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Global Journal of Advanced Biotechnology and Biochemistry Research

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Contents

Environmental Factors Shaping Plant-Microbe Associations: Their Significance To Improve Plant Health And Vegetation Restoration In Degraded Lands

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ABSTRACT

Industrialization has vastly contributed to contamination of environment around the world. The unscientific industrial activities have resulted in landscapes characterized by a multitude of a biotic stresses, specifically low levels of nutrients, organic matter, and moisture and high levels of inorganic and organic contaminants. Wild plants and their associated microbes have been widely used to develop biotechnologies for improving plant health and restoring the degraded lands. However, poor knowledge of environmental factors determining functions of microbial diversity limits the predictability and efficacy of the microbial technologies. Landscape heterogeneity adds to the differential performance of microbial inoculants in a degraded environment. Plant host species differ in their root characteristics especially the exudates, which further make the root environment heterogeneous. All these environmental challenges negatively affect microbial colonization, plant growth, and restoration efforts. Understanding factors affecting microbial functional diversity in the context of the plant-soil feedback (PSF) will help reveal underlying interactions of microbial community functions in the ecosystem. In this context, we propose that the environmental predictors of rhizosphere bacterial communities in the degraded environment would play a significant role improving the microbial inoculation technologies. We developed a research framework to understand the environmental predictors driving the functions of microbial communities at the degraded sites as part of plant-soil feedback. Such studies would also help in developing ecological theories for a contaminated site for application in improving plant growth and restore the degraded lands.

1. Impact of industrial pollution on environment

Developmental activities are modifying the natural ecosystem around the world at a rapid pace with severe negative environmental consequences (Sharma et al., 2016; De Groot et al., 2003). Nations depend on the constant supply of natural resources for maintenance and growth of their economies. Environmental load of waste material keeps on adding due to developmental activities ranging from extracting raw material through mining to the manufacturing products till the consumption and disposal by the end user. Poor management of the waste material pollutes soil, water, and air and destroys the ecosystem (Sharma et al., 2011).

In fact, the areas around the industrial zones remain polluted and degraded for decades without any post-industrial management plan. Mining and power generating plants are the most polluting industrial activities because of the constant requirement of the raw materials and energy for all developmental activities (Rau et al., 2009; Sharma et al., 2011).

Mining industry drives the economic development of human societies around the world; however, it results in degraded ecosystems. In fact, mining has also been identified as one of the major cause of environmental degradation which includes disruption of basic ecosystem service as well as biodiversity loss. In spite of this "abandoned and/or orphan mining sites" are increasing globally due to weak closure and ill-defined post closure provisions (Hoballah, 2010). Partial to complete degradation of the landscape is known to be caused by mining activities. Such degraded ecosystems have a variety of stresses, e.g., poor amount of nutrients, moisture and high amount of toxic elements which result into excessive loss of above and below ground biodiversity (Sharma et al., 2011). Degraded and abandoned land caused by mining are not only an ecological issue but also a challenge for developing nations like India, due to the limiting land resource and increasing environmental pollution levels.

Coal-fired power plants represent another industry which pollutes atmosphere, hydrosphere, and lithosphere and increases toxicity to the biosphere. Coal still meets significant percentage of global electricity demand and serve as the mainstay of electricity in many countries (Farfan & Breyer, 2017). In addition to polluting the air through the production of smoke, coal-based thermal power plants also pollute soil and water through fly ash. Fly ash generated in power plant is mixed with water and dumped in the surrounding low-lying area, which is called fly ash dumps/ponds. The fly ash dumps are one of the most degraded and contaminated landscapes of urban ecosystems, which serve as a source of different pollutants into the environment (Izquierdo & Querol, 2012; Twardowska & Szczepanska, 2002). Fly ash lacks nitrogen and is deficient in other macronutrients. Also, it contains a wide array of toxicants; therefore, the dumps remain barren for decades (Theis & Gardner, 1990; Rau et al., 2009). Fly ash is also known to have high levels of toxic elements like Pb, Cd, Zn, Hg, Cr, Ni, Co (Rau et al., 2009). Such toxic elements pollute nearby water bodies and cause genotoxicity to plants and animals present in the vicinity of ash dumps. Coal ash spreads into the environment (air, water, and soil) from unvegetated dumps and leads to poor air and water quality, and reduces the productivity of ecosystems. The ash spread also causes several respiratory and genotoxic disorders in human and livestock (Primerano et al., 2000; Bertin & Averbeck, 2006; Smith et al., 2006; Raja et al., 2014; Qadir et al., 2016). Therefore, the society at large considers fly ash dumps as a socio-economic and ecological burden (Asokan et al., 2005). Though attempts have been made to utilize fly ash for various purposes, ~70–75% of >780 million t/annum fly ash produced globally is still being managed as open dumps (Izquierdo & Querol, 2012; Wang, 2008).

2. Microbial communities and its importance in ecosystem restoration

The large-scale ecosystem degradation has led to an interest in understanding the movement of toxicants across the different environmental compartments, the transfer of toxicants across the trophic levels and different levels of biological organizations, and impact of toxicants on ecosystems structure and function (Pilon-Smits & Freeman 2006; Sharma et al., 2011; Rawat et al., 2016). In conjunction with waste management practices, the practice of vegetation restoration at degraded ecosystem would play a significant role in reversing the trend of threatening consequences of environmental degradation (Sharma et al., 2016; Sharma et al., 2011; Sharma et al., 2005; Sharma et al., 2002). However, vegetation development at degraded ecosystems is a challenge due to multiple environmental stresses like a low level of nutrients, high level of toxic elements and an insignificant amount of organic matter. Soil microbial communities are essential for the maintenance of soil functions in both natural and managed ecosystems as they drive ecosystem processes like biogeochemical cycles (nitrogen, phosphorus, sulphur, etc.), decomposition of organic matter, and removal of toxic chemicals (Garbeva et al., 2004).

Microbial communities have become the important biological inputs in the science and practice of restoration ecology (Harris, 2009). Soil microbial processes help in soil formation and plant growth promotion (IAA production, HCN production, siderophore production) and thereby govern the vegetation development (Rau et al., 2009; Sharma et al., 2011). Therefore, studying microbial communities, specifically, their ecological functions should be a critical component of the restoration programmes. Though microbial inoculation has helped in vegetation development at degraded lands, it still faces challenges of competition or predation from native soil organisms. However, biostimulation of well-adapted native microbes has been suggested as the ecologically sound option but to develop this strategy the knowledge of environmental determinants of microbial functional diversity is a prerequisite (Figure 1).

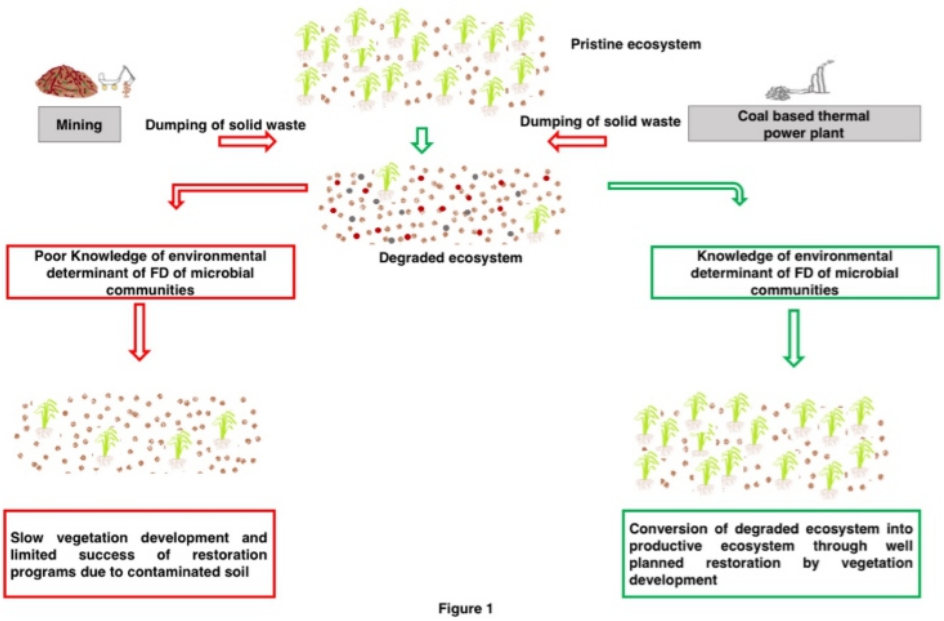

Figure 1: Schematic diagram showing application of environmental determinant to drive microbial functional diversity in restoration of degraded ecosystems

Microbial functional diversity is important for vegetation restoration but the concept of functional diversity and methods for its estimation need to be re-examined. The importance of microbial functional diversity (FD) in plant colonization and ecosystem functioning has been well recognized (Mason et al., 2003; Sharma et al., 2005; Green et al., 2008; Sharma et al., 2008; Rau et. al., 2009; Krause et al., 2014). Though microbial FD is considered ecologically relevant biodiversity measure (Diaz & Cabido, 2001) most of the studies primarily focus on the taxonomic diversity in microbial communities. The taxonomic diversity only reveals the evolutionary history of organisms, whereas understanding FD provides an answer to the basic questions related to ecosystem functioning. Of the limited studies on microbial FD, mostly the studies focused either on determining presence/absence of multiple functional traits or measuring the activity of a functional trait and relating it to the taxonomic diversity of the microbial community. However, this strategy does not consider the dynamics within a single functional trait in a microbial community or among the spatially separated communities. In fact, ecologists have emphasized that variation within each functional trait of microbes in a microbial community can be independent of variations in other traits in a microbial community and its taxonomic composition (Petchey & Gaston, 2002). Such variations within a single functional group can be a result of micro-environmental variations within and between the habitats (Mohmmed et al., 2001; Sharma et al., 2005; Rau et al., 2009; Sharma et al., 2011). Ecologists visualize that "variation within one trait has equal influence on the measure of diversity as variation in any other trait and there is no reason why traits cannot or should not be weighted differently to calculate FD" (Petchey & Gaston, 2002). In fact, the emerging concepts in microbial ecology suggest that to understand the role of microbes in the environment, the functional and taxonomic diversity must be decoupled (Loucas et al., 2016).

Microbial functional group directly involved in plant success at degraded site need priority. In the context of ecological restoration, microbial functional groups like IAA producer form the most important group, which play a central role in plant's ability to colonize degraded site, acquire nutrients and remediate toxicants and thus drive the ecological restoration. Therefore, diversity in these functional groups of the microbial community is critical for the plant recruitment and vegetation development. Microbial indole acetic acid (IAA) is an important plant growth regulator that facilitates host plant colonization via enhancing seed germination, promoting root growth and root hair, initiating lateral roots, and promoting shoot growth. Also, the role of IAA in adapting the host plants and microbes to diverse stresses has also been demonstrated (Bianco et al., 2006). The level of IAA determines the result of plant-bacterial interactions as pathogenesis or symbiosis, but the useful level of IAA may vary with the plant species (Vacheron et al., 2013).

Among other microbial functional group, we recommend to focus on nitrogen fixers as the second most groups, which may play a significant role in the restoration of degraded ecosystems. It may be noted that degraded ecosystems more often characterized with nitrogen limitation, which poses a major challenge for vegetation restoration. Diazotrophic bacteria fix atmospheric nitrogen enzymatically and grow without the available nitrogen. Ecology and diversity (functional and compositional) among diazotrophs have been well investigated in the nitrogen-limiting marine environment (Fernández-Méndez et al., 2016; Affourtit et al., 2001). However, they play equally or even more important role in the nitrogen-deficient terrestrial environment, where free-living or associative bacteria supply fixed nitrogen in rhizosphere and to the host plant. Biology and ecology of diazotrophs symbiotic to different plant species from agriculture fields and desert ecosystems have been investigated (Coelho et al., 2008; Coelho et al., 2009; Wartiainen et al. 2008; Chowdhury et al., 2009; Rau et al., 2009; Sato et al. 2009). Free-living and associative diazotrophs are ubiquitous and contribute significant N for terrestrial ecosystem functions, however, their role in degraded ecosystems is still little understood (Vitousek 1984; Schmidt et al., 2008; Reed et al., 2011).

3. Environmental predictors of functional diversity/ functional performance of soil microbial communities in degraded ecosystems

Taxonomic and functional diversity in microbial communities from degraded ecosystems have been investigated but the factor(s) that govern the microbial functional diversity poorly understood (Rau et al., 2009; Sharma et al., 2011). Most studies focus on the taxonomic diversity of microbial communities, and only a few of them investigated functional diversity. Also, mostly studies on functional diversity of microbial communities of natural ecosystems. However only a few analyzed the degraded ecosystems. Consequently, studies on functional diversity of microbial communities of both natural and degraded systems would strengthen theories of microbial ecology and models of restoration

ecology. In fact, such studies rhizosphere microbial communities of ecologically successful species at degraded sites would further help in explaining rhizosphere processes driving ecological restoration through vegetation development (Harris, 2009).

Environmental factors determine the microbial diversity; however, the principle factor for different species or ecosystem may be context dependent. Microbial ecologists have reported numerous factors (biotic and abiotic) that shape the diversity of microbial communities. These factors include local climate, season, soil structure, physicochemical properties, above ground vegetation, and fauna (Berg & Smalla, 2009). However, these studies provide contrasting reports on the role of principle factor(s) governing the microbial functional diversity. For example, some studies suggested the role of plant host and others demonstrated the soil type as dominant factors affecting the structure and function of microbial communities (Berg & Smalla, 2009; Fierer & Jackson, 2006). On the other hand, some studies indicated the role of soil properties, like pH in controlling microbial diversity (Fierer & Jackson, 2006). Therefore, it becomes pertinent to investigate the principle factor(s) driving the microbial diversity at the degraded ecosystems.

