients by plants to realize their full genetic potential. At present, the use of biological approaches is becoming more popular as an additive to chemical fertilizers for improving crop yield in an integrated plant nutrient management system (Kumar *et al*., 2009). The use of PGPR has been found potential in developing sustainable systems in crop production (Shoebitz *et al*., 2009). A variety of symbiotic (*Rhizobium* sp.) and non-symbiotic bacteria (*Azotobacter*, *Azospirillum*, *Bacillus*, and *Klebsiella* sp., *Pseudomonas* sp. etc. are now being used worldwide with the aim of enhancing plant productivity (Cocking, 2003). Current production methods in agriculture are the improper use of chemical pesticides and fertilizers, creating the environmental and health problems (Leach and Mumford, 2008). Chemically produced nitrogen fertilizers can provide this nitrogen to plant, but it is expensive to produce in addition to being harmful to the environment. This damage to the environment includes changes in the global nitrogen cycle, loss of nitrous oxides to the atmosphere, acid rain, nitrate pollution of ground water and induced leaching of soil nutriens (Vilousek *et al*., 1997). An inexpensive and environment friendly alternative to nitrogen fertilizer is biological nitrogen fixation (BNF).

Auxins are recognized as the most active plant growth stimulators (phytohormone) and indole-3-acetic acid (IAA) is a key substance (Woodward and Bartel, 2005). IAA is essential for the growth and development of plants. It is most common and physiologically most active. It stimulates both rapid (increases in cell elongation) and long-term (cell division and differentiation) responses in plants

(Cleland, 1990; Hagen, 1990). IAA production is also widespread among soil and plant associated bacteria, and its biosynthesis is an integral core trait of symbiotic species within the genera of *Rhizobium*, *Bradyrhizobium*, and *Nostoc* as well as some other plant-associated saprophytic PGPR (Unno *et al*., 2005; Mehnaz and Lazarovits, 2006). It has been estimated that 80% rhizobacteria can produce the plant growth regulator IAA (Patten and Glick, 1996). Tryptophan (Trp) is generally considered as a precursor of IAA. Several IAA biosynthetic pathways such as indole-3-acetamide pathway, indole-3-pyruvate pathway, tryptamine pathway, tryptophan side-chain oxidase pathway, indole-3-acetonitrile pathway and tryptophan-independent pathways have been reported in bacteria (Spaepen *et al*., 2007), although the best-characterized pathways in bacteria for the conversion of Trp to IAA are the indole-3-acetamide pathway and the indole-3-pyruvate (IPyA) pathway (Patten and Glick, 1996).

Promotion of root growth is one of the major characteristics by which the positive infuence of plant PGPR is measured (Patten and Glick, 2002). On the other hand, IAA over production by pathogenic strains of *Agrobacterium*, *Pseudomonas*, *Erwinia* or *Stenotrophomonas* adversely affects the physiology of plant by contributing to the host endogenous auxin pool, and induces the development of diverse plant diseases (Patten and Glick, 2002). At the same time, some strains of *Bradyrhizobium*, *Pseudomonas*, *Alcaligenes*, and *Agrobacterium* are recognized as effective IAA degraders and also affect auxin levels by decreasing high IAA concentrations, thus abolishing its harmful effects on plants (Leveau and Lindow, 2005).

Most *Rhizobium* species have been shown to produce IAA (Badenoch-Jones *et al*., 1983), and many studies indicate that changes in auxin balance are a prerequisite for nodule organogenesis (Mathesius *et al.,* 1998). Nevertheless, the over all role of IAA in the different stages of *Rhizobium*–plant symbiosis is still unclear. Recent evidence suggests that Nod factors (lipo-chitin oligosaccharides or LCOs), which are produced by rhizobia upon triggering of *nod* gene expression by plant-derived flavonoids, act as auxin-transport inhibitors. The expression of the auxin-responsive reporter construct *GH3*::*gusA* in a transgenic white clover plant was rapidly and transiently downregulated after inoculation by *R. leguminosarum* bv. *trifolii* followed by an upregulation at the site of nodule initiation (Mathesius *et al.,* 1998). This suggests that local lowering of the auxin balance is required for the initiation of nodule primordia. Other observations indirectly suggest that microbially released IAA could play a role in rhizobia–plant symbiosis. It was demonstrated that the *nod* inducers, the flavonoids, also stimulate the production of IAA by *Rhizobium* (Prinsen *et al*., 1991) Combined application of *Rhizobium* and *Azospirillum* can enhance nodulation.

Phosphate Solubilization: Phosphorus (P) is a major nutrient after nitrogen (N) that limits plant growth (Gyaneshwar, *et al*., 2002). A large portion of inorganic phosphates applied to soil as fertilizer is rapidly immobilized after application and become unavailable to plants. Generally, added P fertilizer is precipitated by iron, aluminum and calcium complexes present in the soils (Turan *et al*., 2006).Thus, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability. Seed or soil inoculation with phosphate-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yields. These reactions take place in the rhizosphere, because phosphate-solubilizing microorganisms render more phosphates into soluble form than is required for their growth and metabolism and the surplus gets absorbed by plants. However, their establishment and performance are severely affected by environmental factors, especially under stress conditions (Mehta and Nautiyal, 2001).

Phosphorus exists in soil in organic and inorganic forms the availability of which ranges from soluble P (plant available) to very stable (plant unavailable) compounds. There is a dynamic and complex relationship among different forms of P involving soil, plants and microorganisms. Organic P compounds are found in humus and other organic materials including decayed plant, animal and microbial tissues. Phosphorus in labile organic compounds can be slowly mineralized (broken down and released) as

available inorganic phosphate or it can be immobilized (incorporated into more stable organic materials) as part of the soil organic matter (Mckenzie and Roberts, 1990). The process of mineralization or immobilization is carried out by microorganisms and is highly influenced by soil moisture and temperature. Mineralization and immobilization are most rapid in warm and well-drained soils (Busman *et al.*, 2002). Approximately 70 to 80% of P found in cultivated soils is inorganic P (Foth, 1990). Phosphorus ion concentration in most soils ranges from 0.1 to 10 μM (Hinsinger, 2001).

The second major component of soil P is organic matter, which constitute 30–50% of the total phosphorus in most soils, although it may range from 5% to 95% (Paul and Clark, 1988). Organic P in soil is largely in the form of inositol phosphate (soil phytate). It is synthesized by microorganisms and plants and is the most stable of the organic forms of phosphorus in soil, accounting up to 50% of the total organic P (Harley and Smith, 1983). Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters. Organic compounds containing phosphorus are decomposed and mineralized by bacteria and other group of microorganisms. Several bacteria such as *Azotobacter, Bacillus, Clostridium*, fluorescent pseudomonads and rhizobia are reported to solubilize inorganic phosphorus (Gupta *et al.*, 2002; Deshwal *et al.*, 2003a; Sridevi and Mallaiah, 2009).

Phosphate fertilizers can increase P availability initially, but will promote the formation of insoluble P minerals and consequently lead to P buildup. Therefore, P management is important both environmentally and economically. Phosphate solubilizing microorganisms (PSM) may be an answer for maintaining the supply of plant available P because PSM carry out the conversion from labile P to plant available P. The precipitated inorganic phosphate is solubilised by the action of minerals and organic acids produced by soil bacteria (Deshwal *et al.*, 2003a) and other organisms.

Many soil bacteria and fungi have the ability to solubilize phosphate (Pi) minerals and make it available to plants (Egamberdiyeva *et al*., 2003). They are capable of using inorganic and organic forms of P. The population of phosphate solubilizing microorganisms (PSM) varies from soil to soil and ranges from less than 10^2 g^{-1} cfu of soil to 3 x 10^6 cfu g^{-1} of soil (Peix *et al*., 2001).

The principal mechanism for mineral phosphate solubilization is the production of different type of organic acids, whereas different type of enzymes (acid phosphatases) plays a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings. The production of organic acids by phosphate solubilizing bacteria has been well documented. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization. Strains of *Bacillus* were found to produce mixtures of lactic, isovaleric, isobutyric and acetic acids. Other organic acids, such as glycolic, oxalic, malonic, and succinic acid, have also been identified among phosphate solubilizers. Strains from the genera *Pseudomonas, Bacillus* and *Rhizobium* are among the most powerful phosphate solubilizers (Rodriguez and Fraga, 1999). Seshadri *et al*. (2002) reported that the species of *Pseudomonas* and *Bacillus* solubilize more phosphate than others.

Several enzymes, such as nonspecific phosphatases, phytases, phosphonatases, and C-P lyases release soluble phosphorus from organic compounds in soil. C-P lyases cleave C-P links in organophosphonates (Lugtenberg and Kamilova, 2009). The activity of these enzymes in the rhizospheric soils of legumes serves many important roles. They include release of soil organic phosphorous into inorganic phosphorous (Makoi *et al*., 2010), cycling of nutrients, fertilizer use efficiency, soil microbiological activities and act as indicators of soil fertility change (Ndakidemi, 2006).

Makoi *et al*. (2010) have found significant ($P \leq 0.05$) differences in acid phosphatase activity in the rhizosphere of *P. vulgaris* associated with *Rhizobium* inoculation, Mo and lime supply in glasshouse

and field experiments. *Rhizobium* inoculation decreased the levels of acid phosphatase activity in the rhizosphere soil by 22.2% (field) and 20.0% (glasshouse) compared with the uninoculated control. The lower acid and alkaline phosphatase activities exhibited in the rhizosphere of *P. vulgaris* suggest less stress for mineral elements for plant growth.

Phytate, a hexaphosphate salt of inositol, is the major form of P in organic matter contributing between 50 and 80% of the total organic P. Although microorganisms are known to produce phytases that can hydrolyze phytate, which tends to accumulate in virgin soils because it is rendered insoluble as a result of forming complex molecules with Fe, Al and Ca (Alexander, 1977).

Richardson and Hadobas (1997) reported that 63% of culturable soil bacteria such as *Bacillus subtilis, Pseudomonas putida* and *P. mendocina* were able to grow on this substrate as carbon and P sources on agar medium. However, only 39–44% could utilize phytate as a P source in liquid medium, while a very low proportion could use it as a C source in this condition. The group of rhizobia is considered one of the most powerful P-solubilizers and some of them, such as *R. leguminosarum* are able to mobilize phosphorous to plants (Rodriguez and Fraga, 1999), strains nodulating *Phaseolus* have also been reported as phosphate solubilizers (Chabot *et al.*, 1998). Peix *et al.* (2001) observed a greater number of nodules in common bean inoculated with a P-solubilizing *Burkholderia cepacia* strain.

Co-inoculation with *Rhizobium* and PSB had a synergetic effect manifested in increased nodulation, nodule dry weight, shoot dry weight and shoot nitrogen and phosphorus content, increased yield, crude fiber and crude protein compared to uninoculated control (Rugheim and Abdelgani, 2009). It is common to obtain PSM under laboratory conditions, while field performance by the PSM are highly variable; no increase in crop yield or P uptake was found in 70% of field experiments (Gyaneshwar *et al.*, 2002). To find a highly efficient PSM that performs well under laboratory conditions and in soil remains a challenge. To find a medium that predicts the ability of PSM to perform in soil from the laboratory screening is an importment step to approach that challenge. Ivanova *et al.* (2006) reported the rock phosphate solubilization by soil bacteria by producing organic acid.

Zinc Solubilization: Zinc is one of the eight essential micronutrients required for the normal healthy growth and reproduction of crop plants and are required in relatively small concentrations (5–100 mg/kg) in plant tissues. Root cell membrane permeability is increased under Zn-deficiency, which might be related to the function of Zn in cell membranes (Parker *et al.*, 1992). Zn-deficiency is well reported in the soils of much of the world. Indian soils are generally low in zinc and as much as half of the country soils are categorized to be zinc-deficient. Total and available zinc content in Indian soils ranged between 7-2,960 mg kg^{-1} and 0.1-24.6 mg kg^{-1}, respectively with an average deficiency of 12 to 87 %. Crops grown in these soils have low zinc content in shoot and seed. It is estimated that to correct micronutrient problem, India would need fertilizer zinc 324, iron 130, copper 11, boron 3.9 and manganese 22 thousands tones ha^{-1} yr^{-1} by the year 2025 for raising crops successfully otherwise it will lead to multinutrient deficiencies resulting in low yield and nutritional disorders. The overall zinc deficiency is expected to increase from 48% (1970) to 63% (2025), because more and more areas of marginal lands are brought under intensive cultivation without adequate micronutrient supplementation. The states of Punjab, Haryana, Part of Uttar Pradesh and Andhra Pradesh have, however, shown a build of zinc status and decline in its deficiency. Multi nutrient deficiencies are emerging fast in certain location and needs attention. Iqbal *et al.* (2010) reported that all plant growth parameters of legume crop increase when seedlings were inoculated with zinc solubilizing bacteria compared to uninoculated controls.

Potassium Solubilization: Potassium is available in four forms in the soil which are K ions (K^+) in the soil solution, as an exchangeable cation, tightly held on the surfaces of clay minerals and organic matter, tightly held or fixed by weathered micaceous minerals, and present in the lattice of certain K-containing primary minerals. There are several processes that contribute to the availability of potassium in the soil.

Soil solution potassium is already available in the soil for plant uptake; however the concentration of potassium is affected by soil weathering, cropping history and fertilizes use. Thus, the amount present is insufficient to meet crop requirement. Potassium solubilizing bacteria (KSB) such as *Bacillus mucilagenosus* and *B. edaphicus* are example of microorganisms are used in biofertilizer. KSB are able to solubilize potassium rock through production and secretion of organic acids (Han and Lee, 2006).

ACC deaminase production: ACC is the precursor of ethylene which is a well known phyohormone (Glick *et al*., 2007). Some of the ACC is secreted into the rhizosphere and is re-adsorbed by the roots, where it is converted into ethylene. This accumulation of ethylene leads to a downward spiral effect, as poor root growth leads to a diminished ability to acquire water and nutrients, which, in turn, leads to further stress. Thus, PGPR with the ability to degrade ACC in the rhizosphere can help to break this downward cycle and reestablish a healthy root system that is needed to cope with environmental stress. The primary mechanism is used by rhizobacteria to degrade ethylene is the destruction of ethylene via the enzyme 1-aminocyclopropane-1-carboxylate ACC deaminase (EC 4.1.99.4). This enzyme plays a well-understood role in the regulation of a plant hormone, ethylene and, thus growth and development of plants (Glick, 2005) and its presence has been widely reported in numerous microbial species of Gram-negative and Gram-positive bacteria, endophytes (Pandey *et al.,* 2005a; Sgroy *et al*., 2009) and fungi (Jia *et al.,* 1999). It is extensively studied in numerous species of plant growth promoting bacteria. like *Bacillus* (Belimov *et al*., 2001), *Pseudomonas* (Govindasamy *et al*., 2008), *Ralstonia solanacearum* (Blaha *et al.,* 2006), *Rhizobium* (Duan *et al*., 2009), *Variovorax paradoxus* (Belimov *et al.,* 2001) have been isolated from different soils or rhizosphere of different plant.

ACC deaminase can diminish or prevent some of the harmful effects of the high ethylene levels. The ACC deaminase acts on ACC and degrade into α-ketobutyrate and ammonium, (Glick *et al*., 1998). The overproduction of ethylene in response to abiotic and biotic stresses leads to inhibition of root growth and consequently growth of the plant as a whole. Ethylene synthesis is stimulated by a variety of environmental factors/stresses, which hamper plant growth. These ACC deaminase PGPR boost up the plant growth particularly under-stressed conditions by the regulation of accelerated ethylene production in response to a multitude of abiotic and biotic stresses like salinity, drought, waterlogging, temperature, pathogenicity and contaminants.

On the other hand, inoculants applied once in the soil irrespective of any means should be effective for further few years. Introduced strains are re-isolated from the applied field by using suitable markers viz., intrinsic resistance to high level of antibiotic, *Tn*5, *gfp* (green fluorescent proteins), *gus* marker, etc. (Sessitsch *et al*., 1998). Enumeration of applied bacterial strains tagged with any one of markers mentioned above from the field by using serial dilution technique or standard plate count (Dubey and Maheshwari, 2011) is generally used. Other markers used in strain detection are antigenic molecules that locate the cell surface by reacting with specific antibodies. This immunological response can be detected by ELISA, fluorescent-labelled antibodies or immunodiffusion technique (Sessitsch *et al*., 1998). In recent years, molecular tools have been developed to identify and quantify specific microbial activity in mixed population. These tools are especially valuable for analysis of specific rhizobia in complex environmental samples (Topp, 2003). Research has concentrated on the development of marker genes for tapping a particular bacterial species of interest so that the cells can be specially identified and monitored (Lindow, 1995).

HCN Production: Some rhizobacteria (generally Gram-negative) are capable of producing HCN (hydrogen cyanide or cyanide) (Rezzonico *et al*., 2007). PGPR produce HCN which depend on soil and plant characteristics. HCN does not appear to have a role in primary metabolism. In general, cyanide is formed during the early stationary growth phase (Knowles and Bunch, 1986). HCN is a volatile, secondary metabolite that suppresses the development of microorganisms and also affects negatively the growth and development of plants, if produce in excess amount (Siddiqui *et al*., 2006). HCN is a powerful

inhibitor of many metal enzymes, especially copper-containing cytochrome C oxidases and is highly toxic to all aerobic microorganisms at picomolar concentrations. HCN first inhibits the electron transport and the energy supply to the cell is disrupted leading to the death of the organisms. It inhibits proper functioning of enzymes and natural receptors reversible mechanism of inhibition (Corbett, 1974) and it also known to inhibit the action of cytochrome oxidase (Gehring *et al.*, 1993). To date many different bacterial genera have shown to produce HCN, including species of *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* (Devi *et al.*, 2007; Ahmad *et al.*, 2008). HCN production is a common trait within the group of *Pseudomonas* present in the rhizosphere, with some studies showing that about 50% of pseudomonads isolated from rhizosphere are able to produce HCN *in vitro* (Schippers *et al.*, 1990). Production of HCN by pseudomonads is associated with biological control of black root rot studied in tobacco, but other workers observed that it can have a detrimental effect on plant growth (O'Sullivan and O'Gara, 1992). The amount of HCN produced was not measured, which may be a determinant factor in the PGPR behaviour of a strain (Alström and Burns, 1989). Cyanide produced by *P. fluorescens* strain CHA0 has an ecological role in that this metabolite accounts for part of the biocontrol capacity of strain CHA0, which suppresses fungal diseases on plant roots. Volatile compounds may also contribute to inhibition of growth of fungal pathogens. Indeed, several studies have shown the importance of volatiles in the biocontrol of different plant diseases (Gagné *et al.*, 1991).

Production of volatiles in liquid cultures proved inhibitory to spore germination and mycelial growth (Arora and Upadhyay, 1978; Schippers *et al.*, 1987; Defago and Hass, 1990; O`Sullivan and O`Gara, 1992; Dowling and O`Gara, 1994). However, rhizobia do contain low amount of HCN in comparison to that of fluorescent pseudomonads and only 12.5% (Beauchamp *et al.*, 1991) and 3% Antoun *et al.* (1998) strains were found to be HCN producers.

Siderophore Production: Iron is also an abundant metal being the fourth most plentiful element in the Earth's crust. It is vital for most organisms because it is a component of several key molecules that include cytochromes, ribonucleotide reductase and other metabolically linked compounds (Guerinot, 1994). In soil it is unavailable for direct assimilation by microorganisms because ferric iron (Fe III) which predominates in nature is only sparingly soluble and too low in concentration to support microbial growth. However, in oxidative environments and at physiological pH, iron mostly exists in an insoluble ferric (Fe^{3+}) state (Guerinot, 1994), hence is not readily bioavailable. As a consequence, most microorganisms have developed specialized strategies to acquire iron from the environment (Andrews *et al.*, 2003). Some bacteria have developed iron uptake systems (Neilands and Nakamura, 1991) that involve siderophore (*Sid* = iron, *phores* = bearer) an iron binding legend and an uptake protein needed to transport iron into the cell. Siderophores are low molecular weight (400-10,000 Da), ferric-specific ligands produced by microorganisms as scavenging agents in order to combat low iron stress. Iron is an essential macronutrient for all microorganisms, with the exception of the lactobacilli (Pandey *et al.,* 1994), and is associated with a number of important bacterial characteristics such as virulence. There is always a threshold value at which the ambient iron concentration represses siderophore production.

Siderophores are common products of aerobic and facultative anaerobic bacteria and of fungi. Greater attention has been paid to bacterial siderophores especially produced by rhizobacteria and some human pathogens than fungal siderophores because of high chelating affinity for Fe^{3+} and a low affinity for Fe^{2+} ions produced under iron limiting conditions. The chelated form of iron (III) is transported into bacterial cells (Neilands, 1995). It is required as a structural component of a wide variety of haem and non-haem iron proteins. Kloepper *et al.* (1980a) first demonstrated the importance of siderophores in plants. Inside the cell, the siderophore becomes free from the transporter protein and again released outside as free ligand (desferriform) to repeat the cycle. Iron is a component of cell and its deficiency can cause growth inhibition, decrease in RNA and DNA synthesis and inhibition in sporulation and cell morphology change. Siderophores solubilise iron which is then transported into the bacterial cells using

specific receptors. This gives possibility to bacteria to deplete the available iron source from other potentially harmful bacterial strains (Wilson *et al*., 2006).

Depending on the functional groups siderophore are of three types: hydroxymate, catecholates (phenolates) and carboxylates (complexones, polycarboxylates) or mixed (Hofte, 1993). The most effective siderophores have three bidentate ligands per molecule, forming a hexadentate complex and causing a smaller entropic change than that caused by chelating a single ferric ion with separate ligands. A number of fungal siderophores are di- and tri-hydroxamates based on ornithine, the 5-amino group of which has been hydroxylated and acylated to form the hydroxamate ligand (Hofte, 1993). Bacterial siderophores in which the hydroxamate ligands are based on D- or L-ornithine include the ornibactins from *Burkholderia cepacia,* which contain one carboxylate and two hydroxamate ligands (Stephan *et al.,* 1993) and the pyoverdines from *Pseudomonas* spp. which may have one or two hydroxamate groups (Hofte, 1993). It is produced by several PGPR such as *Bacillus* (Singh *et al*. 2008), *R. leguminosarum* bv. *viciae* (Rogers *et al*., 2001) etc. Siderophores produced by root nodule bacteria include carboxylates such as rhizobactin (Smith *et al.,* 1985), citrate (Guerinot *et al.,* 1990) and anthranilate (Rioux *et al.,* 1986), catechols (Roy *et al.,* 1994) and hydroxamates (Persmark *et al.,* 1993). Rhizobactin 1021 from *E. meliloti* (de Lajudie *et al.,*1994), the only root nodule bacterial hydroxamate siderophore whose structure is known, is a dihydroxamate containing a central citrate moiety substituted on one terminal carboxyl group by 1-amino-3-(N-acetyJ-N-ydroxyamino)-propane by 1-amino-3-(N-hydroxy-N-(E)-2-decenylamino) propane on the other (Persmark *et al.,* 1993). The ability to synthesize siderophores appears to be restricted to a limited range of strains rather than widely distributed in root nodule bacteria (Carson *et al.,* 1992). Vicibactin (previously called hydroxamate K) is a trihydroxamate-type siderophore produced by *R. leguminosarum* biovar *uiciae* WSM710 (Carson *et al.,* 1992), the iron complex of which has 1510 M cm and a molecular mass of 828 (Carson *et al.,* 1994).

In addition, siderophores also mediate the iron uptake by plant roots in iron limiting conditions (Wang *et al*., 1993). Siderophore production has been reported by a number of workers in different groups of plant growth-promoting bacteria such as rhizobia (Deshwal *et al.,* 2003a; Vargas *et al*., 2010), pseudamonads (Gupta *et al.,* 2002; Bhatia *et al*., 2003; Singh *et al*., 2010; Khare *et al*., 2011), *Bacillus* spp. (Singh *et al*., 2008; Mehta *et al*., 2010), and *Burkholderia* sp. (Pandey *et al*., 2005a). It was suggested that siderophore production was conserved among the members of legionellae family (Starkenburg *et al*., 2004). Various authors have described the role of siderophores in the inhibition of several fungal pathogens resulting in increased crop yield (O' Sullivan and O'Gara, 1992; Deshwal *et al*., 2003a; Bhatia *et al*., 2003; Pandey *et al*., 2005b).

Oxalate-oxidase enzyme Production: The capability of sclerotia of *Sclerotinia sclerotiorum* to survive for more than four years becomes very difficult to manage the crop from the infection of white mold fungus. The early stage of infection involves the production and the accumulation of a large amount of oxalic acid (OA) which appears to be one of the essential determinants of the pathogenecity. Once produced and accumulated, OA plays a key role, provoking some disease-like symptoms independent of presence of pathogen (Lehner *et al.,* 2008). Maxwell and Lumsden (1970) found that oxalic acid produced by *S. sclerotiorum* acted synergistically with pectic and cellulolytic enzymes for destruction of host tissues. Using oxalic oxidase-producing bacteria for control of *S. sclerotiorum* is a novel biocontrol method. The PGPR producing such enzyme are very usefull to control the stem rot in plants caused by *S. sclerotiorum.* Foliar application of *B. subtilis* reduced white mold of white bean under field conditions (Tu, 1997). *Erwinia herbicola* and *Bacillus polymyxa* inhibited the growth of *S. sclerotiorum in vitro* (Godoy *et al*., 1990a). Oxalic acid is also produced by *R. solani* and its detoxification using *Pseudomonas fluorescens* strain PfMDU2 was first reported by Nagarajkumar *et al*. (2005).

Lytic enzyme Production: Several biocontrol mechanisms have been studied, some of which include disease reduction by lytic enzymes produced by bacteria and fungi such as β-1,3-glucanase (Ruiz Duenas and Martinez, 1996), β-1,4-glucanase (Diby *et al*., 2005), β-1,6-glucanase (Valois *et al*., 1996), cellulases (Chatterjee *et al*., 1995), chitinase (Gupta *et al.,* 2006), chintosanses (Helisto *et al*., 2001, protease (Szekeres *et al.,* 2004) amylase and pectinase. Surfactant and toxins (bacteriocins) are also involved to control phytopathogens (Chin-A-Woeng *et al.,* 2003).

β-glucan degrading enzymes are classified according to the type of β-glucosidic linkages: β-1,4-glucanases (including cellulases), β-1,3-glucanases, and β-1,6-glucanases (Pitson *et al*., 1993). Because β-1,3-glucan is a structural component of fungal cell walls, the production of extracellular β-1,3-glucanases has been reported as an important enzymatic activity present in biocontrol agent. In addition to chitin and glucans, cell walls of filamentous fungi contain proteins. Thus, the production of proteases may play a role in antagonism (Flores *et al*., 1997).

Cellulose is a major polysaccharide constituent of plant cell walls including fungi and one of the most abundant organic compounds in the biosphere. It is an unbranched glucose polymer composed of an β-1,4 glucose units linked by a β-1,4-D-glycosidic bond. Cellulolytic enzymes degrade cellulose by cleaving the β-1,4-D-glycosidic bonds (Han *et al*., 1995). A number of bacteria and fungi are capable of producing multiple groups of enzymes collectively known as cellulases (Akiba *et al*., 1995). In general, bacterial cellulase production is constitutive, whereas fungal cellulase production is inductive, *i.e.* produced only in the presence of cellulose (Suto and Tomita, 2001). Cellulases can be classified into three types: endoglucanases or carboxymethyl cellulases (CMCases) (endo-β-1,4 glucanase, EC 3.2.1.4), exoglucanases or cellobiohydrolases (exo-β-1,4-glucanase, EC 3.2.1.91), and β-glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) (Kang *et al*., 1999). Endoglucanases randomly hydrolyze internal β-1,4-D-glycosidic bonds in cellulose (Siddiqui *et al*., 2000). As a result, the polymer rapidly decreases in length and the concentration of the reducing sugar increases slowly. Exoglucanases hydrolyze cellulose by removing the cellobiose units from the non-reducing end of cellulose (Han *et al*., 1995). In this way besides biocontrol of phytopathogen by degrading cellulose, cellulolytic microorganisms play an important role in the biosphere by recycling cellulose (Beguin and Anbert, 1993).

Chitin is hydrolysed by two main enzymes chitinase (E.C.3.2.1.14) and β-N-acetyl hexosaminidase (E.C.3.2.1.52) (Patil *et al.,* 2000). Chitinases are reported to play a protective role against fungal pathogens (Boller, 1985). Besides its ability to attack the fungal cell wall directly, chitinases release oligo-*N*-acetyl glucosamines that function as elicitors for the activation of defense-related responses in plant cells (Ren and West, 1992). Therefore, formulations based on chitinases offer a potential approach for phytopathogens.

In bacteria, chitinases play a role in nutrition and parasitism. In addition to this potential application, chitinases can be used for the production of chitooligosaccharides, which have been found to function as antibacterial agents, elicitors of lysozyme inducers, and immunoenhancers (Wen *et al*., 2002). Chitinases can also be used in agriculture to control plant pathogens (Dahiya *et al*., 2005). There are so many reports which proof that rhizobial isolates produce chitinase and inhibited the deleterious pathogens (Chernin *et al*., 1955; Mazen *et al*., 2008). The chitinase produced by *Enterobacter* sp. NRG4 was highly active against *Fusarium moniliforme*, *Aspergillus niger, Mucor rouxi*, and *Rhizopus nigricans* (Dahiya *et al*., 2005). The chitinase from *Alcaligenes xylosoxydans* inhibited the growth of *Fusarium udum* and *Rhizoctonia bataticola* (Vaidya *et al*., 2001). Chitinase from *B. cereus* YQ 308 inhibited the growth of plant pathogenic fungi such as *Fusarium oxysporum*, *Fusarium solani* and *Pythium ultimum* (Chang *et al*., 2003). Lee and Kobayashi (1980) reported lysis and abnormal branching in *Rhizoctonia solani* growing in the influence of antifungal metabolites of *P. cepacia*. This strain restricted mycelial growth and caused sclerotial deformities and exhibiting the direct involvement of antifungal compounds during antagonism.