As a functional group comprises of different species, then multiple factors may govern the diversity within a functional group of microbial communities. However, mostly the targeted bacterial strain or microbial community was analyzed for the effect of a single factor on the selected functional trait in in vitro, controlled greenhouse or field environment. Also, these studies do not consider environmental predictors (plant, soil structure, soil properties, etc.) of microbial functional diversity as part of plant soil-feedback that occur in nature (Figure 2). Plant-soil feedback, i.e., plant-induced changes in the structure and function of soil communities that further affect the plant growth and establishment, is a key ecological process for effective and sustainable restoration of degraded ecosystems (Eviner & Hawkes, 2008; van der Putten et al., 2013; Inderjit & Cahill, 2015). In fact, the study of PSF and its implication on microbial functional diversity becomes important as different workers have suggested that functions of microbial communities in polluted soils are linked with the composition of plant community and physicochemical properties of soil (Krumins et al., 2015; Schimel et al. 2007). Bardgett (2011) has also emphasized that understanding the consequences of industrial pollution on ecosystem functioning requires consideration of links between aboveground vegetation and belowground microbial communities. Such studies become a prerequisite in case of the contaminated site as such site may vary in quality and quantity of the contaminants (Krumins et al., 2015). Therefore, lack of such studies limits our knowledge on the complex linkages between plant community, soil properties and microbial functional diversity (Krumins et al., 2015; Grimm et al., 2000). Testing theory of plant-soil-feedback at the contaminated site and its impact on soil microbial functional diversity would provide useful data to develop site-specific restoration models and practices.

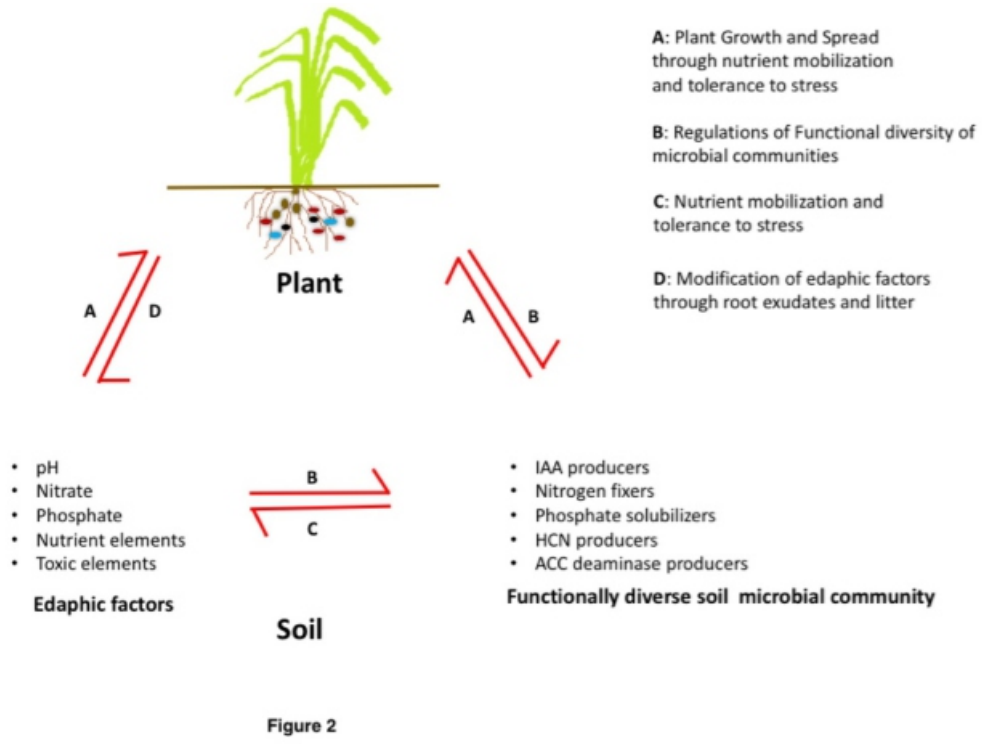

Figure 2: Schematic diagram showing the role of microbial functional diversity driving plant-soil feedback processes

4. Plant-soil feedback and environmental factors determining microbial functional diversity

Since below ground biodiversity governs the above ground biodiversity, it is essential to understand PSF and identify the predictors of microbial functional diversity to develop nature-based site-specific restoration plans (Figure 1, Figure 2) (Eviner & Hawkes, 2008; Cadotte et al., 2011; Zhang & Chu, 2013; Xiao et al., 2016; Malhotra et al., 2017; Smith-Ramesh et al., 2017). PSF is considered direct when plant-induced changes in soil affect individual of same species, on the other hand, PSF is considered indirect, or interspecific when plant induced changes in soil affect individual of other species. PSF can be both positive as well as negative (van der Putten et al. 2013).

Experimental evidence show that positive PSF exists between the plant and symbiotic nitrogen-fixing bacteria during early stages of primary succession. Such feedback results in the increased content of soil N in the ecosystems, which help in vegetation development as the parent material lacks N at the early stages of succession. So both the prevailing soil conditions and the plant existing favors the growth of nitrogen fixers. For example, Lupinus and Lepidus, nitrogen-fixing legume dominated Mount St. Helens after the latest volcanic eruption, and formed "island of fertility," which triggered the vegetation of later successional stages (Titus & del Moral 1998; Corti et al. 2002).

PSFs differ depending upon the stage of succession. Studies on the old field showed that negative PSF plays an important role during early stages of secondary succession, whereas at later successional stage positive PSF became more important. Different workers demonstrated that during early stages of secondary succession, the effects of symbiotic mutualists might have less impact than soil-borne pathogens, but this pattern is reversed in later stages of succession (Kardol et al., 2006; Janos 1980; van der Putten et al. 2013). We suggest that contaminated sites represent degraded sites undergoing succession. Plant-soil feedback plays significant role in the development of all terrestrial communities, including natural and contaminated sites. However, PSF has been investigated from natural ecosystem, but PSF at contaminated sites will reveal useful information about the critical processes responsible for vegetation restoration. Our analyses recommend analyzing environmental factors affecting microbial functional diversity at degraded sites as part of plant-soil feedback.

The present study concludes that existing studies do not reflect the functional diversity within ecologically important soil microbial functional groups, such as IAA producers and diazotrophs, and their environmental predictors (plant host, edaphic factors) are scarcely studied. Understanding of principal factors determining the interactions and dynamics of plant and microbial communities in the background of plant-soil feedback theory would help in improving restoration of vegetation in the degraded environment. The knowledge of such principal environmental factors will serve as the foundation of developing better biostimulation and bioaugmentation technologies for ecological restoration programmes. Also, detailed studies in various degraded environments on several microbial function groups and their environmental determinants are required to improve the existing ecological theories used for making restoration programmes.

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Impact of CO2 on Disease Severity of Fungal Plant Pathogens and Their Management By Modern Day Techniques

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ABSTRACT

Plant diseases are among the major factors limiting crop productivity. The frequency of invasiveness of plant fungal pathogens has risen dramatically in recent years and climate change has played a major role in these plant-pathogen interactions. Climate change leads to variation in temperature, humidity, CO2 concentration, etc. that affects the process of fungal pathogenesis of important crop plants. Due to differences in pathogenicity, significant measures are required for their management as it will affect yield of crop plants thus affecting food security. The present review highlights the key disease management strategies that will help in controlling the spread of pathogens under current climate change scenario. The current review also focuses on the role of modern day techniques such as genomics, transcriptomics, proteomics etc. that will help in elucidating the role of different genes and proteins and thus, aid in disease resistance mechanism.

Keywords: Biosensors, Climate change, CO2, Nanotechnology, OMICS, Pathogenicity

1. Introduction

The gaseous composition of the atmosphere has undergone a significant change in last few years. This change in atmospheric composition is a result of human induced activities such as urbanization that have led to increased fossil fuel combustion and rapid deforestation. Due to urbanization, there are significant changes in land use and management practices that also resulted in considerable change in the gaseous composition of the atmosphere. One of the important effects of urbanization is deforestation that leads to significant rise in CO2 levels. At the same time, a modification in different method fertilizer application leads to contamination of soil and emissions from agricultural fields. It has been recently studied that certain vegetation species emits various harmful biogenic volatile organic compounds that may acts as a precursor for secondary pollutants. All these activities have resulted in an increased emission of carbon dioxide (CO2), methane (CH4) and nitrous oxide (N2O), popularly known as the 'greenhouse gases' (Fig. 1.1a). As a consequence of all such human induced activities,

global atmospheric carbon dioxide (CO2) concentration in atmosphere have risen from pre-industrial values of approximately 280 mmol mol-1 to a current global mean of approximately 380 mmol mol-1 (Keeling and Whorf, 1999) (Fig. 1.1b). The concentrations of CO2 have been projected to increase to 540-958 mmol mol-1 by the year 2100 (IPCC, 2007) (Fig. 1.1b). Increase of CO2 concentration to such a high level will have a great effect on the natural vegetation and their performance under such conditions cannot be predicted. There are reports on how elevated levels of CO2 affecting plant growth, development, and functions have changed in the recent past (Ainsworth and Long, 2005), but how plants will respond to their respective pathosystems with the rapid change in CO2 is still a question of debate.

2. CO2 ENRICHMENT TECHNOLOGIES

One of the important pre-requisite for studying plant responses to elevated CO2 concentrations requires the understanding of various technologies used for CO2 enrichment and exposing plants to increased CO2 and then compare the performance of the plants that were exposed to enriched CO2 with those grown under ambient CO2 conditions.

An advanced technology that were used to study effect of CO2 on plants without altering their natural ecosystem includes Open top chambers (OTCs) and Free-air CO2 enrichment (FACE) derived systems (Leadley et al., 1997; Macháčová, 2010).

2.1. OPEN TOP CHAMBERS (OTCS)

OTCs are the most widely and most thoroughly used experimental method for exposing field grown plants to elevated CO2 and other atmospheric gases. Climatic conditions such as temperature, light, rainfall etc inside the OTCs can be maintained by means of user friendly control systems which costs lesser in amount than that required by complex environmental controls used in closed chambers (Jones et al., 1984) (Fig. 2a).

2.2. FREE-AIR CO2 ENRICHMENT TECHNOLOGY (FACE)

FACE allows the researchers to study plant responses to elevated CO2 under fully natural and atmospheric conditions. There are no closed confined structures in FACE rather it consists of an array of vertical or horizontal vent pipes for CO2 injection located at the periphery of the experimental plots. FACE is a state of art technology where the whole experimental area is fumigated with similar concentration of CO2 depending upon wind speed and direction. Some of the differences between FACE and OTC are discussed below (Table 1).

Table 1: Comparison between FACE and OTCs

S. no.	Free-air CO_2 Enrichment Technology (FACE)	Open Top Chambers (OTCs)
1	Bigger diameter-helps in studying effect of enhanced CO_2 on large area of plant cover	Smaller diameter-useful for working with seedlings but not with tall mature trees
2	It does not negatively affect the plot's microenvironment	OTCs affects the microclimate
3	FACE is very costly due to high consumption of CO_2 during fumigation	OTC is cheap due to a significantly lower consumption of carbon dioxide.
4	Pathogen cannot be artificially inoculated	Artificial inoculation studies can be performed

3. PLANT RESPONSES TO ELEVATED CO2

The yield of most of the C3 crops such as wheat, rice, soybean, and mustard have been projected to increase with increase in atmospheric CO2. Increased yield of crops is mainly due to increased biomass under elevated CO2 (Ainsworth and Long, 2005; de Graaf et al., 2006). As the concentration of CO2 increases in the atmosphere, there will be increased concentration of carbon in plants and greater carbon-to-nitrogen ratios (C: N) of plant tissues (Matros et al., 2006; Taub and Wang, 2008). Effect of elevated CO2 on plant responses has been summarized in Fig. 3.

4. ELEVATED CO2 AND PLANT DISEASES

The three key parameters of the classic disease triangle are a susceptible host, a virulent pathogen, and favourable environment conditions (Fig. 3). Thus, changes in host resistance morphology, physiology and chemistry due to increasing CO2 concentrations will alter disease expression in plants

5. FACTORS AFFECTING HOST RESISTANCE UNDER ELEVATED CO2

5.1. GROWTH AND PHOTOSYNTHESIS

Elevated CO2 enhances the rate of photosynthesis thereby increasing growth and biomass. With significant increase in photosynthesis, plant channelizes most of its resources towards host resistance. Host resistance on one side leads to increased biomass in plants while on the other hand, it attracts some leaf infecting fungi also, considering that more tissues will be available to the pathogen under elevated CO2Therefore, it can be concluded that enhanced growth and photosynthesis can affect the microclimatic conditions favorable for the growth and sporulation of pathogen (Coakley et al., 1999; Chakraborty et al., 2000).