Nitrogen Fixation: Nitrogen is an essential plant nutrient deficiency of which reduces agricultural yields. In nature very little nitrogen is available in mineral form and plants are unable to make use of atmosphere nitrogen. According to FAO (2001), about 42 million tons of N fertilizer is being used annually on a global scale for the production of three major cereal crops (non-legumes), *i.e.* wheat, rice and maize (17, 9 and 16 million tons respectively). Crop plants are able to use about 50% of the applied fertilizer N, while 25% is lost from the soil–plant system through leaching, volatilization, denitrification and many other factors causing not only an annual economic loss of US$ 3 billion but also cause pollution to the environment. BNF system could be good alternative that increases the potential for nitrogen supply because fixed nitrogen would be available to the plants directly with little or no loss. Such a system could also enhance resource conservation and environmental security, besides freeing farmers from the economic burden of purchasing fertilizer nitrogen for crop production (Saikia and Jain, 2007). Plants treated by a mixture of compost and microorganisms showed a significant increase in vegetative growth, total N, P and carbohydrate content and essential oil production thus compost and microorganisms could replace conventional NPK fertilizers in the cultivation of rosemary, and consequently minimize environmental pollution by these compounds (Abdelaziz *et al*., 2007).

Nitrogen fixation ability is widely distributed among phylogenetically diverse bacteria. The conventional nitrogenase is composed of two proteins: the iron (Fe) protein and the molybdenum iron (MoFe) protein. The *nifH* gene encodes the iron protein and the *nifDK* genes encode the molybdenum iron protein. Evolutionarily conserved amino acid sequences within the *nifH* gene have been exploited to design PCR primers to detect the genetic potential for nitrogen fixation in any environment (Mehta *et al.,* 2003). The majority of the world's land based biological nitrogen fixation can be accounted for by the symbiotic nitrogen fixation relationship between leguminous plants and rhizobia and other beneficial rhizobacteria. The advantages of this type of BNF have led to numerous studies investigating the diversity and identity of the associated bacterial population. Globally, terrestrial BNF is estimated at between 100 and 290 million tons of N $year^{-1}$ (Jensen and Hauggaard-Nielsen, 2003). Of this, 40-48 million tons is fixed by agricultural field crops (Jenkinson, 2001). Nitrogen fixing microorganisms could be an important component of sustainable agricultural systems. Biologically nitrogen fixation is performed exclusively by prokaryotes that possess the enzyme nitrogenase. Rhizobia can fix nitrogen in symbiosis with legume plant, while the other non-symbiont beneficial bacteria also fix nitrogen in leguminous plant root and non-leguminous plant roots (Sorokin *et al*., 2008).

For nodulating legumes, nitrogen is provided through symbiotic fixation of atmospheric N_2 by nitrogenase in rhizobial bacteroids. This process of biological nitrogen fixation (BNF) accounts for 65% of the nitrogen currently utilized in agriculture, and will continue to be important in future sustainable crop production systems (Matiru and Dakora, 2004). Important biochemical reactions of BNF occur mainly through symbiotic association of N_2-fixing microorganisms with legumes that converts atmospheric elemental nitrogen (N_2) into ammonia (NH_3) (Shiferaw *et al*., 2004). Rhizobia form intimate symbiotic relationships with legumes by responding chemotactically to flavonoid molecules released as signals by the legume host. These plant compounds induce the expression of nodulation (*nod*) genes in rhizobia, which in turn produce lipo-chitooligosaccharide (LCO) signals that trigger mitotic cell division in roots, leading to nodule formation (Matiru and Dakora, 2004). The N_2 fixed by Rhizobia can also benefit associated non-legumes via direct transfer of biologically fixed N to cereals growing in intercrops (Snapp *et al*., 1998) or to subsequent crops rotated with symbiotic legumes (Deshwal *et al.,* 2006). Common bean crops are of great significance because they produce substantial amounts of organic nitrogen fertilizer resulting from symbiosis between the plant and bacterial symbionts (Jensen and Hauggaard-Nielsen, 2003; Hirsch, 2004). Inoculation with a compatible strain of rhizobia was found to enhance nodulation, dry weight of nodules, nitrogen fixation and yield of common bean and other crops grown in dry land (Abdelgani *et al*., 2003).

Co-inoculation of PGPR and *Rhizobium* spp. increased root and shoot dry weight, plant vigour, nodulation, and nitrogen fixation in various legumes. The presence of PGPR in the root vicinity may also improve ability of rhizobia to compete with indigenous populations for nodulation. Srinivasan *et al.* (1997) reported that *Bacillus* sp. enhanced the nodulation in common bean when co-inoculated with *Rhizobium etli.* Nitrogen fertilization improved dry matter production but depressed nodulation. Phosphate mitigated the depressive effect of nitrogen on nodulation and further enhanced its stimulatory effect on dry matter production (Mahdi and Mustafa, 2005). Single *Rhizobium* inoculation of *P. vulgaris* has showed significant difference for the studied parameters (Mohamed Ahmed *et al.*, 2009).

It is still a matter of controversy whether the stimulation of plant growth and N fixation by coinoculation is due to either an increase in root surface by hormonal effects or nodulation and nutrient uptake. In relation with physiological and biochemical changes, Groppa *et al.* (1998) found that upon coinoculation with *B. japonicum* no significant differences were detected in total dry matter production. Nitrogen content of coinoculated plants was significantly increased by 23% as compared with plants inoculated only with *B. japonicum*. Accordingly, a strong stimulation of acetylene reduction activity and a significant increase (39%) in leghemoglobin content were observed using this treatment. Peoples *et al.* (2009) reported that common bean might be expected to fix <50 kg shoot N ha^{-1}. Rondon *et al.*, (2006) reported that BNF by common beans increases with addition of *Rhizobium* CIAT 899 and bio-char (charcoal, biomass-derived black carbon) and, hence bean yield increased by 46% and biomass production by 39% over the control at 90 and 60 g kg^{-1} bio-char, respectively. Figueiredo *et al.* (2008) conducted a study in a greenhouse to assess the potential of PGPR on nodulation, biological nitrogen fixation and growth of *P. vulgaris* cv. *tenderlake*. The disinfected seeds werre inoculated with *Rhizobium tropici* alone and in combination with PGPR strain. Beans co-inoculated with *R. tropici* and *Paenibacillus polymyxa* had higher concentrations of leghemoglobin, nitrogenase activity and N-fixing efficacy and thus formed associations of greater symbiotic efficiency. Inoculation with *Rhizobium* and *P. polymyxa* strain stimulated nodulation as well as nitrogen fixation. *B. subtillis* DLA co-inoculated with *R. leguminosarum* bv. *phaseoli* increased growth and yield of bean plants under nitrogen-free conditions in comparision to plant inoculated with *R. leguminosarum* bv. *phaseoli* alone (Lee *et al.*, 2005).

BIOCONTROL

Most of pathogenic fungi survive in soil for several years in the form of resting bodies for example sclerotia of *M. phaseolina* survive for 2-15 years in soil depending on environmental conditions (Cook *et al.*, 1973; Short *et al.*, 1980) *F. oxysporum* can survive in soil by means of chlamydospores for about 3 years (Haware *et al.*, 1996), the capability of sclerotia of *S. sclerotiorum* survive for more than 4 years (Fernando *et al.*, 2004). Therefore, it becomes very difficult to manage the crop from the infection by fungi, which markedly reduce the potential of crop rotation as a disease management strategy. Regarding the environmental and health concerns, the extended use of chemicals to control these pathogens; there is considerable interest in finding alternative control approaches for use in biological control strategies for crop diseases (Raupach and Kloepper, 1998).

PGPR, which are sometimes called biopesticides or biocontrol PGPR, which mostly belong to *Pseudomonas* and *Bacillus* spp. Whipps (1997) found several reasons to study the antibiotics produced by *Pseudomonas* spp. They *(i)* are common inhabitants of rhizosphere and phyllosphere environments, *(ii)* are isolated easily from natural environments, *(iii)* utilize a wide range of substrates, *(iv)* are easy to culture and manipulate genetically, making them more amendable to experimentation. Bacterial biocontrol strains not only exhibit a wide range of diversity in the type but also in the number of antibiotics produced, *Bacillus cereus* strain UW85 (Handelsman and Stabb, 1996) and *P. fluorescens* strains CHA0 and Pf5 (Bender *et al.*, 1999) produce multiple antibiotics with overlapping or different degrees of activity against specific pathogenic fungi. Some biocontrol agents secrate antibiotics that may

account for the suppression of specific or multiple plant diseases. Many of the antibiotics produced by bacterial biocontrol agents have a broad-spectrum activity, *e.g.* the broad-spectrum activity of pyrrolnitrin produced by *Pseudomonas* and *Burkholderia* species (Nishida *et al.* (1965). Similarly, DAPG produced by several strains of *P. fluorescens*, not only has activity against a wide range of plant pathogenic fungi but also has antibacterial, anthelminthic and phytotoxic properties (Keel *et al*., 1992; Thomashow and Weller, 1996). Zwittermycin A, produced by *B. cereus* and *B. thuringiensis* adversely affects the growth and activity of a wide range of microorganisms, including several plant pathogenic fungi such as *Phytophthora* and *Pythium* species (Silo-Suh *et al*., 1998). Antibiotic production in bacterial biocontrol agents, for example *Pseudomonas* spp. is modulated by a wide range of endogenous factors, including the two component GacA/GacS regulatory system (Gaffney *et al*., 1994), cell density-dependent regulation via *N*-acyl homoserine lactones and sigma factors (Sarniguet *et al*., 1995). Wang *et al* (2002) reported that *Sinorhizobium morelense* isolated from root nodules of *Leucaena leucocephala* was highly resistant to some antibiotics, such as carbenicillin (1 mg ml^{-1}), kanamycin (500 µg ml^{-1}) and erythromycin (300 µg ml^{-1}). Antibiotics (phenazine and pyrrrolnitrin) extracted from *Pseudomonas chlororaphis* caused inhibition of sclerotial and spore germination, hyphal lysis, vacuolation, and protoplast leakage in a number of plant pathogens, including *S. sclerotiorum, Pythium aphanidermatum, Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii, Fusarium oxysporum, Alternaria solani* and *Botryodiplodia theobromae* (Zhang *et al*., 2004). Several *Bacillus* spp., also produced the antibiotic zwittermicin-A which may be antifungal to *S. sclerotiorum* (Zhang and Fernando, 2004).

Many species of rhizobia promote plant growth and also inhibit the growth of certain pathogenic fungi in both leguminous and non-leguminous plants, such as *M. phaseolina* (Deshwal *et al*., 2003b), *F. oxysporum* (Mazen *et al*., 2008), *F. solani*, *Rhizoctonia solani, Pythium* spp. (Huang and Erickson, 2007) producing several secondary metabolites which were similar to fluorescent pseudomonads in their mode of action. Most of methods for biocontrol of plant diseases have bean used as single biocontrol agents to a single pathogen (Raupach and Kloepper, 1998). This may partially account for the reported inconsistent performance by biocontrol preparations, because single biocontrol agents are not likely to be active in all soil environments in which they are applied, or against all pathogens that attack the host plant. Consequently, application of a mixture of introduced biocontrol agents would more closely imitate the natural situation and might broaden the spectrum of biocontrol activity, enhance the efficacy and reliability of control and allow the combination of various mechanisms of biocontrol without the need for genetic engineering (Janisiewicz, 1988). The biocontrol agents have different mechanisms or combinations of mechanisms which may be involved in the suppression of different plant disease; for example, inhibition of the pathogen by antimicrobial substances (antibiosis) (El-Mehalawy, 2004), or production of diverse microbial metabolites like siderophore, rhizobitoxin (Deshwal *et al*., 2003b) competition for nutrients supplied by seeds and roots and colonization sites (Essalmani and Lahlou, 2002) induction of plant resistant mechanism inactivation of pathogen germination factors present in seed and root exudates and degradation of pathogenicity factors of the pathogen such as toxins parasitism that may involve production of extracellular cell wall-degrading enzymes, for example chitinase that can lyses pathogen cell walls (El-Mehalawy, 2004), or plant growth enhancement in terms of better shoot height, root length, dry weight and root nodulation (Siddiqui and Mahmoud, 2001), and induction of plant defence mechanisms (Abdelaziz *et al*., 1996), through IAA production and other PGP activities (Deshwal *et al*., 2003a). Biocontrol of *M. phaseolina*, *F. oxysporum*, *F. solani*, *Sclerotinia sclerotiorum*, *R. solnai* and *Choletotricum* sp. in dual culture and CFCF (cell free culture filtrate) by PGPR such as *Rhizobium leguminosarum* RPN5, *Bacillus subtilis* BPR7 and *Pseudomonas* sp. PPR8 isolated from root nodules and rhizosphere of common bean respectively have been carried out by Kumar (2012).

CONCLUSION

Common bean is an important pulse crop of India. Due to nutritious nature, its demand is increasing day by day which can be achieved by using either by chemical fertilizers or biofertilizers. But the use of chemical fertilizers is expensive and harmful for soil health. Biofertlizers are the best alternative to chemical fertilizers. In this study we have reviewed different plant growh promoting rhizobacteria including *Rhizobium*, *Bacillus* and *Pseudomonas* (either in monoculture or in combination) that can enhance the growth and yield of common bean by providing growth hormone, minerals to plant and protection from several phytopathogens.

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Microbial Diversity in Coastal Mangrove Ecosystems

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ABSTRACT

Mangroves are salt tolerant plants existing in the inferface between land and sea in the tropical and subtropical latitudes. The mangrove forests are one among the World's most productive ecosystems with great ecological and economic significance. Mangrove ecosystem supports the diverse microbial groups due to its complex forest structure and microhabitats associated with many floral and founal species. The mangrove microbes in particular bacteria were responsible to improve the fertility of mangrove ecosystems. In India, the mangrove ecosystems are known to have a total of 4,011 biological species and no other country in the world has recorded so many species to be present in mangrove ecosystems. Totally 502 microbial species including microalgae (307), fungi (103), bacteria (69), and actinomycetes (23) were reported from Indian mangrove ecosystems. The wide range of physico-chemical conditions of mangrove ecosystem force the microbes to produce vast number of enzymes with unique activities and bioactive ecompounds with new structures and novel properties. Exploitation of microbial diversity from Indian mangrove ecosystems will also pave the way for the discovery of novel metabolites.

Key words: mangroves, microbial diversity, bioactive metabolites, Indian mangroves

INTRODUCTION

Mangroves are the only woody halophytes that grow in tropical and sub-tropical latitudes along the interface between land and sea, bays, estuaries, lagoons, backwaters. The mangroves are unique to thrive in the habitat where no other trees can survive. They make up one of the world's most productive forest ecosystems, often called as 'tidal forests', 'coastal woodlands' or 'oceanic rainforests'(Kathiresan and Bingham, 2001; Kathiresan and Qasim, 2005). The mangroves in association with microorganisms and animals constitute the 'mangrove forest community' or 'mangal'. The mangal and its associated abiotic factors constitute the 'mangrove forest ecosystem' (Kathiresan and Bingham, 2001). In other words, the mangrove forest ecosystem is complex of plant, animal and microbial communities and their non-living environment, interacting as a functional unit.

Mangroves are ecologically important and economically significant in seven major aspects: (i) protecting shores from wind, waves, water currents and natural calmities like flood, cyclone and tsunami; (ii) preventing soil erosion and siltation; (iii) protecting coral reefs, seagrass beds and shipping lanes; (iv) supplying wood and other forest products like honey, medicine *etc.*; (v) providing important nursery grounds, feeding and breeding sites for a variety of organisms including crustaceans, shellfish, finfish, birds, reptiles and mammals; (vi) supporting coastal fisheries and livelihood; and, (vii) serving as accumulation sites for sediment, pollutants, carbon and nutrients (Kathiresan and Qasim, 2005).

The most significant ecological significance of mangroves is that this ecosystem provides a unique ecological niche for coastal biodiversity especially diverse microbial communities, for the reason that the mangrove ecosystem is a detritus-based one, where the activities of microbes are predominant in

decomposing organic matter, making protein rich detritus food for fishes, recycling of nutrients, carbon fluxes as well as climate change. However, the microbial studies in the mangrove ecosystem are scattered and their significance is largely ignored especially for bioprospecting potential. Hence, purpose of this chapter is to consolidate the information about microbes present in the coastal mangrove ecosystem, based on the obvious questions: (i) what are the microbes present in the mangroves?; (ii) How do these microbes influence the ecosystem as a whole?; and (iii) do the microbes in mangroves have bioprospecting potential for their utility?

WHY IS MANGROVE SYSTEM MICROBIALLY RICH?

Mangrove forest ecosystems support diverse groups of microorganisms. This is due to complexity of forest structure and microhabitats, associated with many floral and faunal species. The calm waters in the forests are ideal nursery and breeding grounds for fish and shellfishes, while the aerial roots, lower trunks of trees and forest floor support a varied fauna of oysters, snails, barnacles, crabs and other invertebrates. The forest also supports terrestrial animals like birds, reptiles, insects and mammals. The mangrove systems are biologically diverse especially in terms of microbes, due to the following reasons:

(i) The mangrove systems include diversified habitats such as core forests, litter-forest floors, mudflats, adjacent coral reefs and seagrass ecosystems, and the contiguous water bodies, which consist of rivers, bays, intertidal creeks, channels and backwaters;

(ii) The mangrove systems are located along the interface between land and sea and hence they are exposed to a wide range of environmental conditions; and,

(iii) The mangrove systems support genetically diverse groups of both aquatic and terrestrial species which have tolerance to a wide range of environmental conditions.

MARINE MICROBIAL DIVERSITY

Marine microbes are taxonomically diverse, genetically special and largely unexplored species. It is estimated that there are 10^{29} prokaryotes in the world's ocean, and there should be about 10 times more viruses. The number of prokaryotic microbes is obviously abundant in the ocean, as compared to eukaryotic microbes; however, biomass in both the types is probably the same. With more than a billion individual microbes in a liter of seawater, the microbes constitute more than 90% of oceanic biomass! (Kathiresan and Duraisamy, 2006). Ocean life is thus microbe-based. Marine microbial weight is remarkably equivalent to the weight of 240 billion African elephants. Total diversity of marine microbes is over five millions and this high diversity is due to mutations in the past 3.5 billion years. The marine bacteria are 100 million times more than stars. There are 100 major phyla and 18 million DNA sequences of marine microbes.

In India, the mangrove ecosystems are known to have a total of 4,011 biological species that include 920 floral species and 3,091 faunal species (Table 1). No other country in the world has recorded so many species to be present in mangrove ecosystems. Microbes alone constitute about 85% of total floral species. Of the biological species, the faunal species occupy about 77% and the floral species 23%. Thus, the faunal species component is about 3.5 times greater than the floral component of the mangrove ecosystem. Microbes are present on both surface and inside the plants as epiphytes and endophytes and on body surface and gutflora of the animals. A checklist of floral and faunal species associated with mangrove ecosystems of India is given in the table 1.

Marine Microbes alone constitute 85% of total floral species that is 502 out of 920 floral species present in mangrove ecosystems (Table 2). The mangrove regional diversity of microbes is shown in Table 3. It is clear that larger the mangroves and larger is the marine microbial diversity.

Table 1. Total numbers of floral and faunal species reported to exist in mangrove ecosystems of India (Kathiresan and Qasim, 2005; Kathiresan, 2000).

No.	Groups	No. of Species
	Floral groups	
1	Mangroves	39
2	Mangrove associates	86
3	Seagrass vegetation	11
4	Marine algae (Phytoplankton + seaweeds)	557
5	Bacteria	69
6	Fungi	103
7	Actinomycetes	23
8	Lichens	32
	Faunal groups:	
9	Prawns and lobsters	55
10	Crabs	138
11	Insects	707
12	Mollusks	305
13	Other invertebrates	745
14	Fish parasites	7
15	Fin fish	543
16	Amphibians	13
17	Reptiles	84
18	Birds	426
19	Mammals	68
Total number of species		**4011**

Table 2 Number of species in different marine microbial groups of mangrove ecosystems of India

Marine microbial group	No. of species recorded	% of total
Microalgae	307	61.2
Fungi	103	20.5
Bacteria	69	13.7
Actinomycetes	23	4.6
Total	502	100

Table 3 Mangrove regional diversity of microbes present in mangrove ecosystems of India

No.	*Name of Species*	*East*	*A & N*	*West*
1	Algae	199	41	152
2	Bacteria	58	13	10
3	Fungi	57	59	80
4	Actinomycetes	23	5	11
Total		337	118	253

MICROALGAL SPECIES

'Plankton' are drifting or passively swimming organisms. Plant components of the plankton community are phytoplankton or microalgae. The total biomass of phytoplankton is many times greater than that of the total plants found on land, and they serve as the "pasture grounds" in the aquatic environment. There would be no life in the sea, if phytoplankton are absent. The microalgae form the basis for marine food-web by fuelling energy to the higher trophic levels by providing the products of their photosynthesis. The phytoplankton contribute to the organic production substantially in the mangrove ecosystem. The biomass, productivity and size of phytoplankton form important elements in regulating the diversity and abundance of higher forms of life in the mangrove ecosystem. Microalgae including blue green algae (cyanobacteria) undoubtedly contribute a great deal to overall primary productivity in the mangroves. The contribution of plankton to the total net production in mangroves ranges from 20 to 50% (Robertson and Blaber, 1992). Similarly, benthic microalgal communities also make important contributions to the well-being of the mangrove environment. Nevertheless, the overall ecological significance of microalgae in the mangrove ecosystem is not yet properly understood. There are 307 species of algae under four families in mangroves of India.

BACTERIAL SPECIES

The mangroves provide unique ecological environment for diverse bacterial communities. The bacteria are particularly important in controlling the chemical environment of the mangrove system. Sub-surface bacterial communities (along with epibenthic microalgae) may sequester nutrients and bind them within nutrient-limited mangrove mud (Kathiresan and Bingham, 2001). Next to trees, bacterial flora dominates the biomass and productivity of mangrove forests. A great deal of effort is required to identify the bacteria up to the species level in the mangrove habitat.

Fertility of the mangrove waters results from the decomposition of organic matter by microbes leading to recycling of nutrients. Among the microbes, the bacterial populations in mangroves are many-

fold greater than those of the fungi. The former exists as symbionts with plants and animals, saprophytes on dead organic matter, and often as parasites on living organisms. The bacteria perform different functions in the mangrove ecosystems such as photosynthesis, nitrogen fixation, methanogenesis, magnetic behaviour, human pathogens, production of antibiotics and enzymes - arylsulphatase, L-glutaminase, chitinase, L-asparaginase, cellulase, protease, phosphatase (Kathiresan, 2000a).

Cyanobacteria are an important component of the microbial flora of mangroves. Six species dominantly occur in the root-soil of mangroves and they are *Spirulina subsalsa*, *Phormidium tenue*, *P. fragile*, *Synechocystis salina*, *Oscillatoria willei* and *O. cortiana*. Their counts vary between $3.1x10^3$ and $4.1x10^4$ colony forming units per gram of soil, with the maximum occurrence during summer (May) and the minimum in post monsoon (December) along the east coast of India (Nabeel et al., 2009).

Lactobacilli are beneficial bacteria that occur abundant in the root-soil of mangroves and the lactobacilli do occur in fresh and decomposing marine organisms including sponges, seaweeds, shellfish, crabs and fishes (Ringo et al., 1998). The dominant species of marine lactobacilli are *Lactobacillus delbrueckii*, *L. lactis*, *L. casei*, *L. xylosus*, *L. palntarum* and *L. curvatus*. Their counts vary from $3x10^2$ to $3.1x10^4$ colony forming units per gram of soil with the maximum occurrence in post monsoon (November) and the minimum in summer (May) along the east-coast of India (Thiruneelankantan et al., 2009).

In total, 69 species of bacteria, falling under 26 genera have been reported. East coast is represented by 58 species, and the Andaman and Nicobar Islands by 13 species, whereas only 10 species have been recorded so far from the west coast.

Much more studies are required on purple sulphur bacteria and purple non-sulphur bacteria. Unlike aerobes, photosynthetic anaerobes use H_2S as electron donor in stead of water and they contribute significantly to the productivity of anaerobic domain of mangrove ecosystem.

Marine viruses are actively infecting bacteria in nutrient-rich waters, whereas they are abundant in nutrient poor waters. They control microbial diversity and operate food chain. They activate carbon cycle through enhanced oceanic respiration and also facilitate genetic recombinations among marine bacteria. Studies on marine viruses in mangroves are far from clear.

FUNGAL SPECIES

Marine fungi in mangroves play a significant role in litter decomposition and nutrient cycling, thereby contributing to the fertility of the environment. Fungal biomass along with detritus contributes significantly to the food-chain of detritus-feeding organisms found in mangroves. *Aspergillus* and *Penicillium* are dominant fungi involved in litter decomposition of mangroves. Since the time of Barghoorn & Linder (Barghoorn and Linder, 1944) till now, marine fungi have been extensively studied, and particularly the wood-inhabiting fungi. These have been termed as “lignicolous fungi”, constituting more than 50% of the total 450 species of obligate marine fungi described so far. About 150 species are found exclusively on decaying mangrove wood, aerial roots and seedlings, and are categorised as “Manglicolous fungi”; most of the species belong to the class of Ascomycetes.

Of the 150 species, more than 30 species of marine fungi are found in the mangroves of the New World. Only a few seem to be host-specific Linder (Kohlmeyer and Kohlmeyer, 1979). For example, Avicennia *germinans* specifically acts as a host for the fungal species - *Rhabdosphora avicenniae*, and *Mycosphaerella pneumatophorae*, whereas *Rhizophora mangle* forms another host for *Didymosphaeria rhizophora* and *Leptosphaeria australensis.*

Marine fungi infest submerged roots, stems, and twigs as well as sessile animals and algae. They are quite abundant in the mangroves due to easy availability of wood as their bait. Large numbers of fungi are found on mangroves such as *Avicennia marina, A. officinalis, Rhizophora mucronata, R.*

apiculata, and *Sonneratia alba.* The fungal biodiversity in the mangroves may reflect the age of the plants. A well-developed mangrove habitat provides a larger number of fungal species than the new mangrove sites.

Mangrove ecosystems are rich in fungal biodiversity. In Kerala, a total of 17 pathogenic fungi, 32 endophytic fungi and 14 wood-degrading fungi (lignicolous fungi) have been recorded (Mohanan, 2008). *Cytospora* species are associated with the die-back disease of *Sonneratia caseolaris* whereas both *Cytospora* and *Endothia* species are responsible for stem infection in *Rhizophora mucronata*. Three lignicolous fungi are widely distributed and they are *Hexagonia apiara*, *Microporus xanthopus* and species of *Phellinus*. All the mangrove roots are in association with arbuscular mycorrhizal fungi ranging from 2% in *Kandelia candel* to 80% in *Aegiceras corniculatum*. The mycorrhizal fungi occur in the root-soil of mangroves in a range from 1.9 to 31.4 per gram of soil. Among the mycorrhizal fungi, *Acaulospora*, *Glomus* and *Gigaspora* are the widely distributed genera. The dominant endophytic fungi are *Cladosporium cladosporides*, *Colletotrichum gloeosporioides* and species of *Phoma*, *Phomopsis*, *Phyllosticta* and *Nigrospora* (Mohanan, 2008).

Yeasts are a group of basidiomycetes and ascomycetous fungi. The dominant species in the root-soil are *Candida tropicalis*, *C. albicans*, *Cryptococcus dimennae*, *Debaryomyces hansenii*, *Geotrichum* sp., *Pichia capsulate*, *P. fermentans*, *Pichia salicaria*, *Sachharomyces cerevisiae*, *Rhodotorula minuta*, *Trichosporan* sp., and *Yarrowia lipolytica*. Their counts range from $1x10^2$ to $1.4x10^4$ colony forming units per gram of soil with the maximum occurrence during post monsoon (November) and the minimum in Summer (May) (Manivanan and Kathiresan, 2009). Altogether, there are 103 species coming under 67 genera. East coast is represented by 57 species, and Andaman and Nicobar Islands with 59 species, whereas the west coast has been reported to have 80 species.

ACTINOMYCETES SPECIES

Actinomycetes are unique microorganisms having both characteristics of bacteria and fungi. They are best known for their ability to produce antibiotics, vitamins, enzymes, pigments, nutrients *etc*. The actinomycetes are Gram-positive and produce cotton-like structures. Of these, streptomycetes form the dominant group, whereas all other genera include rare forms of secondary importance.

Mangrove habitat, by virtue of its fluctuating physical and chemical conditions, may become a potentially high source for isolating the actinomycetes capable of producing novel and useful metabolites. However, little work has been done so far on the biodiversity of actinomycetes in mangroves. Some of the studies carried out in India are of Sivakumar, 2001; Kala and Chandrika, 1993; Balagurunathan, 1992; Lakshmanaperumalsamy, 1978.