5.2. CARBOHYDRATE CONCENTRATION

One of the important factors required by the pathogen for their growth and survival inside the host is the concentration of sugars. Most of the pathogens such as mildews and rusts require high amount of sugars for invading into host cells. Previous studies have very well shown that there is higher accumulation of carbohydrates in leaves under elevated CO2 due to increased photosynthetic rates (Ainsworth and Rogers, 2007; Leakey et al., 2009). Most of the biotrophic pathogen requires living host tissue to attack and studies have revealed that attack of rusts is increased by higher sugar availability under elevated CO2 (Manning and Tiedemann, 1995). On the other hand, attacks by mildews get decreased by the high sugar concentration under elevated CO2. Atmospheric CO2 enrichment has also been shown to enable certain plants to use scarce nutrients more efficiently (Luxmoore et al., 1986; Norby et al., 1986).

5.3. NUTRIENT STATUS

There is a complex relationship between nutrition and plant diseases. Nutrition influences both the host plant and the pathogen which subsequently affect the incidence and severity of diseases. This variation in the absorption rates of different nutrients is mainly due to lowered transpiration rates and stomatal conductance under elevated CO2 which led to increased water use efficiency of plants. This whole process of nutrient uptake from soil depends on mass flow (Kabata-Pendias, 2011). The most notable effect of elevated CO2 is observed in nitrogen which got lowered (Cotrufo et al., 1998; Jablonski et al., 2002; Taub and Wang, 2008). Due to decreased concentration of nitrogen in plants, pathogen attack also varied considerably (Coviella et al., 2002; Matros et al., 2006). Under elevated CO2, there will be increase in carbon based defense compounds while decrease in nitrogen based compounds due to decreased concentration of nitrogen. Thus, there will be significant effect on leaf quality in terms of nutrients that may resist growth of particular type of pathogen or possibly favor some other pathogens.

5.4. LEAF CHEMISTRY

Plants grown under elevated CO2 showed altered leaf tissue chemistry (McElrone et al., 2005). Due to changes in leaf tissue chemistry, plant's susceptibility to various pests and pathogen also varied. As observed from the past studies, significant increase in the concentration of carbon led to increased C/N ratio in plant and dilution of other nutrients such as nitrogen. Most of the carbon containing defense metabolites in plants belongs to class phenylpropanoids that provide resistance against various pests and pathogen (Dickinson, 2003). Due to significant increase in the concentration of carbon there is increase in concentration of carbon based defense compounds such as phenols, salicylic acid, lignin etc while significant decrease in nitrogen based defense compounds such as quinones etc. that affect fungal

5.5. PLANT ARCHITECTURE

Plants grown under elevated CO2 shows better plant architecture in terms of size, number of leaves, number of branches and canopy. This increase in vegetative growth also affects microclimatic conditions of leaf surface such as temperature and humidity. These two features are very imperative for the attack by foliar pathogens (Burdon, 1987). It has been observed that elevated CO2 not only affects micro climatic conditions, it also affects plant's structural characteristics. Increased waxy coating on leaf surface and higher concentration of leaf epicuticular waxes prevents germination of conidia of Erysiphe graminis and germinated only when the waxy coating from the leaf surface was removed (Hibberd et al., 1996).

5.6. CHANGES IN GENE EXPRESSION

The interactions between plant and pathogens can influence the expression of gene at diverse levels (Garrett et al., 2006). Due to the changes in the transcriptome, stress responses may occur in the plant leading to a change in the ecological interactions of the plant. The alteration of the biological processes incurred due to these stress responses has a direct impact on the defense related trade-offs faced at the time of pathogen attack. Down regulation of gene expression in response to elevated CO2 has been observed in Arabidopsis. The change in response has been observed in various biological processes related to photosynthesis including Calvin cycle, Photosynthesis, PS I and II subunits, light harvesting and electron transport (Li et al., 2008). However, up regulation was also observed in processes that include carbon metabolism and utilization, the synthesis of enzymes for cellulose, cell wall proteins, glycolysis, trehalose metabolism, biosynthesis of callose and fructokinase involved in starch/sucrose degradation (Li et al., 2008).

All of the above factors affect disease incidence and severity in different plant species that will rely upon diverse host-pathogen interactions. As reviewed by Chakraborty et al. (2000) due to disparity in disease severity in both biotrophic fungi and necrotrophic fungi envisaging the effects for unstudied pathosystems has become crucial. Some of the recent studies that showed the effects of elevated CO2 on disease incidence and severity have been listed in Table 2.

Table 2: Effect of elevated atmospheric CO2 on plant pathosystems with special reference to fungal pathogens

Effect on disease severity/ disease system	Effects on host	References
Lower disease severity or incidence		
Phyllosticta leaf spot of maple	No change in stomatal density; reduced g_s; altered leaf chemistry; reduced nutritive value of leaf tissue (elevated C:N ratios)	McElrone et al., 2005
Downy mildew of Soybean	No changes in stomatal densities; hypothesized changes in g_s: some changes in cuticular structures	Eastburn et al., 2010
Alternaria leaf blight	Increased concentration of epicuticular waxes; Decrease in stomatal density, length and width of stomatal aperture; Higher concentration of Glucosinolates	Mathur et al., 2013
Leaf spot severity of Eucalyptus	Increased plant height and more shoot production	Silva and Ghini, 2014
Powdery mildew of Japanese oak	Increased photosynthetic rates (P_N)	Watanabe et al., 2014
Powdery mildew of Barley	Increased photosynthetic rates (P_N)	Mikkelsen et al., 2015
Increased disease severity or incidence		
Brown spot of soybean	Increased plant height and canopy density	Eastburn et al., 2010
Crown rot of wheat	Increased biomass under elevated CO_2	Melloy et al., 2010
Cercospora leaf spot of red bud and sweet gum	No changes in host chemistry	McElrone et al., 2010
White rust	Increased concentration of carbohydrates	Mathur et al., 2013
Rot of maize	Lesser fumonisin production	Vaughan et al., 2014
No effect on disease severity and incidence		
Pyrenopeziza betulicola on silver birch	Reduced stomatal conductance under elevated CO_2, but no changes in stomatal density or stomatal index	Riikonen et al., 2008
Spot blotch of Barley	Increased photosynthetic rates (P_N)	Mikkelson et al., 2015

6. FUNGAL DISEASE MANAGEMENT STRATEGIES

Fungal diseases threaten commercially important plants and hence, pose problem to food production and food shortage leading to global food security. Significant reduction in both quality and yield has been observed in commercially important plants due to fungal diseases which lead to the loss of an entire plant. Common fungal plant diseases are anthracnose, rusts, leaf spots, wilts, curls, blight, scab, smuts, galls, cankers, root rots, damping off, dieback and mildews (Anderson et al. 2004). In order to minimize the losses due to fungus, it is essential to detect and identify the pathogens at an early stage by developing advanced, speedy and accurate disease detection technologies. Early detection and accurate identification of pathogens can control the spread of infection (Ray et al. 2017). The current article highlights a number of modern scientific tools which are expected to play a major role in resolving the

issues of food safety posed by climate change. Examples include rapid pathogen and contaminant detection using novel techniques (including nanotechnologies, biosensors); understanding of the molecular association between pathogen and the host plant using new molecular biological tools (like transcriptomics, proteomics and metabolomics) (Fig. 5.). The application of green synthesized nanoparticles (NPs) to control phytopathogens, as well as formulation of nanopesticides and their smart delivery systems to enhance effectiveness has also been addressed.

6.1. "OMICS" APPROACH TO ELUCIDATE THE ROLE OF DIFFERENT GENES AND PROTEINS

Development of a disease-resistant cultivar is an important factor in public health as well as in commercial cultivation around the world. This is a critical challenge for each plant breeder, because each desirable fungal affected trait might reduce the strength of selection for all desirable traits in a population. Breeding for disease control is considered to be reliable and environmentally friendly approach which increases yield as well as selection intensity for desirable genotypes. Use of modern tools like transcriptomics, proteomics and metabolomics have revealed the role of different genes and proteins and, thus, increased our understanding of the molecular association between pathogens and the host plants. Recent study of wheat defence response to Fusarium head blight (FHB) has been found associated with secondary cell wall thickening due to accumulation of phenolic glucoside, hydroxycinnamic acid and flavonoid as evident through differentially activated defence responses such as transcriptomics, proteomics and metabolomics (Shah et al. 2017). The functional genomics sequence and proteomic resources are increasingly used to investigate the biochemical composition of fungi and their biological interactions. Use of molecular markers in crop breeding has also been reported to increase the efficacy of indirect selection for traits of interest for both simple traits as well as QTLs (Gupta et al. 2010).

6.2. NANOTECHNOLOGY IN CONTROLLING FUNGAL PATHOGENS THROUGH GREEN SYNTHESIZED NANOPARTICLES

To control plant diseases from different phytopathogens, farmers usually spray various bactericides, fungicides and various chemicals. Huge applications of these chemicals ecotoxicological effects and their repeated use make the disease causing microbial flora more resistant to these agrochemicals. In some cases, they also enter in the food chain and get accumulated in the human body (Jayaseelan et al. 2012). Nano particles are nano scale compounds of less than 100 nm which are made up of organic and inorganic elements. It exhibits opto-electronic and dimensional attributes superior to their bulk

counterparts. Organic NPs include carbon in form of fullerene, liposomes, dendrimers, and inorganic nanoparticles consist of magnetic, noble metals and semiconductors. Green synthesized nanoparticles (NPs) are used to control plant disease – caused by phytopathogens especially bacteria and fungi, and offer a novel, easy, environmentally benign and cost-effective approach (Saratale et al. 2017). Generally, application of NPs takes place in two different ways to control plant pathogens. In direct application, NPs are sprayed on seeds or foliage of plant to inhibit direct invasion of plant pathogens. In the second approach, NPs are being developed which act as carriers for pesticides, herbicides and fertilizers for their controlled release, efficiently and safely under swamped conditions (Alghuthaymi et al. 2016; Bouwmeester et al. 2009; Bergeson 2010). In addition to this, in agriculture, NPs are exploited as biomarkers or as a rapid tool for the diagnosis and also in the detection of phytobacteria, viruses, and fungi as well as to monitor various environmental stresses such as salinity and drought and to detect the level of soil nutrients and heavy metals (Singh et al. 2010; Wang et al. 2010; Alghuthaymi et al. 2016; Ghormade et al. 2011). Singh et al. (2010) studied the effect of nanogoldbased immunosensors for the detection of Karnal bunt disease in wheat (Tilletia indica) by using the technique of surface plasmon resonance (SPR). In addition, Wang et al. (2010) developed an electrochemical sensor, using gold electrode with copper NPs, for sensing the levels of salicylic acid in oil seeds to detect or find out the pathogenic fungus Sclerotinia sclerotiorum. Recent studies have shown that AgNPs act as strong antimicrobial agents and have a broad-spectrum against different phytopathogen such as Biploaris sorokinniana, Botrytis cinerea, Colletotrichum gloeosporioides, Fusarium culmorum, Phythium ultimum, Phoma, Megnaporthe grisea, Trichoderma sp., Scalerotinia sclerotiorum, Sphaerotheca pannasa, and Rhizoctonia solani (Jayaseelan et al. 2012; Bryaskova et al. 2011; Krishnaraj et al. 2012; Alghuthaymi et al. 2016; Ghormade et al. 2011). Furthermore, antifungal activity of Ag-SiO2 NPs on Botrytis cinerea, combined effect of fluconazole and AgNPs on Phoma glomerata, P. herbarum, Fusarium semitectum, Trichoderma sp., and Candida albicans, as well as of Ag2S nanocrystals and ZnO and ZnTiO3 nanopowders on Aspergillus niger were also documented (Jayaseelan et al. 2012).

6.3. RNA INTERFERENCE (RNAI)

RNAI has been exploited in the development of new genomic tools, which allow the targeted silencing of genes of interest by involving small RNA molecules that leads to sequence-specific MRNA degradation in many eukaryotes (Fire et al. 1998). RNAI has been reported in all four eukaryotic kingdom (Baulcombe, 2015). RNAI has been found to control Fusarium Head Blight disease and mycotoxin contamination in cereals (Machado et al. 2017). Fusarium graminearum affects several part of cereals including stem base, seedling and flower. Fusarium Head Blight (FHB) is one of the major disease caused by Fusarium graminearum. FHB affects yield and quality of cereals and produce

mycotoxins like deoxynivalenol (DON) which are harmful to different organisms. A transgenic technology known as host-induced gene silencing (HIGS) is now being used to silence fungal genes in plants. Here, mobile small interfering RNA (siRNA) is produced by the host plant which is complementary to the targeted fungal gene. The greater use of RNAI for fungal disease control will further increase the understanding of the genes and pathways controlling the phenomena of the trans-kingdom RNAI. This would further help in the construction, deployment and re-use of HIGS multi-gene cassettes for the sustainable control of plant diseases.

6.4. FUNGAL DETECTION BY THE USE OF BIOSENSORS

Fungal diseases threaten commercially important plants leading to significant economic losses worldwide. Food shortage and the damage to food production caused by fungal pathogens also pose an enormous problem for global food security. To evaluate the seriousness of fungal plant diseases, it is essential to develop advanced, speedy and accurate disease detection technologies. Biosensors have been found to significantly enhance detection capabilities of analytical devices (Ray et al. 2017). It helps in early detection of the plant diseases which is not possible through conventional and present day techniques. A variety of biosensors have been reported, and many have shown high sensitivity and low detection limits. High-throughput detection efficiency of biosensor can be achieved by integrating nanomaterials, nanofabrication technologies and DNA sequencing with microfluidic and microelectronic platforms. The integration of existing biosensing mechanism with emerging nanotechnologies and DNA sequencing technologies will unlock the full potential of biosensor technology, thereby addressing the challenges associated with fungal detection in the near future.