In total, there are 23 species coming under 4 genera. Most of the species (20) belong to the genus - *Streptomyces*. Only three rare actinomycetes have been recorded. Two new species, reported for the first time from India, are: *Actinopolyspora indiensis* and *Streptomyces kathirae* (Sivakumar, 2001). East coast is represented with all the 23 species, whereas from the Bay of Bengal Islands, no published information is available. From the west coast, only 11 species (under 2 genera) have been recorded.

CONCLUDING REMARKS

The biggest challenge is how to culture the unculturable microbes. Our ability to culture and study them under laboratory conditions is relatively difficult and only 0.1% is culturable. Reason for this may be that the microbes growing in oligotrophic conditions of the ocean area grown under high nutrients conditions in laboratories. Among the culturables, some of the microbes especially rare actinomycetes are slow growing. There is an urgent need to develop new culture techniques to isolate the slow-growing microbes and those microbes that are unique in production of novel natural products (Jensen et al., 1996). The genome sequencing makes it possible to visualize potential metabolic and biochemical capabilities of

even unculturable marine microbes. One of the future research trends will be focused on bio-active substances derived from non-culturable marine microorganisms.

Main difficulty is identification of marine microbes and this is due to lack of microbial taxonomists and non-availability of proper monographs. Genetic tools are available for a negligible number of the known marine microbes. The use of rRNA sequencing to identify marine bacteria provided significant information on the phylogeny of marine taxa, especially those of the ecologically important Vibrionaceae group. Immunoflorescent-epiflorescent techniques are useful in sensitive detection of human pathogens in coastal environments. Future development of marine microbiology depends on new methodologies and instruments yet to be constructed.

The marine microbes occur in varied physicochemical conditions of mangrove ecosystems. These wide ranges of conditions force the microbes to produce a vast number of enzymes with unique activities and pharmaceutical active compounds of new structures and novel properties. Traditionally, antibiotic-producing organisms were isolated from the terrestrial sources. Only recently the potentials of marine microbes are known. An intensive search for antibiotics from marine microbes is now felt necessary for treating infectious diseases that are caused by pathogenic bacteria and fungi. This poses a serious challenge for current pharmacology. The microbial secondary metabolites can be brought in use in three different ways: (i) the bioactive molecule can be produced directly by fermentation; or (ii) the fermentation product can be used as starting material for subsequent chemical modification; or (iii) the molecules can be used as lead compounds for a chemical synthesis. But there also exist major weakness in the technology for conducting drug screens and industrial fermentation with marine microorganisms as it is estimated that at least 99% of marine bacterial species do not survive on laboratory media. Furthermore, available commercial fermentation equipments are not optimal for use in saline conditions, or under high pressure (Lene, 1996). The greatness of smallness deserves much attention.

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Studies on Cadmium Biosorption by Cadmium Resistant Bacteria from Uppanar Estuary, Cuddalore, Southeast Coast of India

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ABSTRACT

This paper described the study on isolation, characterization and screening of cadmium resistant bacterial strains isolated from Uppanar estuarine environment in Cuddalore, Tamil Nadu and also investigated biosorption status of those cadmium resistant strains. On the basis of morphological, physiological and biochemical characteristics, six strains were identified as *Bacillus* spp., *Enterobacter* sp., *Aeromonas* spp.,and *Pseudomonas* sp., A representative strains of *Bacillus* sp., *Enterobacter* sp., *Aeromonas* sp. and *Pseudomonas* sp. were chosen for analysis of biosorption study. The biosorption rates of those resistant strains were assessed in the initial concentration of 100 ppm containing marine broth. All the strains were reduced cadmium from broth at different concentrations. The uptake of cadmium by these isolates from broth was increased as the incubation increased. From this experiment *Pseudomonas* sp. showed higher absorption activity than other cadmium resistant bacteria.

Key words: Uppanar estuary, cadmium biosorption, bacteria

INTRODUCTION

Microorganisms play a vital role in the detoxification or biotransformation of toxic chemicals into non-toxic form and also play a promising role in environmental cleanup processes. Cadmium is toxic heavy metals cause damage to living creatures, including human beings. Cadmium is relatively rare element found in natural deposits as ores containing other element which is extensively used for electroplating, paint pigments, plastics, silver–cadmium batteries, and coating operations, including transportation of equipment, machinery and baking enamels, photography and television phosphors. It is also used in nickel–cadmium batteries (Volynskii et al., 2006), solar batteries and in pigments. Industrial aqueous effluents contain heavy metals (mercury, cadmium, lead, Zinc and Copper etc.,) and other toxic wastes which are discharging from chemical, pharmaceutical and other industries. The impact of these industrial wastes on the aqueous environment has been extensively studied by many researchers. Conventional chemical processes for removing these wastes may be ineffective or extremely costly. Some bacteria are able to resist heavy metal contamination through chemical transformation, reduction, oxidation, methylation and demethylation (Nascimento et al., 2003). The uptake, adsorption and accumulation of heavy metals by microbial biomass are receiving in increasing attention (Roig et al., 1997; Williams et al., 1998). Biological processes have been employed in bioremediation, including metal recovery are potentially low cost. The uptake of metals by microbes is an efficient process for decreasing the concentration of metal from aqueous solution. Uptake of metals is possible on both dead and living biomass (Baillet et al., 1997). The cadmium uptake capacities have been studied in many microorganisms: *Citrobacter* (Macaskie et al., 1987), *Arthrobacter, Pseudomonas, and Enterobacter* (Scott et al., 1990), *Streptomyces* (Mattuschka et al., 1993), *Bacillus circulans* (Sahoo et al., 1992), *Thiobacillus ferrooxidans* (Baillet et al., 1997) etc., in pure culture, or in consortium. Both antibiotics and metals resistant microorganisms have been isolated from metal-contaminated environments such as estuaries (Allen et al., 1977; Timoney et al., 1978), soils (Lighthart et al., 1979) and sewage (Varma et al.,

1976). Metal contamination in natural environments could have an important role in the maintenance and proliferation of antibiotic resistance (Summers et al., 1993; Alonso et al., 2001). The purpose of this study is to investigate the cadmium removal activity of cadmium resistant isolates from Uppanar estuary and also determine whether such bacteria also exhibit resistance to antibiotics.

MATERIALS AND METHODS

Study area and sampling site: The Uppanar estuary is located at Cuddalore (Lat. 11º 43’ Long. 79º 49’) and it originates from the north eastern part of the Shervarayan hills and opens into the Bay of Bengal near Cuddalore. Uppanar estuary receives untreated and partially treated industrial effluents containing toxic wastes from SIPCOT (State Industrial promotion Corporation of Tamilnadu Limited) industrial complex. In addition to the industrial wastes, the estuary also receives the municipal wastes and domestic sewage from Cuddalore old town and also waste from coconut husk retting. In the present study two stations were selected in Uppanar estuary namely station I (Naduthittu) and station 2 (Thaikkal thonithurai). Station 1 is the upstream of the estuary where the discharge of effluents starts and station 2 is close to the mouth where all the discharged effluents are mixing and diluting (Fig.1).

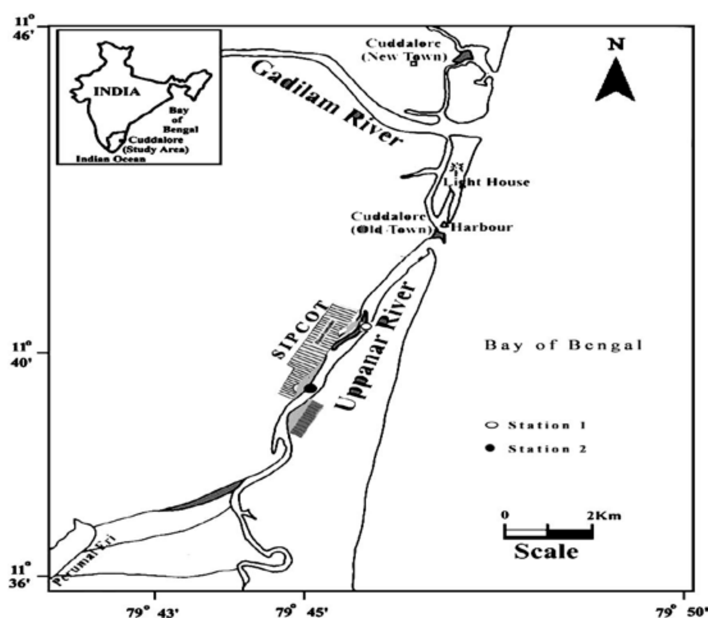

Fig.1. The map shows the study area

Screening and identification of cadmium resistant bacteria: The water samples were collected from polluted sites of Uppanar estuary in station 1 and 2 in Cuddalore and were subjected to serial dilution and plating technique to enumerate the microbial population. The isolated colonies were plated on marine agar amended with 10 ppm $CdCl_2$ to screen the ability for resistant to cadmium ions. Bacterial colonies which showed better growth in $CdCl_2$ plates was taken and streaked on agar slant and stored. The cadmium resistant bacterial colonies were identified based on morphological and biochemical characteristics for further studies. The isolated cadmium resistant bacterial strains identified according to Bergey’s manual of determinative bacteriology (Buchanan and Gibbons, 1974).

Antibiotic susceptibility by resistant isolates: Of these resistant isolates, the representative cadmium resistant isolates were tested for its sensitivity to the 3 antibiotics (Ampicillin, Penicillin, and Tetracycline) (Bauer et al., 1966). The cadmium resistant isolates were swabbed onto Muller Hinton Agar

plates. Antibiotic disc were placed on the plates and were incubated in room temperature at 48 hours. Antibiotic susceptibility patterns were read after incubation.

Bacterial growth rate and Biosorption experiments: All the identified resistant colonies were grown in marine broth. Cadmium stock solution (as $CdCl_2$) to a final concentration of 100 ppm was added to the marine broth prior to inoculation which is the master flask. The flasks in duplicate were incubated on a rotary shaker (120 rpm) at room temperature (approximately 28°C ± 2°C) for 120 hour. One ml samples were withdrawn from master flask aseptically at an interval of 24, 48, 72 and 120 hours and the supernatant and cells were separated by centrifugation at 12,000 rpm for 15 min. The supernatant were digested separately (De et al., 2008). Cadmium removal from medium was analyzed from the supernatants using an Atomic absorption spectrometry.

The cadmium removal rate (η_{cd}) was measured by the following formula (Deng et al., 2007)

$\eta_{cd} = [(a-b)/a] \times 100\%$

Where, a – Mass of cadmium in the solution without bacteria

b – Mass of cadmium in the solution with bacteria

From the duplicate flask, the bacterial growth rate was determined by measuring the optical density of the cell suspension at 600 nm at 24 hours time interval (0, 24, 48, 72 and 120 hours) using UV-visible spectrophotometer.

RESULTS AND DISCUSSION

Totally twenty one different colonies were isolated from the two stations of Uppanar estuary, Cuddalore. Altogether 10 colonies were isolated from station – 1 and remaining 11 colonies from station – 2. Among the 21 colonies, six were resistant to cadmium chloride i.e. shows better growth and remaining strains are sensitive (no growth). Hence, the six colonies were taken to further studies. The six cadmium resistant bacterial isolates were identified as. *Bacillus* sp., *Enterobacter* sp., *Aeromonas* sp. and *Pseudomonas* sp. by using Bergey's manual. Of these, *Bacillus* sp. and *Aeromonas* sp. were found in both stations. From these resistant isolates, gram negative bacteria were mainly present (*Enterobacter* sp., *Aeromonas* sp, and *Pseudomonas* sp.) than the gram positive species *Bacillus* sp. As a result, Gram negative bacteria have higher tolerance ability to heavy metals particularly cadmium than gram positive bacteria (Eagon et al., 1984; Vasanthy et al., 2004).

Antibiotic sensitivity test: The results of this study shows, all the isolated resistant strains were sensitive to Tetracycline (30 mcg) but slightly different from other two antibiotics (Table: 1). Of these *Bacillus* sp., *Enterobacter* sp. *Aeromonas* sp. were resistant against both Ampicillin and penicillin i.e. no zone formation around the disc.

Table 1: Antibiotic profile for cadmium resistant isolates.

Antibiotic	*Bacillus sp.*	*Enterobacter sp.*	*Aeromonas sp.*	*Pseudomonas sp.*
Ampicillin (10 unit)	R	R	R	R
Penicillin (10 unit)	R	R	R	R
Tetracycline (30 mcg)	S	S	S	S

Where, R = resistant, S = sensitive

Under heavy metal stress environmental condition, the microorganisms might have developed various mechanisms to resist antibiotics and tolerate metals. Microbes surviving in polluted soils usually change intrinsic biochemical and structural properties, physiological and genetic adaptation (Zeng et al., 2009).

In cadmium stress condition, growth rates of resistant bacterial strains were entirely different (Fig.2). *Pseudomonas* sp. has outstanding growth against cadmium stress compare than other resistant bacterial strains.

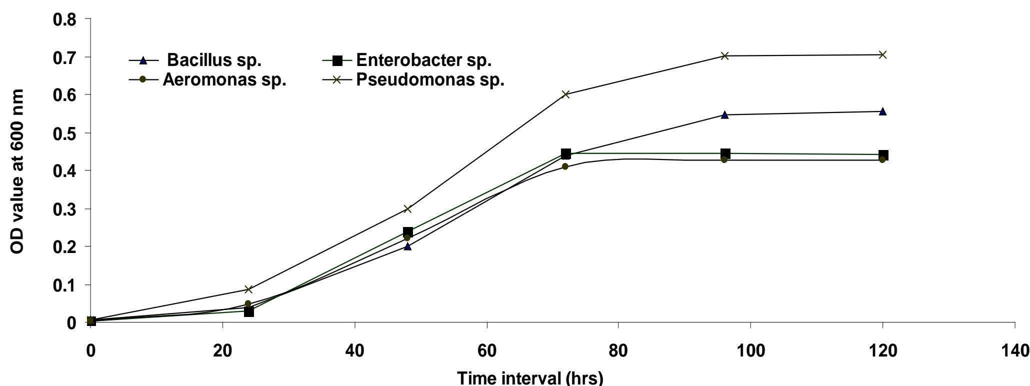

Fig. 2: Growth rate of resistant bacterial isolates under cadmium stress condition

From this study, the cadmium removal activities of four bacteria stains were slightly different. All the resistant isolates absorbed cadmium from the medium rapidly i.e within 24 hours. The maximum removal activity was found in *Pseudomonas* sp. (99 % removed from medium) compare to other isolates. The maximum biosorption of Cd by *Pseudomonas* sp. was recorded in 120 hours of incubation (Fig.3). As a result, the incubation period is increased the percentage of biosorption status were also increased (Parameswari et al., 2009).

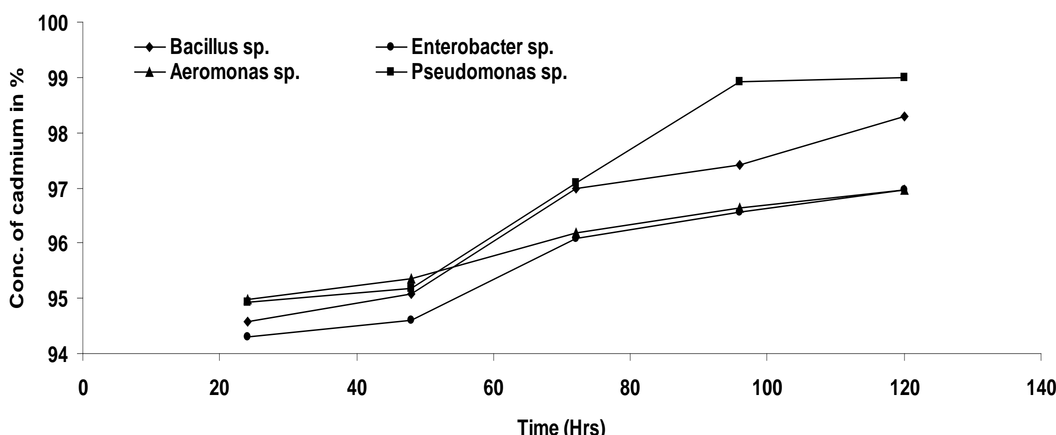

Fig. 3. Percentage of Cd removel from 100 ppm of $CdCl_2$ aqueous solution

Many researchers reported that the rate of absorption was observed in two phases, an initial phase of faster absorption followed by the phase of slower absorption. The initial faster uptake might be due to the availability of abundant metal species and empty metal binding sites of the microbes. The slower phase might be due to saturation of metal binding sites (Garnham et al., 1992). The present study established that resistant bacterial isolates are efficient in the cadmium removal from solution. These results suggest that *Pseudomonas* sp is very efficient for cadmium biosorption based bioremediation.

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Isolation and Characterization of Plant Growth Promoting Rhizobacteria (PGPR) from Vermistabilized Organic Substrates and their Effect on Plant Growth

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ABSTRACT

Plant growth promoting bacteria are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly. The ability to produce or change the concentration of plant growth regulators like indole acetic acid, gibberellic acid, cytokinins and ethylene, asymbiotic N_2 fixation, antagonism against phytopathogenic microorganisms by production of siderophores. Due to the awareness of deleterious effects of chemical fertilizers on soil and environment, in recent years there is an increasing emphasis to develop technology to produce organic manures from agro-industrial and other organic wastes. Hence, the present study is aimed to treat the paper mill sludge by bioconversion and to investigate the physicochemical parameters, in the vermicompost using *Eisenia fetida*. Total bacterial count, bacterial genera and Plant Growth Promoting Rhizobacteria (PGPR) were analyzed in the vermicompost. The effect of the inoculation of plant growth promoting bacteria and vermistabilised organic substrates on the black gram (*Vigna mungo*) was studied at lab scale. The results of the study revealed that the vermicompost showed increased bacterial population count in paper mill sludge + cow dung medium than worm un-processed (control) medium. The results of plant growth revealed a beneficial effect of vermicompost and PGPR isolates on *V. mungo*.

Key words: Earthworms, PGPR, plant growth, vermicompost, *Vigna mungo*.

INTRODUCTION

Microorganisms are important in agriculture in order to promote the circulation of plant nutrients and reduce the need for chemical fertilizers as much as possible. Plant growth-promoting rhizobacteria (PGPR) are able to exert a beneficial effect upon plant growth. N_2-fixing and P-solubilizing bacteria may be important for plant nutrition by increasing N and P uptake by the plants, and playing a significant role as PGPR in the biofertilization of crops. Soil bacteria that colonize plant roots and promote growth when added to seeds, roots or tubers have been termed PGPR (Kloepper et al., 1980). Different plant-growth promoting rhizosphere bacteria, including associative bacteria such as *Azospirillum, Bacillus, Pseudomonas* and *Enterobacter* groups have been used for their beneficial effects on plant growth (Kloepper and Beauchamp, 1992). The mechanisms of plant growth stimulation by associative bacteria are mobilization of nutrients (Lifshitz et al., 1987), stimulation of root growth by production of phytohormones (Bothe et al., 1992; Kloepper et al., 1980) and antagonism against soil borne plant pathogens.

However, much of the work in microbial-earthworm interactions has focused on their functional significance, and little is known about the effects of earthworms on microbial diversity (Karmegam and Daniel, 2009a, b; Prakash et al., 2009; Prakash and Karmegam, 2010 a, b). However, the studies on the vermistabilization, nutrient, microbiological status of vermicompost of paper mill sludge and influence of plant growth have not been reported. Hence, the present study has been carried out to establish the

nutrient, microbiological status of the vermicompost produced from paper mill sludge and influence the plant growth of black gram (seed bacterization with PGPR) using the earthworm, *Eisenia fetida*.

MATERIALS AND METHODS

Paper mill sludge and earthworms: The raw material, paper mill sludge (PMS) was collected from Paper Packaging Ltd., Chennai, India. The earthworm, *Eisenia fetida* Mich. for the study, collected from culture bank of the Department of Biology, Gandhigram Rural University, Tamilnadu, India was mass multiplied in cow dung and used for the study.

Vermicomposting of paper mill sludge (PMS): The paper mill sludge (PMS) were subjected to vermicomposting trials in plastic troughs. The trials were carried out using PMS spiked with CD in three different combinations, i.e., 25:75, 50:50 and 75:25 respectively for each substrate, in six replicates and introduced into standard plastic tubs of size 45 cm x 35 cm x 15 cm accommodating 2.0 kg of the materials. Simultaneously, control sets were also maintained without earthworms for all the combinations. The vermibed materials were maintained with a moisture content 70±5% by sprinkling water and the vermibed substrates were subjected to mixing and turning once in 15 days. All the experiments were carried out at an ambient temperature (26±3°C) (Karmegam and Daniel 2009 a).

Physico-chemical analysis: Determination of pH was done by a digital pH meter, electrical conductivity by a conductivity meter (Elico) using 1:10 (w/v) compost - water (double distilled) suspension. The moisture content was determined after drying at 105°C for 24 hrs. Total organic carbon (TOC) was measured and using the method (Walkley and Black, 1934). Total Kjeldhal Nitrogen (TKN) was determined after digesting the sample with concentrated H_2SO_4 and concentrated $HClO_4$ (9:1, v/v) (Tandon, 1993). Total phosphorus (TP) was analysed using colorimetric method with molybdenum in sulphuric acid. Total potassium (TK) and total calcium (TCa) were determined, after digesting the samples in diacid mixture (conc. HNO_3: conc. $HClO_4$, 4:1 v/v), by flame photometer (Tandon, 1993). Total Fe and Zn were determined by means of atomic absorption spectrophotometer after digestion of the sample with concentrated HNO_3 (4:1, v/v) (Tandon, 1993).

Microbiological analysis: One gram of each sample was transferred to test tubes containing sterilized water, mixed thoroughly with a vortex mixer for 20 min, serially diluted and 1-mL aliquots were plated in triplicate. This was used as inoculum and 1.0 mL was plated in triplicate on Nutrient agar media, Rose Bengal agar and Kenknight's media for the enumeration of bacteria, fungi and actinomycetes, respectively using pour plate method, and incubated for 24, 72 h and one week.

Plant growth studies using vermicompost of paper mill sludge: Plant seeds (black gram – *Vigna mungo* L. Hepper) were obtained from the Govt. District Agriculture institute, Kancheepuram, Tamilnadu, India. A lab scale (Pot) experiment was carried out in the red Soil of Kancheepuram during season of November 2009 to March 2010 to study the effect of PMS vermicompost and NPKS fertilizers on growth, yield and yield components of *V. mungo*.

Seed bacterization: PGPR was isolated from the rhizosphere soil of chickpea and selected for this experiment based on its *in vitro* performance. The bacterial species includes *Bacillus, Pseudomonas, Azotobacter* and *Rhizobium* species were selected from among 30 bacterial strains for this study, because of its unique ability to produce plant growth hormones like indole acetic acid production, ammonia production, catalase production, siderophore production also solubilizes P, and inhibition of pathogens by antimicrobial compounds.

The method of Weller and Cook (1983) was followed for seed bacterization; of Black gram (*Vigna mungo)* were surface sterilized with 1% NaCl for 3–5 min, washed in sterilized distilled water (SDW) 3–4 times and air dried. Bacterial cells of *Bacillus sp., Pseudomonas sp., Azotobacter sp.* and *Rhizobium sp.* were grown in King's B broth (protease peptone 20g + $K_2HPO_4.3H_2O$ 1.908g +

$MgSO_4.7H_2O$ 1.5g + glycerol 15 mL + distilled water 985 mL) for 24 h at 28 ± 1°C under shaking conditions and finally cells in the exponential phase were centrifuged at 7000 rpm for 15 min at 4°C. Pots were sterilized with 20% sodium hypochlorite solution, filled with soil and seeded. The pots were watered to 60% water holding capacity and were maintained at this moisture content by watering to weight every 2–3 days.

There were 4 treatments *viz*., T0= control, T1 =25% paper mill sludge vermicompost, T2= 50% paper mill sludge vermicompost, T3 = 75% paper mill sludge vermicompost. Data on growth yield (Data on root length shoot length, no. of leaves, no. of branches and no. of nodules) were recorded in every 15 days by using standard procedures. Yield contributing parameters i.e., number of flowers, number of pods per plant, pod length, number of seeds per pod, 100 seed weight (fresh and dry weight) and seed yield were recorded using standard procedures and analyzed.

RESULTS

The results of the study showed that the physico-chemical characteristics of PMS during 60 days of vermicomposting using *E. fetida* (Table 1). Figures 1-3, show the total microbial population count of vermicomposting with reference to the control. The vermicompost of *E. fetida* worked compost showed maximum population of bacteria, fungi and actinomycetes whereas in control decreased numbers. The list of morphological and biochemical characterization for PGPR is shown in Tables 2 and 3. A total of four PGPR species was identified in the vermicompost. The increase of bacterial population in vermicomposts is because, the casts are usually rich in ammonia and partially digested organic matter and thus provide a good substrate for growth of microorganisms. The results on the effect of inoculation of PGPR along with an uninoculated control in *V. mungo* which were treated with organic inputs like vermicompost which were vermistabilised of 25% PMS, 50% PMS and 75% PMS have been presented the data in Table 4 and 5.

Table 1. Physico-chemical characteristic of paper mil sludge (PMS) vermicompost prepared using the *Eisenia fetida*.

S. No.	Parameter	25% PMS	50% PMS	75% PMS
1	pH	7.80	7.32	7.65
2	Electrical conductivity (dS/m)	1.32	1.40	1.42
3	Bulk density	0.50	0.60	0.58
4	Pore space (%)	73.52	79.22	81.24
5	Moisture content (%)	62.3	68.3	68.20
6	Organic carbon	25.6	24.6	25.6
7	Nitrogen %	1.96	1.88	1.92
8	Phosphorus %	4.39	3.98	3.98
9	Potassium %	1.80	1.60	1.72
10	Calcium (%)	3.13	3.02	3.62
11	Magnesium (%)	0.67	0.60	0.72
12	Iron (ppm)	199.0	189.0	192.0
13	Copper (ppm)	19.0	18.0	21.0
14	Zinc (ppm)	63.0	60.0	64.0
15	C/N ratio	13.06	13.08	13.33
16	C/P ratio	5.83	6.18	6.43

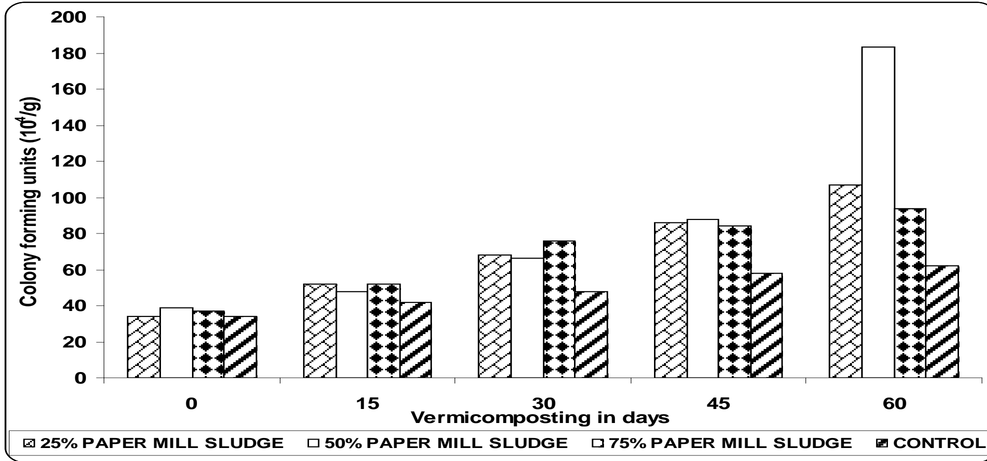

Figure 1. Total bacterial population in 25, 50 and 75% paper mill sludge vermicompost

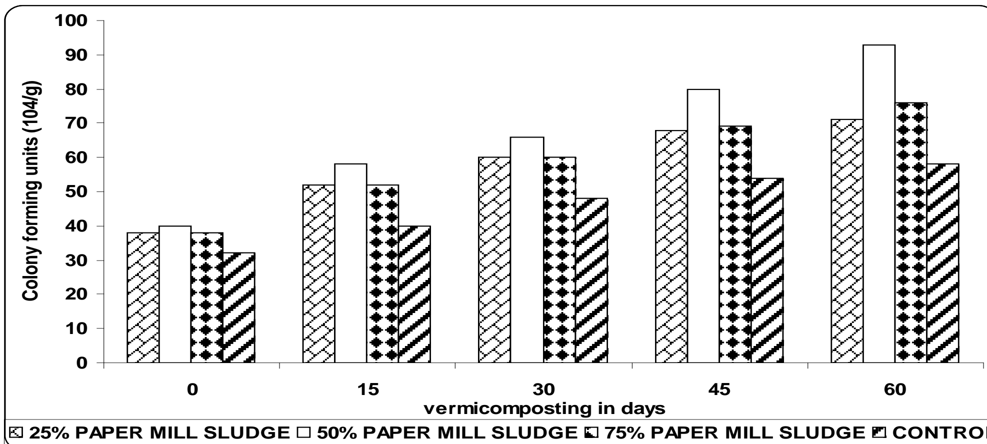

Figure 2. Total fungal population in 25, 50 and 75% paper mill sludge vermicompost

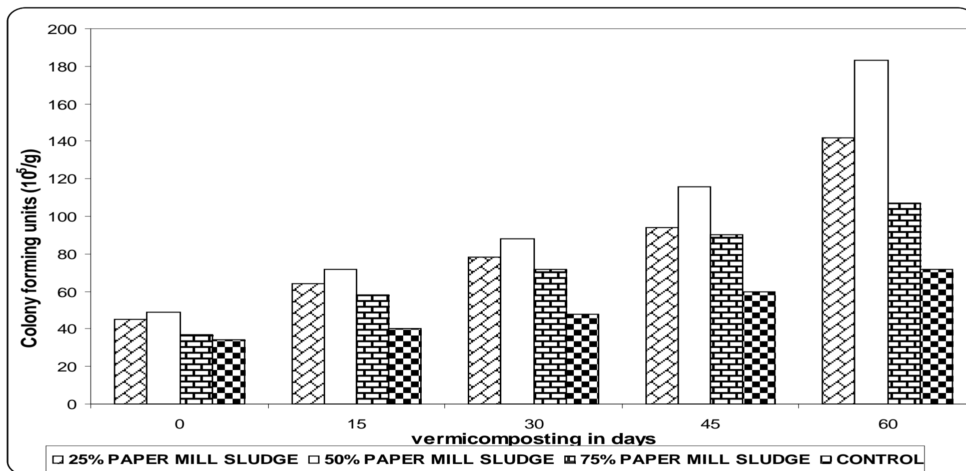

Figure 3. Total actinomycetes population in 25, 50 and 75% paper mill sludge vermicompost

Table 2. Morphological and biochemical characterization of PGPR isolates.