7. CONCLUSIONS

Plants grown under elevated CO2 generally exhibit greater photosynthetic capacity, growth and yield. Elevated CO2 modify host susceptibility by changing leaf defense chemistry subsequently affecting the initial establishment of the pathogen on the host. Incidence and severity of plant disease epidemics and disease pressure on natural and crop plant system is likely to alter with changes in environmental conditions. There appears to be limited knowledge of how climate change will influence the biology of a pathogen and their interaction with the host. Knowledge of the host, pathogens and their interactions will be efficient tools for planning new and useful approaches to control disease. With the recent advancement in molecular biology and biotechnology, constant efforts have been made to study and improve crop productivity in current climate change scenario.

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1. INTRODUCTION

Heterogeneous group of plant growth promoting rhizobacteria (PGPR) can be found in the rhizosphere, i.e., "the layer of soil under influence of the root, which is much wealthier in bacteria (10 to 1000 times higher) than the surrounding bulk soil" or "at root surfaces and in association with roots, which can improve the quality of plant growth directly or indirectly. Diverse bacterial species like Klebsiella, Enterobacter, Alcalisens, Pseudomonas, Azospirillum, Azotobacter, Arthobacter, Burkholderia, Bacillus and Serratia have reported to augment plant growth (Sakthivel and Karthikeyan, 2012). PGPR is a very small portion (about 2–5%) of the total rhizobacterial community (Autoun and Kloepper, 2001). They are also considered as a group of beneficial free living soil bacteria for sustainable agriculture and environment (Babalola, 2010).

Due to the growth of human population fertilizers are used to increase crop production and meet the rising demands for food, but increases in the production cost and the hazardous nature of chemical fertilizers for the environment has led to a repetition of interest in the use of biofertilizers for enhanced environmental sustainability, lower cost production, and good crop yields. In the context of increasing international concern for food and environmental quality, the use of PGPR for reducing chemical inputs in agriculture is a potentially important issue (Lugtenberg and Kamilova, 2009). Phosphorous is found in surplus, but cannot be taken by plant roots due to insoluble precipitated complexes (Rengel and Marschner, 2005). PGPR have the ability to promote the growth of a plant by a direct and indirect mechanism (Gupta et al., 2000). The direct mechanism includes the production of some plant growth promoting composite like enzymes or acids from different bacterial species, which felicitate the uptake of certain plant nutrients from the environment. These mechanisms can be activated independently at different stages of plant growth. Among these are biological nitrogen fixation, phosphate solubilization, phytohormone production, and improvement of other plant nutrients uptake (Zaidi et al., 2009). Indirect mechanisms are the production of metabolites like antibiotics, siderophores, and so forth that decrease the growth of phytopathogens by producing some chemicals like hydrogen cyanide (Glick, 1995). Therefore, PGPR activities have a reflective influence on plant growth by persuading various mechanisms. Hence, the current study was commenced to isolate organisms from rhizospheric soil to study various PGPR activity under laboratory condition that helps to enhance plant growth by acting as a biofertilizer.

2. MATERIALS AND METHODS

2.1. ISOLATION, SCREENING, AND CHARACTERIZATION OF BACTERIAL STRAIN H_1WN_2

Soil samples were collected from the rhizosphere of wheat plant from different regions of Haldwani. Soil adhering to plant root was removed carefully from 15-20 cm deep and kept in sterile bags at low temperature. The isolation of single colony was done by streak plate method, while screening was done on the basis of its phosphate solubilization efficacy on pikovskaya's agar medium at 35°C for 4-5 days. A clear halo zone demonstrated the positive result of the isolate. The qualitative analysis was done by means of measuring their phosphate solubilizing index (PSI) in clear halo zone around their colony in pikovskaya's agar plates{PSI = (colony diameter + halo zone)/ colony diameter}. The microscopic identification was carried out by gram's staining using a light microscope. Biochemical and morphological tests of the isolated strain H_1WN_2 was carried out, as summarized in Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Several other important biochemical activities such as carbohydrate utilization, nitrate reductase tests were performed by using biochemical test kits (KB001, KB002 HiAssortedTM).

2.2. QUANTITATIVE ESTIMATION AND OPTIMIZATION OF PHOSPHATE SOLUBILISATION

Further, for quantitative estimation 1 ml of an overnight culture of H_1WN_2 was inoculated on 100 ml of pikovskaya's broth. The inoculated flask was incubated at 35°C for 6 days. The amount of inorganic phosphate released in the broth was estimated by sampling broth culture after every 24 hours. Each day about 10 ml of broth culture was centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the cell growth and insoluble phosphate. The available phosphate in the supernatant was estimated by phosphomolybdic blue color by using Jackson's method (1964). Simultaneously, a standard curve was prepared using various concentration of phosphate solution. The amount of phosphorus solubilized by the isolates was calculated from the standard curve. Furthermore, optimization of temperature and pH on the phosphate solubilization was determined by preparing two sets of the test tube containing pikovskaya's broth. One set of the test tubes containing pikovskaya's broth with constant pH = 7 were inoculated with strain H_1WN_2 and incubating the culture broth at different temperatures ranging from 15-40°C. Another set of test tubes containing pikovskaya's broth with a wide range of pH ranging from 4-9, were inoculated with strain H_1WN_2 the culture broth at temperature 35°C were incubated. The solubilized phosphates were estimated by the same method (Jackson, 1964).

2.3. QUANTITATIVE ESTIMATION AND CONFIRMATION OF IAA PRODUCTION

Quantification of IAA, by strain H_1WN_2, was done by inoculating bacterial culture into conical flask supplemented with tryptophan at a concentration of 2 mg / ml. The amount of IAA produce in the broth was estimated by sampling broth culture after every 24 hours. Broth culture was centrifuged at 7500 rpm for 10 minutes. The supernatant aliquot (2 ml) was taken from the culture and mixed with two drops of orthophosphoric acid and 4 of salkowski reagent (1 mL 0.5 M FeCl3, 50 mL, 35% perchloric acid;) (Noori and Saud, 2012) and incubated for 25 minutes at 30°C. Absorption was read at 530 nm and the concentration of IAA in the bacterial strain was determined and quantified by comparison with a standard curve of IAA (Reddy *et al*., 2011). Confirmation of IAA production was performed by thin layer chromatography (TLC) by using Nailwal et al., method (2014).

2.4. OPTIMIZATION OF IAA PRODUCTION AT DIFFERENT PARAMETERS

Three sets of the conical flasks containing nutrient broth were prepared to optimize the IAA production at different parameters. One set of the conical flask was maintained at constant pH7 and temperature 35°C, with varying concentrations of, tryptophan 2 to 10 mg / ml. The second set of the conical flask was maintained at constant temperature 35°C and concentration 2 mg / ml, with varying pH 4 to 9. The third set of the conical flask contains constant concentration, 2 mg / ml and pH7 with varying temperature 20 to 45°C. All the sets were inoculated with 500 µl of the active culture (A_{600} = 0.6) and incubated for 48 hours. Subsequently, the cultures were centrifuged at 7500 rpm for 10 minutes; afterwards, 1 ml of supernatant and 2 ml of Salkowski's reagent was mixed and incubated at 35°C for 25 minutes. Optical density was taken at 530 nm by using UV spectrophotometer to measure the amount of IAA production in all sets of the flask.

2.5. DETECTION OF SIDEROPHORE, HCN AND AMMONIA PRODUCTION

Production of HCN was assessed on King's B medium containing 4.4 g / L of glycine. Freshly grown broth cultures of the strain H_1WN_2 was spread on King's B medium containing glycine. A Whatman filter paper No. 1 soaked in 0.5 % picric acid solution (2 % sodium carbonate) was placed inside the lid of the plate. By using parafilm, plates were sealed and incubated for 4-5 days at 35°C. Orange to red color development indicates HCN production. The freshly grown bacterial culture was tested for the production of ammonia by inoculating them into peptone water broth tubes and incubated for 48-72 hours at 35°C. Subsequently, Nessler's reagent was added to each tube, development of brown to yellow color indicates the production of ammonia by isolates. Siderophore production was detected by

Chrome Azurol S (CAS) assay. The high affinity of siderophore for ferric iron to form a complex is the base for releasing the free dye. Iron-dye complex makes the medium blue in color. When siderophore is released, it connects to the ferric iron, releasing the dye, free which is orange in color. Hence, change blue to orange color indicates the presence of siderophore.

2.6. ANTIBIOTIC SENSITIVITY TEST

A fresh bacterial culture was inoculated in the 10 ml of nutrient broth and incubated at 35^0C for 24 hours. 0.1 ml of growing bacterial culture from test tube was spread on the nutrient agar plate. Simultaneously, an antibiotic disc was placed on the prepared nutrient agar plate and incubated them at 35^oC for 24 hours. Subsequent to incubation, the clear zone indicates the antibiotic sensitivity and zone size indicates their effectiveness. The antibiotic used here are, Cefoperazone (CPZ), Piperacillin (PI), Levofloxacin (LE), Gentamicin(GEN), Amikacin (AK), Colistin (CL),

2.7. BIO-FORMULATION AND SHELF LIFE STUDIES OF BACTERIAL STRAINS

Bio-formulation of H_1WN_2 was obtained by mixing broth culture with previously sterilized talc powder. Talc was composed of talcum steatite, talc fine powder, and hydrous magnesium silicate. Shelf-life of the formulations was studied by drawing samples at regular interval up to 25 days from the date of mixing and the colony forming unit (CFU) was counted by serial dilution agar plate method by using Sah et al., method (2011).

3. RESULTS AND DISCUSSION

3.1. ISOLATION, SCREENING AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL STRAIN H_1WN_2

Strain H_1WN_2 was small in size with entire, circular margin, cream color with a smooth surface. Screening on pikovskaya's agar plates represents the activity of phosphate solubilization (Fig. 1A). Furthermore, the strain was analyzed for metabolic properties by observing their response to diverse biochemical reactions using specific HiMedia test-kits for carbohydrate and by performing different another test like Casein hydrolysis test, (Fig. 1D), Starch hydrolysis test, Gelatinase test, Imvic tests, (Table 1). This performance put forward their unique metabolic potential and categorized them conditionally into the group of *Pentoea* sp.

Table 1: Biochemical test of bacterial strain H_1WN_2

S.N.	BiochemicalTest	H_1WN_2 Strain
1	Oxidase	+ve
2	Catalase	+ve
3	Indole	-ve
4	MethylRed	+ve
5	VogesProskauer	-ve
6	CitrateUtrilization	+ve
7	Dextrose	+ve
8	Lactose	-ve
9	Sucrose	+ve
10	Urease	-ve
11	NitrateReduction	+ve
12	Adonitol	+ve
13	Arabinose	-ve
14	Sorbitol	+ve
15	Mannitol	+ve
16	Rhamnose	+ve
17	H_2SProduction	-ve
18	GelatinHydrolysis	-ve
19	StarchHydrolysis	-ve
20	CaseinHydrolysis	+ve

3.2. DETECTION OF SIDEROPHORE, HCN, AMMONIA PRODUCTION AND ANTIBIOTIC SENSITIVITY TEST

Siderophore productions of the strain H_1WN_2 was confirmed by CAS assay. The appearance of a reddish-brown zone on CAS plates suggests the positive result for siderophore production. This low molecular weight iron binding protein is well known to exhibit antagonistic activity against phytopathogenic fungi. Color change in the CAS agar plate was the confirmation for siderophores production (Fig. 1B), and the color intensity can be the consequence of siderophore concentration. The availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting. Since it is the fourth most abundant element in the earth's crust it is largely required by all living organisms for direct microbial assimilation. The responsibility for such iron-chelating

siderophores in plant growth promotion is well-known (Felestrino et al., 2017). Ammonia production of the strain H_1WN_2 was found negative; the negative result of ammonia production is exposed by no color change in the brown medium. The production of HCN was checked for the strains H_1WN_2 and found negative. All the antibiotics were found effective against growth of the strain H_1WN_2, but the effectiveness is varied, out of these Cefoperazone (CPZ), Piperacillin (PI), were less effective compared to the rest antibiotic used Levofloxacin (LE), Gentamicin (GEN), Amikacin(AK), Colistin (CL(Fig. 1C).

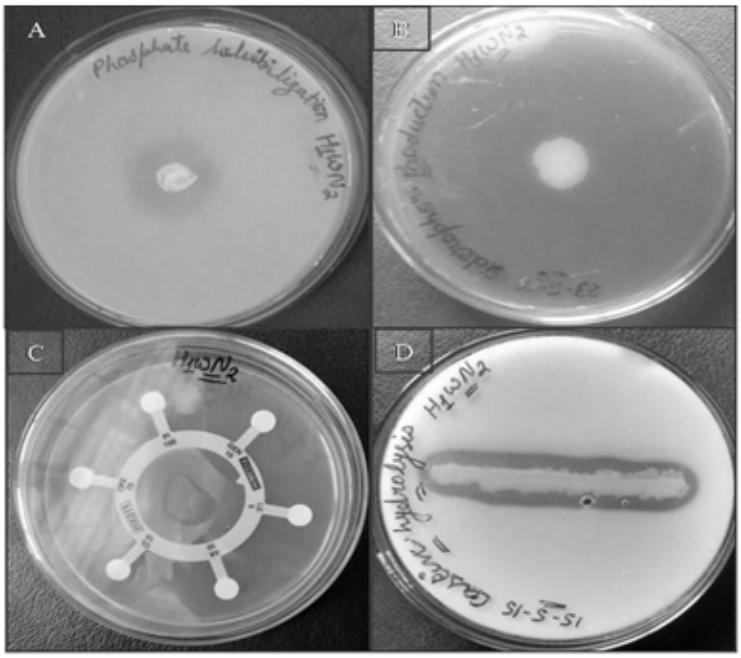


Fig. 1: The halo zone of inorganic solubilized phosphate (A); Siderophore production (B); Antibiotic Senstivity Test (C); Casein Hydrolysid (D).