Morphological and biochemical characterization	***Bacillus sp.***	***Pseudomonas sp.***	***Azotobacter sp.***	***Rhizobium sp.***
Grams reaction	G +ve	G -ve	G -ve	G -ve
Shape	rods	rods	rods	rods
Pigments	-	+	+	+
Lactose	+	-	+	+
Dextrose	+	+	+	-
Sucrose	+	+	+	-
Mannitol	+	-	+	+
Oxidase	-	+	+	+
OF test	-	+	+	-
H_2S production	-	+	-	+
Indole	-	-	+	+
Methyl red	-	-	+	+
Vogues Proskauer	+	-	+	+
Citrate utilization	+	+	+	-
Nitrate reduction	+	+	+	+
Starch hydrolysis	+	+	+	+
Gelatin hydrolysis	+	-	-	-

Table 3. Characterization of PGPR isolates

PGPR Characterization	***Bacillus* sp.**	***Pseudomonas* sp.**	***Azotobacter* sp.**	***Rhizobium* sp.**
IAA Production	+	+	+	+
Ammonia Production	+	+	-	+
Catalase Production	+	+	+	+
Siderophore Production	-	+	-	-

Root length: All the treatments were significantly increasing their efficiency on the growth of roots. Here, this experiment showed the best performance of T3 (24.9 cm) followed by T1 (22.8cm) and T2 (22.5) than T0 as control having 22 cm in 90th day of plant growth.

Shoot length: This simple experiment was conducted to find out the effect of plant growth promoting rhizobacteria which increased the shoot length over un inoculated control. In this experiment, the treatment T3 (41.8 cm) showed the best performance than the T1 (41.2 cm) followed by T2 (39) and treatment T0 as a control (32cm).

Number of root hairs: PGPR increased the number of root hairs growth over the control. In this experiment, the T3 (79) and T1 (79), showed the good performance than the T2 (76) and T1 as a control(70).

Table 4. Effect of vermicompost and PGPR isolated from vermistabilised organic substrates on the growth of *V. mungo*

Treatment	No. of days	Shoot length (cm)	Root length (cm)	No. of root hairs	No. of root nodules	No. of branches	No. of leaves
T0	15	7.2	3.5	5	0	2	3
T1		9.6	5.9	14	0	3	4
T2		8.8	4.8	7	0	3	4
T3		9.8	5.9	11	0	3	4
T0	30	14.5	8	13	13	5	8
T1		17	9.5	28	16	6	8
T2		14.8	9	20	15	6	7
T3		17.2	9.2	28	15	6	8
T0	45	18.8	11.9	30	14	7	12
T1		21	16.4	48	19	8	14
T2		21.2	13.5	45	22	10	12
T3		22.4	16.5	48	16	8	14
T0	60	21.8	16.2	48	14	8	18
T1		23.8	18.2	58	19	9	21
T2		24	18	56	22	11	20
T3		24.5	19.9	58	19	10	24
T0	75	25.8	19.6	60	15	9	28
T1		35.8	21.2	72	19	10	29
T2		32	20.4	65	22	12	24
T3		37	22.5	72	19	11	29
T0	90	32	22	70	15	10	28
T1		41.2	22.8	79	20	11	34
T2		39	22.5	76	22	13	30
T3		41.8	24.9	79	19	14	36

Values are mean of three replicates; T0 = Red Soil; T1 = 25% PMS; T2 = 50% PMS; T3 = 75% PMS

Number of root nodules: All the treatments were significantly increasing their efficiency on the root nodules of black gram plant. Here, this experiment showed the best performance of T1, T2 and T3 than T1 as control.

Number of branches and leaves: PGPR increased the number of branches and leaves on plant growth over the control. Here, this experiment showed the best performance of T1, T2 and T3 than T0 as control.

Effect of vermicompost on the yield of black gram (*Vigna mungo*): The number of main parameters on the yield of black gram was significantly influenced by application of different treatments (Table 5). The number of flower per plant ranged form 2 to 12. The highest number (24) of pods produced per plant by the application of vermicompost T3 (75% Paper Mill Sludge) on the 90th day. The plant having no vermicompost produced the minimum number (16) of pods per plants on the 90th day. The highest number (8) of seeds present per pod of plant by the application of vermicompost T3 than T0 control. The fresh weight and dry weight of 100 seeds were higher in the treatments T1 to T3 than in T0 (control) (Table 5).

DISCUSSION

Organic compound can be used as carbon and energy sources by microorganisms, microbial growth and activity is particularly intense in rhizosphere. In general, the number, diversity, and activity of soil organisms are influenced by soil organic matter properties (Kobabe et al., 2004). Organic matter

content may have contributed to the development of different microbial community structures in the soils (Clegg et al., 2003). Also, the growth and metabolic activity of soil microorganisms are limited by the availability of nutrients (Welbaum et al., 2004). Mineral nutritional factors can affect the number of bacteria in the rhizosphere (Marschner et al., 1999).

Table 5. Effect of Vermicompost on the yield of *V. mungo*

Treatment	No. of days	No. of flowers	No. of pods per plant	No of seeds per pod	Weight of 100 seeds (fresh wt., g)	Weight of 100 seeds (dry wt., g)
T0	15	-	-	-	-	-
T1		-	-	-	-	-
T2		-	-	-	-	-
T3		-	-	-	-	-
T0	30	-	-	-	-	-
T1		-	-	-	-	-
T2		-	-	-	-	-
T3		-	-	-	-	-
T0	45	0	0	0	-	-
T1		2	0	0	-	-
T2		2	0	0	-	-
T3		2	0	0	-	-
T0	60	6	2	5	6	4
T1		6	4	6	6.8	4.5
T2		10	6	8	7	4.8
T3		8	12	8	7.2	4.8
T0	75	7	6	6	6.8	4
T1		8	16	8	7.8	5
T2		8	12	8	7.8	5
T3		8	16	8	7.8	5
T0	90	6	16	8	7	5
T1		8	21	8	7.8	6
T2		8	22	8	8.2	6
T3		12	24	8	8.2	6

Values are mean of three

Some of the intestinal mucus secreted during passage through the earthworm gut is ingested with the casts, where it continues to stimulate microbial activity and growth (Barois and Lavelle, 1986; Scheu, 1991). This kind of increased microbial population in casts has been reported by many workers (Daniel and Anderson, 1992; Marinissen and Dexter, 1990). No doubt that the casts of the earthworm, *E. fetida* is applied to agricultural soil, definitely enhance and harbour beneficial soil microorganisms whereby increase the soil fertility status. This data revealed that vermicomposting (using *E. fetida*) is a suitable technology for the decomposition of leaf litter in to value-added material. Hence, the vermicompost obtained from leaf litter decomposition clearly indicates its utility as soil conditioner and good source for plant nutrients in agriculture.

In the pot, trial PGPR inoculation increased the weight of both tops and roots throughout the season, although the difference in weight of tops and root diminished during the end of the growing period (Tables 4 and 5). At the final harvest in pots, leaf, root and seed weight of black gram were significantly greater than the control in all treatments. This work indicated that growth promotion effects

were seen early in plant development, and these subsequently translated into higher yields. The similar results showing that inoculation was found to affect early plant and root development, plant and root dry weight, grain yield and the N-uptake efficiency of plants (Dobbelaere et al., 2002). PGPR inoculation strongly influenced the weight of root, leaf and sugar during the early stages of growth.

The results of the trials reported in this study indicate that from soils with PGPR inoculation a higher yield potential can be expected. In view of environmental pollution due to excessive use of fertilizers and high costs of the production of N fertilizers, bacteria tested during this study may well be used to achieve more sustainable and environmental friendly agricultural production. The experiment revealed that the PGPR inoculation was an effective treatment to improve the parameters measured of black gram, especially with reference to the increase growth responses early in the season. As free living bacteria depend on soil organic matter as a food source, addition of organic matter to the soil may be increased nitrogen fixing and plant growth-promoting activity of PGPR. The favorable effect of the inoculation on plant growth and improved N and P nutrition may be due to growth promoting substances by plant growth rhizobacteria.

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Isolation of Thermo Tolerant *Escherichia coli* from Drinking Water of Namakkal District and Their Multiple Drug Resistance

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ABSTRACT

The main sources of faecal contamination in surface water were open defecation, domestic waste water and sewage discharge. Animals acts as major source however human are also a significant contributor of water contamination. In the present study incidence of thermo tolerant *Escherichia coli* from drinking water of different points of Namakkal District was analysed. The percentage of thermo tolerant *Escherichia Coli* were found to be 92% positive. The prevalence rate was 80% from main storage reservoir, 93% from distribution line and 93% from consumer taps. Among the 25 samples tested against 20 antibiotics, the thermo tolerant *Escherichia coli* isolates were resistant to eleven antibiotics such as vancomycin, carbenicillin, rifampicin, nystatin, cephalexin, bacitracin, nalidixic acid, mezlocillin, penicillin G, neomycin, and amikacin and sensitive to three antibiotics, such as streptomycin, ciprofloxacin and co-trimaxazole and slightly sensitive to six antibiotics, such as kanamycin, cephotaxime, norfloxacin, gentamicin, tobramycin and chloramphenicol. Hence attributor to poor sanitation, low level of hygienic sanitary conditions and irregular disinfections by the local people are needed to be changed their behaviours and adopt better sanitation and hygienic practices and avoid open defecation to prevent faecal pollution in water sources. Detection of high counts of thermo tolerant coliform implies a serious health concern.

Key words: *Escherichia coli*, Water sample and Antibiotics

INTRODUCTION

Over a billion people in the world do not have access to safe water. Many dreadful diseases and illness are associated with water, directly or indirectly impure drinking water leads to various water borne diseases. Water gets contaminated by human and animal faecal matter and spreads the infections. Waterborne diseases are among the leading causes of morbidity and mortality in developing countries and every year around 2.2 million people die due to basic hygiene related diseases like coliform diarrhoea. Interventions in hygiene, sanitation and water supply proved to control these diseases. Universal access to safe drinking water and sanitation has been promoted as an essential step in reducing these preventable diseases (Tambekar and Banginwar, 2005). The normal inhabitant of human intestine, *Escherichia coli* has central place in water microbiology as an indicator of faecal pollution whereas certain strains of pathotype *E. coli* can also cause diarrhoea (Nataro and Kaper, 1998). Resistance to antimicrobial drugs is increasing day by day worldwide in almost all bacterial genera and to almost all drug classes. The use, misuse and abuse of antibiotics are held to be responsible far this development.Pandey and Mussarat (1993) worked on antibiotic resistant coliform bacteria in drinking water in the urban area of Aligarh City and revealed the presence of multiple drug resistance *E. coli.* Parveen *et al.,* (1997) recorded association of multiple antibiotic resistance profiles with point and non point sources of *E. coli* in Apalachicola Bay. The use, misuse and abuse of antibiotics are held to be responsible far this development. (Austin *et al.,* 1999, Bronzwaer et *al,,* 2002), water-borne pathogens i.e., *E. coli*, since it has been used as an indicator of water quality and to assist the control of water- borne diseases (Ejaz and Ahmad, 2001*;* Kjrschner *et al.,* 2004*;* Araujo *et al.*, 1997). We aimed to evaluate the bacteriological quality of drinking water by isolating thermo-tolerant *Escherichia coli* (indicator of fecal contamination) in drinking water of Namakkal District and their possible association with drug-resistance.

MATERIALS AND METHODS

A total of 50 drinking water samples were collected from different places in Namakkal district and processed by using membrane filter method on Eosin Methylene Blue agar and incubated at 44.5°C for 24-48 hours.

Membrane filter Method: Membrane filter with pore size 0.45 mm and diameter 47nm is used for filtering the water sample collected. At first the filter apparatus was dissembled aseptically and membrane filter was inserted. Then the water samples were shaken well and 100 ml of sample was poured into the funnel and sample was filtered under vaccum. When the entire sample was filtered the inner surface was washed with 100 ml of sterile distilled water. Then the filter was removed from filter holder and it is placed on EMB agar medium. Then the plates were incubated in an inverted position at 44.5°C for 24 hours. After incubation the filter paper was observed for the presence of red or magenta colonies on it.

Isolation of thermo tolerant *Escherichia coli* using selective media: Red or magenta colonies on filter paper of Eosin Methylene Blue agar after incubation at 44.5°C for 24 hours were taken and it is directly streaked on EMB agar medium and incubated at 44.5°C for 24 hours for the isolation of *Escherichia coli.* After incubation green metallic sheen colonies on Eosin Methylene Blue agar incubated at 44.5°C were taken as thermo tolerant *Escherichia coli.*

Characterization and identification of thermo tolerant *Escherichia coli*: All the isolates were identified as thermo tolerant *Escherichia coli* by biochemical reactions.

RESULTS

In this study, a total of 50 samples from various places of Namakkal district were analyzed for water quality. Water samples were collected from main reservoir (5), distribution lines (15) and consumer taps (30) for the isolation and detection of thermo tolerant *Escherichia coli.* Out of 50 samples tested 46 were found to be positive for thermotolerant *E.coli* on Eosin Methylene Blue Agar (EMB) when incubated at 44.5°C for 24 hours (Table 1 and 2). The isolated organisms were confirmed by biochemical reactions (Table 3). The antibiotic susceptibility of these isolates was tested by using different antibiotics (Table: 4).

Table.1: Number of samples collected from different places of Namakkal district

S. No.	Name of place	Number of samples
1	Velur(Distribution line)	8
2	Paramathi	7
3	Chitalandur	6
4	Mohanur	5
5	Tiruchengode	2
6	Rasipuram	4
7	Olappalayam	3
8	Nanjai Edayar	3
9	Namakkal	7
10	Main reservoir(velur)	5
	Total	**50**

DISCUSSION

Faecal coliforms or thermo tolerant coliforms are sub group of micro organisms that are capable of growth and fermenting lactose with the production of gas and acid at 44.5°C. Because they are almost exclusively found in the waste of warm blooded animals, this group of bacteria more accurately reflects the presence of faecal contamination than those the total coliform group. The antimicrobial resistance of *Escherichia coli* is of particular concern because it is the most common Gram negative pathogen in humans and the most common cause of urinary tract infection. A very high percentage of samples tested (92%) were positive for the presence of thermo tolerant *Escherichia coli* by membrane filtration method, which is the indicator of faecal contamination incubated at elevated temperatures 44.5°C.

Table 2: Prevalence of thermo tolerant *Escherichia coli* isolated from different points of Namakkal district

S. No.	Point where samples were collected	No. of samples	No. of positive samples	Percentage
1	Main reservoir	5	4	80%
2	Distribution line	15	14	93%
3	Consumer taps	30	28	93%

Table.3: Biochemical reactions of thermo tolerant *Escherichia coli*

Organism	Oxidase	Catalase	Indole	Methyl red	Voges proskauer	Citrate	Glucose	Lactose	Sucrose	Maltose	Mannitol	TSI
Thermo tolerant *Escherichia coli*	-	+	+	+	-	-	A^+G^+	A^+G^+	A^+G^+	A^+G^+	A^+G^+	A^+/A^+G^+ $H2S^-$

+ (Positive), - (Negative), A+ (Acid positive), G+ (Gas positive), H2S-(H2S negative)

The prevalence of *Escherichia coli* was highest in consumer taps (93%) and in distribution line (93%). This is to be expected because of cross contamination of sewage water due to leakage of water distribution pipes at various points were contamination of faecal material is often recorded. Water from municipal reservoir (main reservoir) with a high prevalence of *Escherichia coli* (60%) indicates the inadequate disinfection treatment of water. This indicates that drinking water used in Namakkal district is of poor quality. It also acts as a major source of water borne disease such as coliform diarrhoea gastroenteritis and dysentry. The quality of drinking water is also affected by seasonal variation. High coliform count can be detected in rainy season. It is necessary to determine the prevalence of them tolerant *Escherichia coli* isolated from drinking water in order to prevent faecal contamination on regular basis. Among 20 antibiotics tested the thermo tolerant *Escherichia coli* were resistant to eleven antibiotics amikacin, penicillin, vancomycin, cephalexin, rifampicin, nystatin, bacitracin, mezlocillin, carbenicillin, neomycin and nalidixic acid and slightly sensitive to six antibiotics, such as kanamycin, cephotaxime, norfloxacin, gentamycin, tobramycin and chloramphenicol and sensitive to three antibiotics such as streptomycin, co-trimaxazole and ciprofloxacin. The thermo tolerant *Escherichia coli* recovered form various water sources have multiple antibiotic resistance. Mostly drinking water is contaminated by human faecal matter and non human faecal matter.The emergence of antimicrobial resistance in bacteria has been a major problem through out the world. This study was supported by Thambekar *et al*., (2006) who reported the resistance of *Escherichia coli* isolates to ofloxacin followed by novabiocin cefdixin and ciprofloxacin but azithromycin, gentamicin, amikacin, chloramphenicol, co–trimaxazole and tetracycline were the effective antibioltics. Similar study was also carried out by Shar *et al*., (2009) reported that multidrug resistant *Escherichia Coli* from drinking water. Thermo tolerant *Escherichia coli* isolated were resistant to sixteen antibiotics tested and sensitive to cravat, nalidixic acid, neomycin, cefotaxime,

tobramycin and cefoperoxone.The variation occurred in antibiotic sensitivity pattern of *Escherichia coli* isolated from drinking water confirmed the emergence of antibiotics and antibiotics resistance of *Escherichia coli* species in drinking water. Due to this indiscriminate use of antibiotics the resistance in bacteria increased and infections. Therefore, the precautions should be taken not to abuse or treat infection indiscriminately with antibiotics. Among the twenty antibiotics tested streptomycin, cotrimaxazole and ciprofloxacin are the best choice of drugs while amikacin, mezlocillin, pencillin, bacitracin, nystatin, rifampicin, vancomycin, cephalexin, carbernicillin, neomycin and nalidixic acid should be avoided against *Escherichia coli* diarrhoeal infections in Namakkal district.

Table 4: Antibiotic response against isolated thermo tolerant *Escherichia coli*

Name of the antibiotic	Concentration (mcg)	Resistant (%)	Sensitive (%)
Ciprofloxacin (Cf)	5	--	100
Co-trimoxazole (Co)	25	8	92
Streptomycin (S)	25	12	88
Chloramphenicol (C)	30	40	60
Norfloxacin (Nx)	10	44	56
Gentamicin(G)	10	52	48
Kanamycin (K)	30	52	48
Cephotaxime (CE)	30	56	44
Tobramycin (Tb)	10	56	44
Nalidixic acid (NA)	30	64	36
Neomycin (N)	30	80	20
Carbenicillin (Cb)	100	84	16
Mezlocillin (Mz)	75	92	8
Amikacin (Ak)	30	92	8
Penicillin (P)	10	100	--
Bacitracin (B)	10	100	--
Nystatin (Ns)	100	100	--
Rifampicn (R)	5	100	--
Vancomycin (Va)	30	100	--
Cephalexin (Cp)	30	100	--

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Biosynthesis of Silver Nanoparticles Using Extremophilic Actinomycetes Isolated from Alkaline Soil

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ABSTRACT

Actinomycetes are classified as prokaryotes due to their close affinity with Mycobacteria and coryneforms. Extremophiles are organisms that thrive under extreme conditions such as temperature, pH and pressure. These organisms have developed numerous special adaptations to survive such extreme habitats. Nanotechnology can be defined as a research for the design, synthesis & manipulation of particles with dimension smaller than 100 nm. Biosynthesis of nanoparticles is an exciting recent addition of nanotechnological research and it offers an economically alternative tool for chemical & physical methods. The present study was investigated on the biosynthesis of silver nanoparticles using extremophilic actinomycetes. Nine different soil samples were collected from Vellore, serially diluted, plated on starch casein agar with pH 9 and incubated for 7 days at 27°C. Colonies were identified on the basis of their colony morphology, microscopic observation and biochemical tests. Five strains exhibited their potential for reduction of silver ions in to nanoparticles.

Key words: Screening, Extremophiles, Actinomycetes, *Streptomyces*, Nano particles

INTRODUCTION

Actinomycetes are aerobic & gram positive organisms. They are one of the major groups of soil population and are very widely distributed (Kuster, 1968). The name Actinomycetes was derived from Greek 'aktis '(a ray) and mykes (fungus). The number and types of actinomycetes present in a particular soil would be greatly influenced by geographical location such as soil temperature, type, pH, organic matter content, cultivation, aeration & moisture. Among microorganisms, Actinomycetes gained greater importance for the production of bioactive compounds and enzymes. Therefore, the present study is mainly focused on the biosynthesis of nanoparticles using actinomycetes isolated from alkaline soil.. The objectives of this study was, to isolate the extremophilic actinomycetes from soil with a pH range of 8-9, to identify the different strains of actinomycetes by colony morphology and by microscopy, to identify the genus by biochemical tests, to screen the actinomycete isolates for the biosynthesis of silver nanoparticles.

MATERIALS AND METHODS

Collection of soil sample: The soil samples were collected from Allapuram, Sathuperi, Senbakkam, Thorapadi and in Arcot Taluk includes, Mangadu, Muppaduvetti, Mulluvaadi, Mambakkam & Ponnaipaadi of Vellore district. Nine different soil samples were collected at a depth of 5 – 10 cm with a sterile spatula & transferred to a sterile container for transportation to the laboratory. The soil samples were pretreated to inhibit unwanted microorganism. Moist heat treatment in a water bath at 50°C for 10 minutes was employed to select various actinomycetes groups.

Medium used for isolation: Brain heart infusion agar, Sabouraud's dextrose agar, Starch casein agar

Isolation of actinomycetes: One gram soil sample was weighed and diluted serially in sterile distilled water. The dilutions (10-3, 10-4 & 10-5) were plated on the starch casein agar media (pH 9) supplemented with Actidione and nystatin (0.050 mg/ml) as an antibiotic. The plates were incubated at 25°C for 2- 7 days and growth of typical actinomycetes colonies were noted up to 10 days.

Morphological characterization: Colonies were identified on the basis of their morphology and colour.

Colour of aerial mycelium was determined from mature, sporulating actinomycetes colonies on starch

casein agar (Pridham, 1964). Microscopic observation was done using Gram staining of smears from colonies up to 10 days. The observed structure was compared with Bergey's manual of Determinative Bacteriology. Microscopic characterization was done by cover slip culture method. The structure, colour and arrangement of conidiospore on the mycelium were observed through the oil immersion microscope.
Microbial synthesis of Silver nanoparticles: For the synthesis of silver nanoparticles, the actinomycetes was grown in 250 ml Erlenmeyer flasks containing 50 ml MGYP medium which is composed of malt extract (0.3%), glucose (1 %), yeast extract (0.3%) and peptone (0.5 %). Sterile 10 mg sodium carbonate was used to adjust the pH of the medium; the culture was grown with continuous shaking on a rotary shaker (200 rpm) at 50 ^{o}C for 96 h. After incubation, the cells were separated from the culture broth by centrifugation (5000 rpm) at 20° C for 20 min (Murali Sastry et al., 2003). The culture supernatant was added with 1 mM of silver nitrate and incubated on an shaker (dark condition) for 96 h at 28°C (Sathya Sadhasivam et al., 2010).

UV – visible spectral analysis: Bioreduction of the Ag + ions in the solution was monitored by colour change. The absorption maxima of reduced silver ions were recorded using UV- Visible spectrophotometer at the range of 200 – 700 nm.

Characterization of silver nanoparticles: The biosynthesized silver nanoparticles are yet to be characterized by X- ray diffraction, TEM and SEM analysis.

RESULTS & DISCUSSION

Morphological and cultural characteristics of isolates: A total of 11 isolates were isolated from nine different soil samples, and it was morphologically identified Colonies having characteristics features such as powdery appearance with convex, concave or flat surface and colour ranging from white, gray to pinkish were selected. Colonies observed at 1st and 2nd day were eliminated because actinomycetes are considered as slow grower (Currie, 2006). . All the strains were suspected as Streptomycetes (Table 1).

Table 1. Morphological Characteristic of the Isolates

Colony characteristics on starch-casein agar	Microscopic characteristics	Actinomycetal isolate	No. of Isolates
Powdery colony appears convex, concave or flat surface; white, gray to pinkish color colony.	Filaments long highly branched and non fragmenting; aerial filament with spirali,coils, or multiple branching & long chains of Spores.	*Streptomyces*	11

Microbial synthesis of Silver nanoparticles: Among 11 isolates screened for the biosynthesis of silver nanoparticles 5 isolates exhibited the bioreduction of silver ions. The silver nanoparticles synthesis was confirmed by color change. Appearance of yellow to brown colour is an indication of silver nanoparticles and this was due to excitation of surface plasmon vibrations in the particles.

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BCL-2 Expression in Systemic Lupus Erythematosus Cases in Chennai, Tamil Nadu

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ABSTRACT

Systemic lupus erythematosus (SLE) is a prototype inflammatory autoimmune disease resulting from autoimmune responses against nuclear antigens. During apoptosis many lupus auto antigens congregate inside the cells and are susceptible to modifications. Modified nuclear constituents are considered foreign and dangerous. Therefore, apoptotic cells have to be efficiently removed to avoid the accumulation of apoptotic debris and the subsequent development of the autoimmune responses. Hence, apoptosis and clearance of apoptotic cells/material are considered key process in the etiology of SLE. Therefore in this study, probably for the first time Bcl-2 involvement has been assessed from SLE patients of Chennai, Tamilnadu.

Keywords: SLE, Bcl-2, ELISA.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototype inflammatory autoimmune disease resulting from autoimmune responses against nuclear autoantigens. The primary clinical and pathological manifestations are consequences of local inflammatory events initiated by wide spread immune complex (Mok and Lau,2003).

Bcl-2 has been considered as a candidate gene for SLE because of its role in apoptosis in the lymphoid system. Cell survival is enhanced by Bcl-2 through the inhibition or delaying apoptosis. The present study is aimed at expression of Bcl-2 among the systemic lupus erythematosus patients from Chennai, Tamilnadu.

MATERIALS AND METHODS

Seventy-three patients fulfilling the 1997 revised criteria of the American College of Rheumatology (ACR) for SLE were enrolled from July 2008 to July 2009 in Government General Hospital and Sri Ramachandra Medical College and Research Institute, Chennai. Bcl-2 expression was analyzed using ELISA kit from Bender Med Systems, Austria.

RESULTS

Out of 73 cases tested for the expression of Bcl-2 ,42 (57.53%) showed less expression of Bcl-2.The remaining 31 samples were found to be normal for Bcl-2 assay (Table-1;Figure-1).

Table-1: Bcl-2 Expression level among systemic lupus erythematosus cases

S.No	Gender	Low	Normal	Total
1	Male	1	3	4
2	Female	41	28	69
Total		42 (57.53%)	31 (42.46%)	73

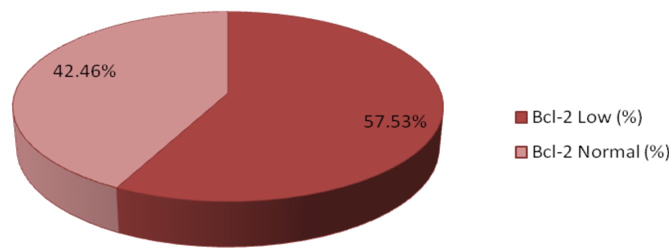

Fig. 1: Bcl-2 Expression level among systemic lupus erythematosus cases

DISCUSSION

Indian literature on Bcl-2 involvement in SLE is scanty.Bcl-2 has been considered as one of the candidate gene for SLE because of its role in apoptosis in the lymphoid system. The expression of Bcl-2 in SLE patients has been reported in several studies with conflicting results.In the Present study Bcl-2 expression was seen 57.53% in our SLE patients.This finding is an agreement with several earlier studies (Chan *et al*., 1997; Kumagai *et al*., 1994; Rose *et al*., 1995). It has recently been suggested that a failure of apoptosis wherein autoreactive lymphocytes continue to survive is a major factor in the pathogenesis of SLE. Other than Bcl-2, Fas antigen is also increased on peripheral blood lymphocytes from human SLE patients while Bcl-2 protein, which inhibits apoptosis, is highly expressed in peripheral T lymphocytes of SLE patients. Many studies have shown lowered Bcl-2 quantities in SLE lymphocytes suggesting that the decreased Bcl-2 expression is consistent with the increased rate of apoptosis.

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