3.3. ANALYSIS OF PHOSPHATE SOLUBILIZATION AND THEIR OPTIMIZATION

The strain H_1WN_2 was found to be potent phosphate solubilizer in qualitative analysis by means of measuring their PSI, 3.44 cm in clear halo zone around their colony in pikovskaya's agar plates (**Fig. 1A**). Meanwhile, quantitative examination of phosphate solubilization liberated in broth from tricalcium phosphate solubilization was deliberate by means of KH_2PO_4 curve at 600 nm to be (234 µg / ml) upon 6^{th} days of growth. The statistics to reveal a time-dependent augment in the sum of solubilized phosphate (**Fig. 2A**). Intermittent examination of pH of the culture filtrate showed a notable decrease from pH 7.0 to 3.96 (**Fig. 2C**). Thus, the results of improved phosphate solubilization with the screened bacterial strain could be due to higher acid production which honestly dissolves the rock phosphate, production of organic acids by phosphate solubilizing bacteria have been reported earlier (Vikram et al., 2007). The pH optima of the strain H_1WN_2 for phosphate solubilization was found to be 7.0 followed by pH 6 and 8. From the data obtained for pH stability studies that phosphate solubilization

was some extent more pronounced in the pH range from pH 6 to 8. Phosphate solubilization showed a decline in activity as pH increases over pH 8.0 suggesting that it best at neutral to slightly acidic to an alkaline range (Fig. 2D). Comparable results related to phosphate solubilization activity on different pH were supported by Jena and Chandi (2013).

The temperature optimum of the strain H_1WN_2 for phosphate solubilization was obtained at 35°C. Phosphate solubilization activity increases as the temperature increases from 15°C to 35°C, but a decline in activity abruptly as temperature cross 35°C (**Fig. 2B**). Parallel kinds of results related to phosphate solubilization activity at different temperature were supported by Sagervanshi et al., (2012).

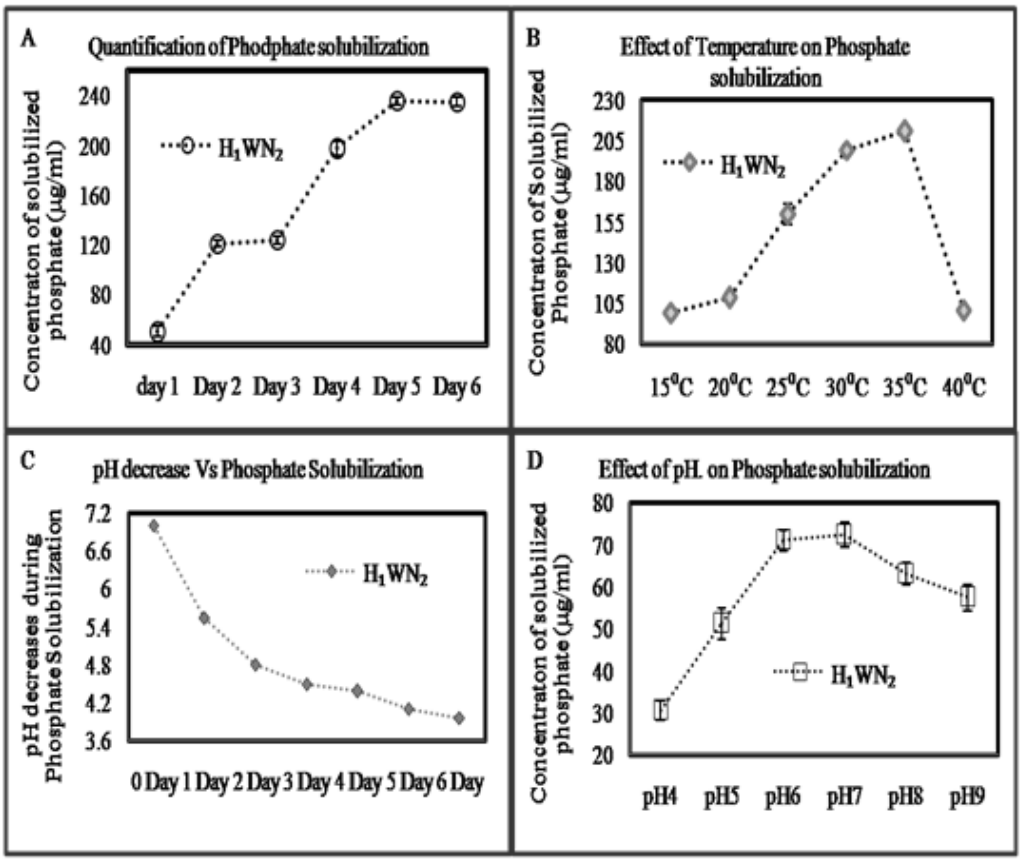


Fig. 2: Quantification of phosphate solubilization (A), Effect of temperature (B), Reduction of pH (C), Effect of pH (D) on phosphate solubilization

3.4 . PRODUCTION OF IAA, QUANTIFICATION, AND CONFIRMATION

Estimation of IAA produced by strain H_1WN_2 was carried out using the calibration curve of IAA. The extended incubation of culture up to 6 days and the culture filtrate at different time intervals showed a a linear and time dependent increase in IAA production. Enhanced production of IAA (44 µg / ml) was observed in the presence of 2 mg / ml tryptophan (Fig. 3A). Similar findings related to AA quantification were documented by Sakthivel and Karthikeyan. (2012).

Confirmation of IAA production was done by TLC method; chromatograms of spots were sprayed with the salkowski reagent that shows almost the same Rf value. It seems that almost the same Rf values are in agreement with other reports (Sudha et al., 2012).

The optimum temperature for IAA production was 35°C (54.06 µg / ml), followed by 40°C. The range of IAA production is quite consistent with the temperature series from 30°C to 45°C and slightly depleted as the temperature shifted to a lower temperature (Fig. 3B). Furthermore, optimum pH for IAA production was obtained at pH 6 (33.26 µg / ml), followed by pH 5. The range of IAA production is dramatical changes as the series of pH increases or decreases (Fig. 3D). Subsequently, the optimum tryptophan concentration, for IAA production of the strain H_1WN_2 was attained at 8 mg/ ml. The range of IAA production is a dramatical increase as the concentration of tryptophan increases from 2 mg / ml to 8 mg / ml and unexpectedly decreases as the concentration of tryptophan increases from 8 mg/ ml to 10 mg/ ml (Fig. 3C). The same optimization findings are in agreement with reports by another scientist (Walpol *et al*., 2013). IAA production by the bacterial strain has a cascade effect on the plant development due to its ability to influence root growth as well as shoot development, which in turn affects the nutrient uptake and ultimately the plant productivity. These trends coincided with the previous reports indicating IAA formation in the stationary stage of culture (Verma *et al*., 2016).

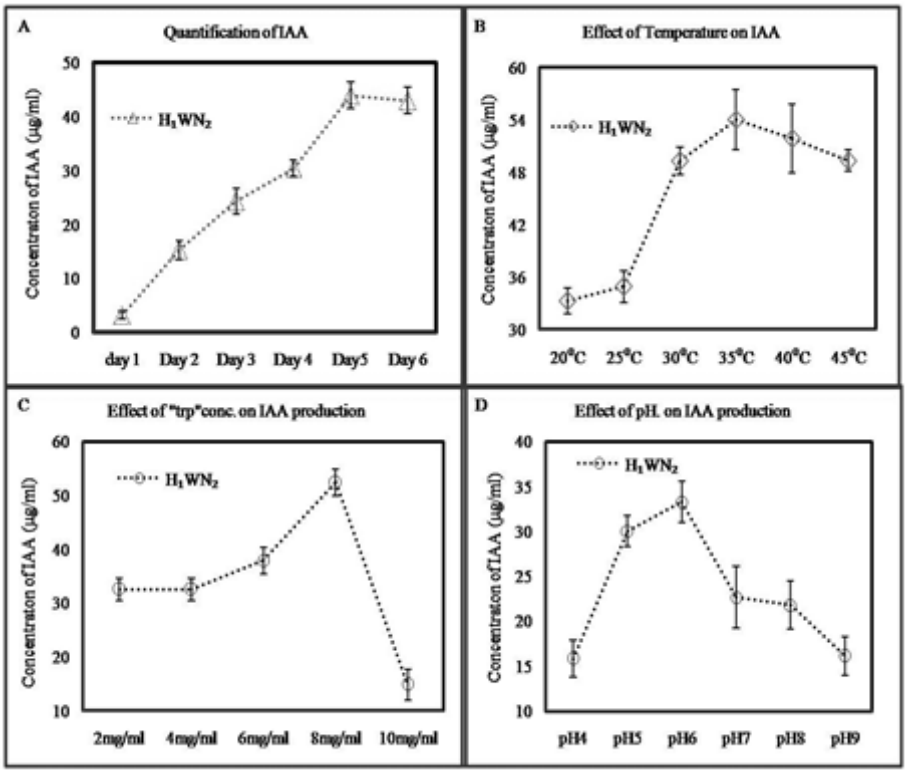


Fig. 3: Quantification of IAA production (A), Effect of temperature (B), Effect of tryptophan conc. (C), Effect of pH (D), on IAA production

3.5. ANALYSIS OF SHELF LIFE OF WBT1 UNDER BIO FORMULATION

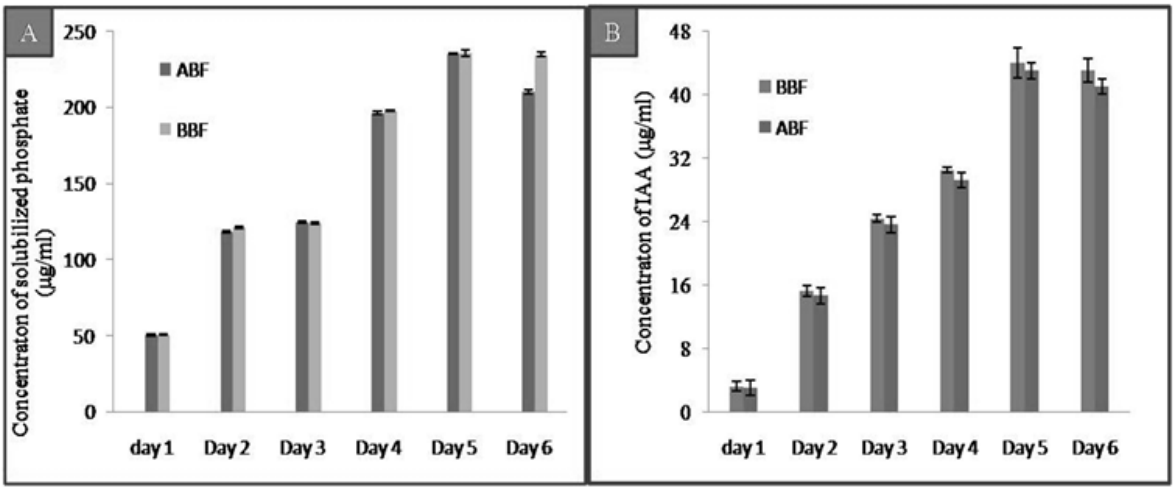
Further, the strain H_1WN_2 was used for the preparation of bio-formulation using talc powder as carrier material and evaluated for its viable cell count during the storage period of 25 days. The strain showed sustained viability with the progression of storage while showed a reduction in cell counts from 170 X 10^6 to 32 X 10^6 after storage for 25 days. The strain registering approximately counts of 170 X 10^6 cfu / ml after 3rd day with the succession of storage; it showed a slight decrease in the viability 132 X 10^6 cfu / ml after 7th day. The remarkable decrease was found after 14th day (102 X 10^6 cfu / ml). Finally, the count is reduced to 32 X 10^6 cfu / ml for CPP-1 (Table 2). Parallel results of viability counts were also conducted on PGPR bio inoculants using sawdust as a carrier (Arora et al., 2008).

Table 2: Total viable count of strain H_1WN_2 under bioformulation

Strain	Dilution Factor	3rd	7th	14th	20th	25th
H1S2	106	170±1	132±1	102±1	75±1	32±1

3.6. FUNCTIONAL CHARACTERIZATION OF CARRIER BASED FORMULATION

For further commercialization, the potential efficacy of phosphate solubilization and IAA production activity were measured periodically before and after bio-formulation, the results obtained are almost similar in both the cases. By analyzing the results, it is concluded that after storage of bio-formulated strain up to 25 days, the phosphate solubilization (Fig. 4A) and IAA production potential remains active with an insignificant alteration(Fig. 4B).



BBA= Before Bioformlation, ABF=After Bioformulation

Fig. 4: Comparable results of phosphate solubilization (A) and IAA production (B) before and after bio-formulation

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Characterization of Indigenous Potential Bacteria to Reduce Fluorine Concentration and Chemical Fertilizer Utilization from the Soil Environment

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAH) are group of environmental pollutant that are composed of carbon and hydrogen with fused benzene ring. PAHs occur as common constituents of petroleum, and coal tar but are most frequently formed by incomplete combustion of fossil fuels, waste incineration. PAH continuously increases and accumulate in soil, water and sediments and finally affect the environment and crop production. The effect of fluorene on germination and physiological and biochemical characteristics in different plant species is negatively correlated. Thus, appropriate management is requisite to diminish the concentration and toxicity of these substances. Bioremediation, a protected environment- friendly, method, uses the ability of an organism to lessen the concentration of PAH to a satisfactory level by transforming them into a less toxic form. This study examined the ability of PAHs (fluorene) degrading as well plant growth promoting activity of three bacterial strains (MHR_1, MB_1 & MB_4) isolated from crude oil polluted soil from Haldwani and Bhowali in Uttarakhand region. These strains showed considerable growth over fluorene, as the sole carbon source with 100-500 ppm concentration in Mineral Salt Medium (MSM) agar plates after 24 hours. Although, all the strains are screened out towards plant growth promoting activity, that subsequently replace the use of chemical fertilizer. Comparative study of fluorene degradation was found prominent, 86.2% in strain MHR_1 followed by strain MB_4, 68.7%and strain MB_1, 65%. Further, the efficiencies of various PGP activities like Phosphate solubilization, HCN, Siderophore, Ammonia and IAA production were analyzed and detected high in strain MB_1 followed by MB_4 and MHR_1. Therefore, the study suggests that these strains are extremely useful, possessing novel characteristic like environemental clean by removing fluorene concentration from the oil contaminated environment as well as eliminate utilization of chemical fertilizer by providing diverse PGP activity.

Keywords: PAH, Fluorene degradation, Siderophore, phosphate solubilization, IAA production

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a recalcitrant group of contaminants and are known to be highly persistent in the environment**.** There are many sources of PAH contamination in soils. These include creosote, petroleum products ((Huang *et al*. 2004**)**. The soil contaminated with aromatic compounds is of demanding ecological anxiety as they demonstrate cancer and mutation causing properties properties (Mrozik and Seget, 2010). PAHs have a low hydrophilic property, and highly lipophilic and frequently persist in soil and sediments **(**Kanaly and Harayama, 2010). Fluorene is a typical byproduct of coal conversion and energy related industries and is commonly found in vehicle exhaust emissions, crude oils, motor oils, coal and oil combustion, waste incineration, and industrial effluents (Salam and Obayori, 2014). Unlike many of the lower molecular weight PAHs such as naphthalene, phenanthrene, and anthracene, degraders are not as readily isolated from the different environment. Microorganisms play an important role in the degradation of aromatic hydrocarbons in terrestrial and aquatic ecosystems. Microbial metabolism of lower-molecular weight aromatic hydrocarbons is well established (Gibson et al., 1984). The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots, stimulate plant growth indirectly by inhibiting other deleterious microbes or root pathogens (Singh et al., 2013). The addition of PGPR increased the organic pollutant (PAHs and creosote) removal probably by enhancing plants germination. Ethylene is important for plant growth **(**Deikman, 1997), while excessive ethylene promoted by stresses can depress growth (Morgan and Drew, 1997). Now a day, there is increased interest to detoxify PAH-contaminated sites. Bioremediation which is based on microbial transformation and degradation is one of the most promising methods applied in the field of environmental biotechnology for cleanup of contaminated environments (Verma et al., 2017). However, the achievement of bioremediation has been inadequate by the shortage of microorganisms capable of degrading a broad range of PAHs. The rhizosphere, representing the thin layer of soil surrounding plant roots and the soil occupied by the roots(Bisht et al., 2015), supports large active groups of bacteria known as PGP (Singh et al 2013), that can stimulate plant growth directly and indirectly (Paliwal *et at*. 2016). Several bacteria have been isolated in purpose to utilize fluorene as the only carbon and energy source (Sokolovská et al., 2002). In this article, we described the degradation of fluorene in addition to analysis of plant growth promoting activity under in vitro condition by applying isolated indigenous microbial strains.

2. MATERIALS AND METHODS

2.1. ISOLATION AND SCREENING OF FLUORENE DEGRADING BACTERIAL STRAINS

Soil samples were collected from crude oil polluted soil near fuel filling stations from Bhowali (29.3823° N, 79.5196° E) and Haldwani (29.2183° N, 79.5130° E) in Uttarakhand region. Bacterial strains were isolated by enrichment culture techniques using 5 g of petroleum contaminated soil as inoculum in 50 ml minimal salt media (MSM) comprises {$CaCl_2$ 0.001g, $MnSO_4$ 0.0001g, KH_2PO_4 0.0005g, $(NH_4)_2SO_4$ 2.5g, Na_2HPO_4 1.0g, $MgSO_4$ 0.5g, Fe_2 (SO_4) 0.01g, $CoCl_2$ 0.005g, and $(NH_4)_6Mo_7O_{24}.4H_20$ 0.0001g per liter distilled water, pH=7)} sterile medium in a flask incorporated with 100-500 ppm fluorene (fluorene was added after autoclaving). Flasks were incubated at 35°C for 14 days in a shaker. 5 ml aliquot was transferred to 50 ml of a fresh MSM having same fluorene concentration and incubation. Following repeated successive enrichments, the culture suspension were plated onto the MSM agar plate comprising above fluorene concentration and incubation after serial dilution. For screening, isolated colonies were selected on the basis of clear zones formation in MSM agar plate having above fluorene concentration and incubation. Furthermore, well isolated single colony was picked up from MSM plate and streaking them on nutrient agar medium (Peptone 5.0g, Beef extract 3.0g, Potassium nitrate 5.0g per liter with 2% agar, pH= 7.2) and incubate them at 35°C for 3 days.

2.2. CHARACTERIZATION AND IDENTIFICATION OF SCREENED STRAINS

The selected strains were characterized by colony morphology on nutrient agar, gram staining, and different biochemical analysis was carried out according to Bergey's manual of systematic bacteriology (Holt *et al*. 1994). Other important biochemical properties were performed by using biochemical test kits (KB001 HiIMViC™ Biochemical Test Kit).

2.3. OPTIMIZATION OF BACTERIAL GROWTH AGAINST DIFFERENT PH AND TEMPERATURE

Two set of test tubes containing MSM medium incorporated with 0.5 mg L^{-1} fluorene concentration were prepared. One set of test tubes having different pH5 to 8, were inoculated with 500 µl of the active culture (A_{600} = 0.6) of bacterial cells and incubated them at 35°C. Another set of test tubes having a specific fixed pH 7, were inoculated with 500 µl of the active culture (A_{600}= 0.6) of bacterial cells and

incubated them at a different temperature from 25, 35, 40 and 45°C. Growth was monitored by taking OD after 6 days using spectrophotometer at 620 nm.

2.4. DEGRADATION ASSAY

Culture condition was optimized based on growth assay at pH 7 with 1 mg L^{-1} fluorene incubated, incubated at 35°C for 6 days. 1 ml liquate from the assay medium was extract off and acidified to pH 2 with 6 N HCl to inhibit bacterial activity by addition of 2 ml ethyl acetate using a shaker. The organic and water layers from the acidified medium were separated by centrifugation at 10,000 rpm for 10 minutes. The water layer was discarded while the organic layer was analyzed with UV-VIS spectrophotometer at 238 nm OD. Percent degradation was intended by applying the formula, % degradation = (Abs (b) - Abs (a) /Abs (b)) X 100. To where Abs (a) was the maximum absorbance and Abs (b) was the absorbance of the medium before incubation after the 6^{th} day of the incubation period. Mukesh *et al*. (2012) earlier also reported same type of result for percent degradation of fluorene.

2.5. HYDROGEN CYANIDE, AMMONIA, AND SIDEROPHORE PRODUCTION

Production of HCN was assessed on King's B medium containing 4.4g l^{-1} of glycine. Freshly grown broth cultures of all the strains were spread on King's B medium containing glycine. A Whatman filter paper No. 1 soaked in 0.5 % picric acid solution (2 % sodium carbonate) was placed inside the lid of the plate. Plates were sealed with parafilm and incubated at 35°C for 4-5 days. Development of orange to red color indicates HCN production. The freshly grown bacterial culture was tested for the production of ammonia by inoculating them into peptone water broth tubes and incubated them for 48-72 hours at 35°C. Subsequently, Nessler's reagent was added to each tube, development of brown to yellow color indicates the production of ammonia by isolates. Siderophore production was detected by Chrome Azurol S (CAS) assay. The high affinity of siderophore for ferric iron to form a complex is the base to releasing the free dye. Iron-dye complex makes the medium blue in color. When siderophore is released, it connects to the ferric iron, releasing the dye, free which is orange in color. Hence, change blue to orange color indicates the presence of siderophore.

2.6. PHOSPHATE SOLUBILIZATION AND ITS QUANTIFICATION

Separate pikovskaya's agar plate was inoculated in the center with each bacterial isolate and incubated for 4 to 5 days at 35°C. Phosphate solubilization activity of the isolates was detected by clearing zone in the plate. Further, by taking 1 ml of overnight culture of each isolates and inoculated it in 100 ml of

pikovskaya's broth (Pikovskaya, 1948), and incubated at 35°C for 6 days for quantification of phosphate solubilization potential. The amount of inorganic phosphate released in the broth was estimated by sampling broth culture after every 24 hours. Each day about 10 ml of broth culture was centrifuged at 10,000 rpm for 10 minutes to separate the supernatant from the cell growth and insoluble phosphate. The supernatant contain the available phosphate was estimated by using Jackson, (1973) of phosphomolybdic blue colour method. 50 ml volumetric flask containing 10 ml of chloromolybdic acid was mixed thoroughly with 1 ml of the supernatant. The volume was made up to just about three fourth with distilled water and 0.25 ml chlorostannous acid was added to it. Immediately, the volume was made to 50 ml with distilled water and mixed thoroughly. After 15 minutes, the blue colour developed was quantified at 610 nm spectrophotometrically. At the same time, by using various concentration of phosphate solution, a standard curve was prepared. The standard curve helps to calculate the amounts of phosphorus solubilized by the isolates in the medium.

2.7. OPTIMIZATION OF PHOSPHATE SOLUBILIZATION AT DIFFERENT PARAMETERS

Conical flask containing pikovskaya's broth was prepared in two sets, to optimize the solubilized phosphate at different parameters. One set of the conical flask containing constant pH7, with varying temperature 15 to 35°C and second set of conical flasks containing constant temperature 35°C, with varying pH 5 to 9. Subsequently, both the set of the conical flask was inoculated with 500 µl of the active culture (A_{600} = 0.6) of bacterial culture and incubating them for 48 hours. The available phosphate in the supernatant was estimated by the phosphomolybdic blue color method.

2.8 PRODUCTION, QUANTIFICATION, AND CONFIRMATION OF INDOLE-3-ACETIC ACID (IAA)

All the isolates were inoculated into 10 ml of nutrient broth supplemented with 0.5 mg ml^{-1} of tryptophan and incubated at 35°C in shaking incubator for 48 hours. Broth cultures were then centrifuged at 7500 rpm for 10 minutes. Then 1 ml of the supernatant was taken in another tube and 2 ml of salkowski's reagent was added and incubated at 35°C. Development of dark pink to orange color indicates production of IAA. Furthermore, to quantify the IAA produce in tubes, the absorbance was taken at 530 nm and the concentration of IAA in each bacterial strain was determined and quantified by comparing with a standard curve of IAA (Anwar *et al*. 2014).The confirmation of IAA production is executed by means of thin layer chromatography (TLC), for which single bacterial colony was inoculated to 10 ml of nutrient broth containing 1 mg ml^{-1} of tryptophan and incubated at 35°C for 5

days on a shaker. Bacterial cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 minutes. The supernatant was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted with ethyl acetate. Extracted ethyl acetate fraction was evaporated in a rotatory evaporator at 40°C. The extract was dissolved in 1 ml of methanol and kept at -20°C. In a glass chamber mixture of benzene: n butanol: acetic acid at 4:5:1 proportions were prepared as solvent system for the chromatogram. 1 µl of the extract was spotted and marked at the lower portion of the TLC plate. Similarly, a control spot of IAA (1 mg ml^{-1}) was placed. The plate was placed inside the glass chamber and was covered. Once the solvent front reached the top layer of the plate, the plate was dried naturally. IAA was identified by spraying the plates with salkowski's reagent. Test and control sample were compared by spot size and Rf value.

2.9. OPTIMIZATION OF IAA PRODUCTION AT DIFFERENT PARAMETERS

Three sets of the conical flask containing nutrient broth were prepared to optimize the IAA production at different parameters. One set of conical flask contains constant pH7 and temperature 35oC, with varying concentrations of, tryptophan 1 to 7 mg ml-1. The second set of conical flask contains constant temperature 35oC and concentration 2 mg ml-1, pH 5 to 9. The third set of conical flask contains constant concentration, 2 mg ml-1 and pH with varying temperature 15 to 40oC. All the setting were inoculated with a 500 µl of the active culture (A600 = 0.6) of the volume of culture and incubate for 48 hours. Subsequently, the cultures were centrifuged at 7500 rpm for 10 minutes afterward 1 ml of supernatant and 2 ml of Salkowski's reagent was mixed and incubated at 35oC for 25 minutes. Optical density was taken at 530 nm by using UV spectrophotometer to measure the amount of IAA production in all set of the flask.

3. RESULTS AND DISCUSSIONS

3.1. ISOLATION, SCREENING AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL STRAINS

Isolated strains MHR1, MB1 and MB4 were successfully isolated from crude oil polluted soil by applying enrichment technique. All the isolated strains were gram negative, short rod shaped white color with a smooth surface while the strain MHR1 has a rough surface (Figure 1A, 1B, and 1C). The screening was done on the basis of clear halo zone formation on MSM plates incorporated with fluorene. Similar pattern of isolation of fluorene degrading bacteria were done by Salam and Obayori, 2014. All the strains were analyzed for metabolic properties by observing their response to diverse biochemical reactions using specific HiMedia test-kits for carbohydrate and by performing different another test (Table-1).

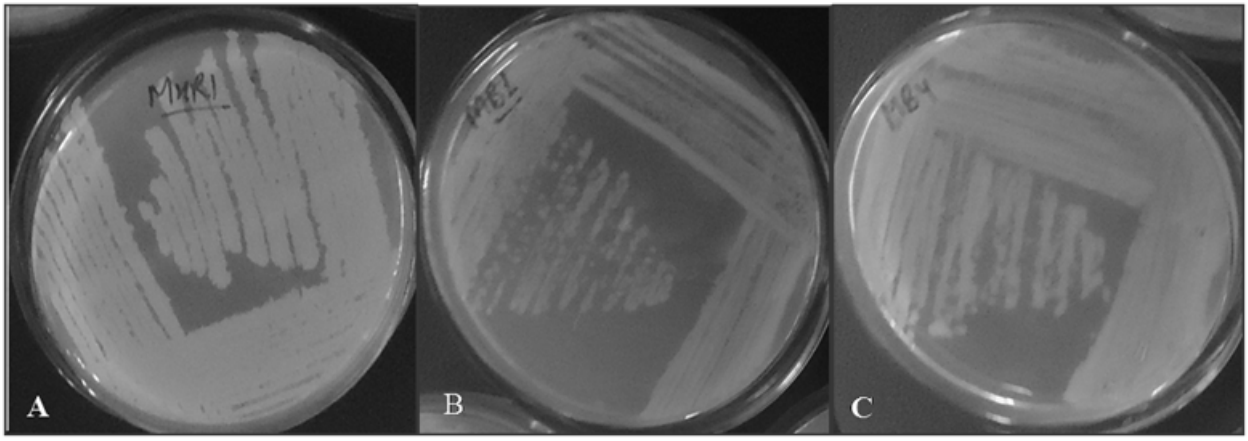

Figure 1: Morphology of isolated bacterial strains, MHR1(A), MB1(B), MB4©

Table 1: Biochemical test of bacterial strains

S.N.	Biochemical Test	Strain MB_1	Strain MHR_1	Strain MB_4
1.	Oxidase	+Ve	+Ve	+Ve
2.	Catalase	+Ve	+Ve	+Ve
3.	Indole	+Ve	-Ve	+Ve
4.	Methyl Red	+Ve	-Ve	+Ve
5.	Voges Proskauer	-Ve	-Ve	-Ve
6.	Citrate Utrilization	+Ve	-	+Ve
7.	Dextrose	+Ve	+Ve	+Ve
8.	Lactose	-Ve	-Ve	-Ve
9.	Sucrose	-Ve	+Ve	+Ve
10.	Urease	+Ve	+Ve	+Ve
11.	Nitrate Reduction	-Ve	-Ve	-Ve
12.	Adonitol	-Ve	+Ve	-Ve
13.	Arabinose	-Ve	-Ve	-Ve
14.	Sorbitol	-Ve	-Ve	-Ve
15.	Mannitol	+Ve	-Ve	-Ve
16.	Rhamnose	+Ve	-Ve	-Ve
17.	H2S Production	-Ve	-Ve	+Ve
18.	Gelatin Hydrolysis	+Ve	+Ve	+Ve
19.	Starch Hydrolysis	-Ve	-Ve	-Ve
20.	Casein Hydrolysis	-Ve	+Ve	-Ve

3.2. OPTIMIZATION OF BACTERIAL GROWTH AGAINST DIFFERENT PH AND TEMPERATURE

Optimal growth of all the bacterial strains was found higher at temperature 35ºC, followed by 40ºC, 30ºC =45ºC, and 25ºC with the exception of strain MHR1, it shows higher density almost in all the temperature range from 45ºC to 35ºC while less in 25ºC. So we concluded that all the strains have the capability to grow, in a wide range of temperature, but the requirement of temperature is different. Similarly, the optimal growth for pH of all the bacterial strain was found higher at pH 7 followed by pH 8 and lower at pH 5 (Figure 2). A similar type of optimization of bacterial growth in presence of hydrocarbon was reported (Gomare and Lahane, 2011).

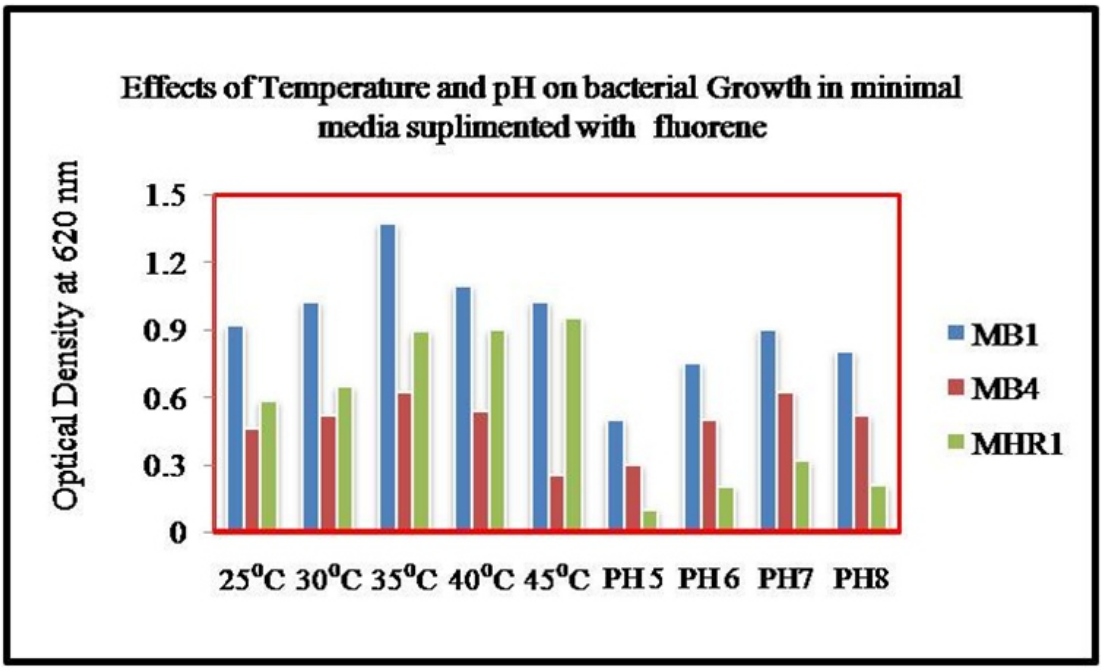

Figure 2: Optimization of Bacterial growth in response to various temperatures and pH in minimal media supplemented with fluorene.

3.3 FLUORENE DEGRADATION ASSAY

Minimal media incorporated with fluorene concentration 0.5 mg L-1, with neutral pH are inoculated with all the strains and subsequently incubated for 6 days at their optimal condition of temperature 35oC. Percent degradation of fluorene was measured by the taking optical density at 238 nm. Percent degradation of fluorene was found high (86.2%) in case of strain MHR1 followed by strain MB4 (68.7% and MB1 (65%) respectively (Table- 2). The percent fluorene degradation is also reported by (Salam and Obayori, 2014).

Table 2: Percent degradation of fluorene

Strains	OD in 238 nm	% degradation= (Abs (b) - Abs (a)/Abs(b))X 100
MB_1	0.028	(0.08 - 0.028/0.08) X 100 = 65%
MB_4	0.025	(0.08 - 0.025/0.08) X 100 = 68.7%
MHR_1	0.011	(0.08 - 0.011/0.08) X 100 = 86.2%

OD of control at 238nm = 0.08, (a) = OD of treated sample at 238nm, (b) = OD of control at 238nm,

3.4. SIDEROPHORE, AMMONIA AND HCN PRODUCTION

CAS assay, confirmed the siderophore productions by all the strains. The appearance of a reddish-brown zone on CAS plates suggests the positive result. This low molecular weight iron binding protein is well known to exhibit antagonistic activity against phytopathogenic fungi. The changes in color in the CAS agar plate by the strains recommend the production of siderophores (Figure 3 C), and the color intensity can be the consequence of siderophore concentration. Ammonia production of all the strains was found positive; the result of ammonia production is exposed by brown to yellow color. The production of HCN was checked for all the strains. The strain MB1 and MB4 found positive while strain MHR1 is negative. The results of HCN production revealed a remarkable change in color from yellow to brown against the control (Figure 3A, and 3 B). The availability of iron for microbial assimilation in environments such as the rhizosphere is extremely limiting. Since it is the fourth most abundant element in the earth's crust it is largely required by all living organisms for direct microbial assimilation. The similar results of siderophore production were supported by Vandana et al 2018. The responsibility of such iron-chelating siderophores in plant growth promotion is well-known (Felestrino et al., 2017). Likewise HCN, is recognized as a biocontrol agent, based on its ascribed toxicity against plant pathogens, suppress the pathogenic population in rhizospheric environment of the plant as well as it also involved in geochemical processes in the substrate (e.g., chelation of metals), indirectly increasing the availability of phosphate (Rijavec and Lapanje, 2016). Diazotrophs released Ammonia is one of the most important PGPR's traits which benefits the crop (Kundu, 1987, Vandana et al 2018).

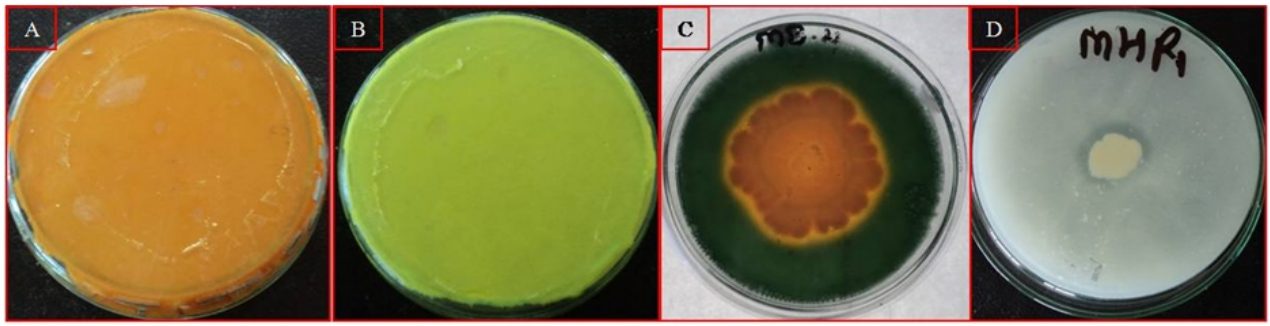

Figure 3: In vitro PGP activity of Isolated Strains (A) Positive HCN production (B) Control of HCN production (C) Positive Siderophore production (D) Positive Phosphate Solubilization

3.5. QUANTITATIVE MEASUREMENT AND OPTIMIZATION OF PHOSPHATE SOLUBILIZATION

Halo zone formation around the inoculum in pikovskaya's agar plate (Figure 3D) indicates the qualitative phosphate solubilization. Subsequently, quantitative examine was performed in pikovskaya's broth. The magnitude of soluble phosphate liberated in broth from tri-calcium phosphate, solubilization was measured by means of KH2PO4 curve upon 5 days of growth at 600nm. Relative account of solubilized phosphate was found high in case of MHR1 and MB4 (200 µg ml-1) followed by MB1 (191 µg ml-1). (Figure 4A). Intermittent examination of pH of the culture filtrate showed a notable decrease from pH 7.0 to 3.8 (Figure 4C). The optimum temperature of phosphate solubilization was obtained at 35°C for all the strains. Comparative account of solubilized phosphate was found high in case of MHR1 (166 µg ml-1) followed by MB1 and MB4 (116.34 µg ml and 115. 67) respectively (Figure 4A). The results are very interesting, all the strains solubilized phosphate succeeding, as the temperature increases up to attaining an optimum temperature, and abruptly reduction is started beyond the optimum temperature (Figure 4B). The pH optima of all the strains for phosphate solubilization were found to be 7.0 (95 -159µg ml-1) (Figure 4D).). The similar results of phosphate solubilization activity on different temperature were supported by (Sagervanshi et al., 2012) and on different pH were supported by Pandey and Putatunda 2018. We strongly believe that the phosphate solubilization aptitude of the bacterial strains could have played an imperative role in plant growth promotion.The production of organic acid, phosphatase enzyme and its role in phosphate solubilization is well known (Anwar et al., 2014).

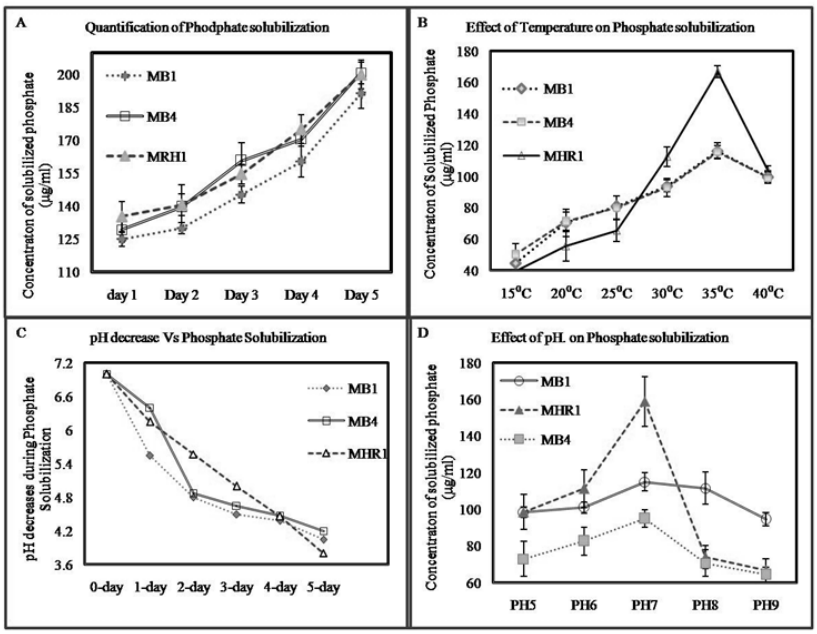

Figure 4: Quantification of Phosphate solubilization (PS) (A), Effect of temperature on PS (B), Reduction of pH during PS (C), Effect of pH on PS (D)

3.6. IAA PRODUCTION: CONFIRMATION AND ITS QUANTIFICATION

IAA production was found negative in the case of MHR1 while the extended incubation of the two strains MB1 and MB4 for a period of 5 days demonstrated a few degree of decrease in cell viability. The filtrate of the two strains at varying time periods confirmed a straight and time-dependent augmentation in IAA formation. IAA production is found high (11.23 µg ml-1) at 5th days in the case of MB4 by comparing with MB1 (Figure 5A) (Khan et al. 2014). This result corresponds with the earlier observations indicating IAA production in the stationary phase of culture (Verma et al. 2016; Ambawade and Pathade 2018). Furthermore, the confirmation of IAA was done by TLC method, in which the culture filtrate of all the strains was used to extract IAA for characterization. The spots of ethyl acetate extract of culture and standard IAA were tested in the solvent mixture of chloroform: ethyl acetate: formic acid (5:3:2). Chromatograms of spots were, sprayed with the salkowski reagent that shows almost the same Rf value as obtained by the standard IAA solution. Thus, it validates the intrinsic aptitude of isolated strains to produce phytohormone. The same TLC findings are in agreement with reports by another scientist (Walpol et al. 2013). The optimum temperature for IAA production was 25°C (44.36 and 39.63 µg ml-1) of the strains, MB4, and MB1, respectively, followed by 20°C in case of MB1 and 30°C in case of MB4. The range of IAA production is quite consistent with the temperature series from 20°C to 30°C and slightly depleted as the temperature fluctuates to higher and lower temperature (Figure 5B). While optimum pH for IAA production (43 µg ml-1) was obtained at pH 8, in case of MB1 while (42.93 µg ml-1) at PH7 in case of MB4. The range of IAA production is dramatical changes as the series of pH increases or decreases from pH 7 to pH 9 or to pH5 (Figure 5D). Subsequently, the optimum tryptophan concentrations for IAA production of both the strains were attained at 5 mg ml-1. But the concentration of IAA production was attained high for MB4 (60.34 µg ml-1) followed by MB1 (53.13 µg ml-1). The range of IAA production is dramatical increases as the concentration of tryptophan increases from 1 mg ml-1 to 5 mg ml-1 and similarly noticeably decreases as the concentration of tryptophan increases from 5 mg ml -1 to 7 mg ml-1 (Figure 5C). The same optimization findings are in agreement with reports by another scientist (Walpol et al. 2013). IAA production by the bacterial strain has a cascading effect on the plant development due to its ability to influence root growth as well as shoot development, which in turn affects the nutrient uptake and ultimately the plant productivity. These trends coincided with the previous reports indicating IAA formation in the stationary stage of culture (Verma et al. 2016).

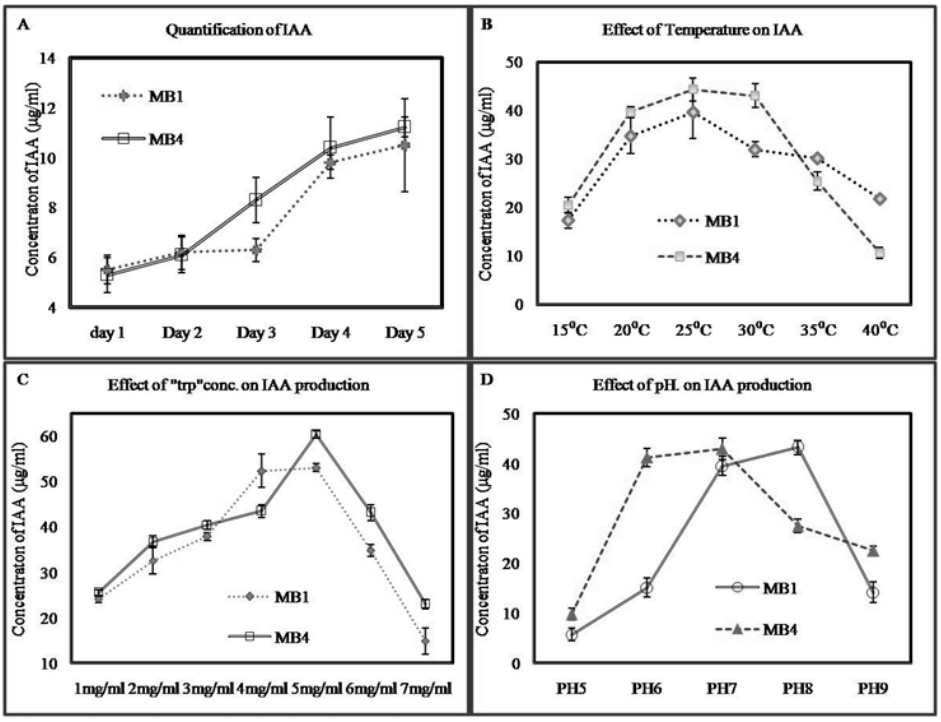

Figure 5: Quantification of IAA production (A), Effect of temperature on IAA production (B), Effect of tryptophan conc. on IAA production (C), Effect of pH on IAA production (D)

4. CONCLUSION

Numerous bacteria strains present in petroleum contaminated region have been shown to acquire fluorene biodegradation as well as plant growth promoting activity. These strains have quite a lot of characteristics which help them to promote plant growth and their yield. They have some characteristics like the ability to produce phytohormones, for root and shoot development, some enzyme/acid production to make the availability of solubilized phosphate that easily takes up by the plant, HCN, and siderophore production that also act directly or indirectly on plant growth by fighting against phytopathogenic microorganisms or providing ferrous availability. Moreover, the biodegradation potential of fluorene provides nutrient to the microbial flora that make the soil more permeable and in due course increases their water holding ability. So, in this context, potential bacterial strains (MHR1, MB1 & MB4) against fluorene biodegradation activity and PGP activity were isolated, screened and characterized. Hence, the present isolates can act as potential candidates for plants growth as well as best fluorene depredator.

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1. Introduction

Plant growth is reinforced by plant nutrition and it is essential for the production of crops. The zone of interactions of plant roots and microorganisms are generally called as rhizosphere and micro-organisms resides in this zone are called rhizospheric bacteria (Cleyet- Marcel et al., 2001 and Kloepper, 1994). The association between organisms and roots can be beneficial (water uptake, soil stabilization, growth promotion, N2 fixation, bio-control, antibiosis, symbiosis), harmful (infection, phytoxicity) or neutral (nutrient flux, free enzyme release, attachment, allelopathy, competition) (Weller et al., 1988), or indirectly via their ability to remove a broad spectrum of bacterial, fungal and parasitic infections, also provide protection against viral disease. Many studies show the diversity of

microbial agents involved in the biological control (Ahmad et al., 2008). There are lots of species with PGP potential are: Pseudomonas, Bacillus, Rhizobium, Burkholderia, Micrococcus, Azotobacter and Erwinia. (Siddiqui et al., 2006). Advancement of PGP bacteria was recounted by nitrogen fixation, phytohormone synthesis, resistance against environmental stress, inhibition of ethylene production, as well as accumulative accessibility of nutrients like phosphorus, iron and minor elements, and growth enrichment by volatile compounds. There are some studies suggested enhancement of efficacy of different agro-inputs by employing the best combinations of bacterial microbes (Chang et al., 2005).

Rhizosphere has been generally partitioned into the following three precincts (Pratibha et al., 2013). (1). Endorhizosphere: that consists of the root tissue including the endodermis and cortical layers; (2). Rhizoplane: is the root surface where soil particles and microbes adhere. It consists of epidermis, cortex and mucilaginous polysaccharide layer; (3). Ectorhizosphere: that consists of soil immediately adjacent to the root. Stimulation of microbial proliferation around the root due to the release of various organic compounds by the roots is known as the rhizospheric effect. According to a report by Prakash and Karthikeyan, (2013), secretion of vast array of compounds into rhizosphere, with nearly 49% of all photo synthetically fixed carbon being transferred to the rhizosphere, is a remarkable feature of plant roots.

To fulfil the requirements of food for world's increasing population there should be an alternative of chemical fertilizer because of its toxic effect and is also caused soil infertility. The alternative should not only overcome this issue but also increase crop production with minimum efforts and the main objective for fertilizers should be cost effective. The best alternative to overcome this problem could be bacterial inoculum; bio-fertilizer is a fertilizer in which bacterial culture use to enhance crop productivity. Definition of bio-fertilizer according to Vessey (2003), an inoculum which contains mixture of living microorganisms, when applied to field, colonizes the rhizosphere and promote the growth by increasing the solubility of insoluble nutrients to host plant. Bio-fertilizers have a natural mechanism to supply nutrients to plants by solubilizing phosphorus, nitrogen fixation and by synthesis of plant growth promoting substances. There are microbes present in bio-fertilizers that increase the soil natural nutrient cycle and help in building soil organic matter and maintain the soil fertility. This study investigate candidature of potent isolated strain HBYM-1 on the parameter of PGPRs.

2. MATERIALS AND METHODS:

2.1. ISOLATION, SCREENING, AND BIOCHEMICAL CHARACTERIZATION

Soil sample was collected from rhizosphere of mustard plant from different sites of haldwani (Uttarakhand). Soil adhering to plant root is removed carefully from 15-20 cm deep and kept in sterile bags. The isolation of single colony was done by streak plate method while screening was done on the basis of its phosphate solubilisation efficacy on pikovskaya's agar medium at 37°C for 4-5 days. A clear halo zone was demonstrated the positive result of the isolate. The microscopic identification was carried out by gram's staining using a light microscope. Morphological and biochemical characterization of HBYM-1 was carried out accordingly Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). All important biochemical activities such as carbohydrate utilization, nitrate reductase tests were performed by using biochemical test kits (KB001, KB002 HiAssortedTM).

2.2. QUANTITATIVE ESTIMATION AND OPTIMIZATION OF PHOSPHATE SOLUBILIZATION

After screening quantitative estimation was done by inoculating 1 mL of overnight culture of HBYM-1 into 100 mL of pikovskaya's broth. The inoculated flask was incubated at 37°C for 6 days. The amount of inorganic phosphate released in the broth was estimated by sampling broth culture after every 24 hrs each day 10 mL of broth culture was centrifuged at 10,000 rpm for 10 min to separate the supernatant from the cell growth and insoluble phosphate. The available phosphate in the supernatant was estimated by phosphomolybdic blue colour by using Jackson's method (Jackson 1964). Simultaneously, a standard curve was prepared using various concentration of phosphate solution. The amount of phosphorus solubilized by the isolate was calculated from the standard curve. Furthermore, optimization of temperature and pH on the phosphate solubilisation was determined by preparing two sets of test tube containing pikovskaya's broth. One set of pikovskaya's broth test tubes having constant pH 7 was inoculated with strain HBYM-1 and incubating the culture broth at different temperatures ranging from 20-40°C, in another sets of containing pikovskaya's broth test tubes the pH ranging from 4-9 were inoculated with strain HBYM-1 and incubating the culture broth at temperature 37°C. The solubilized phosphates were estimated by same method described above (Jackson 1964).

2.3. DETECTION OF SIDEROPHORE AND HCN PRODUCTION

The analysis of siderophore production by the bacterial strain HBYM-1 was performed by Chrome

presence of siderophore. The CAS assay is the universal chemical assay for the detection of siderophores. It is based on the high affinity of siderophores for ferric iron, where by ferric iron bound to dye, is released from the dye. The blue colour of the medium is due to the dye iron complex. When siderophore is added, the siderophore binds to the ferric iron, releasing the free dye, which is orange in colour.

Fe^{3}+ +dye (blue) + siderophore → Fe^{3} siderophore + dye (Orange)

Change in colour from blue to orange confirms the production of siderophore.

Production of HCN was assessed on King's B medium containing 4.4g/l of glycine. Freshly grown broth culture was spreaded on King's B medium containing glycine. A Whatman's filter paper no. 1 soaked in 0.5% picric acid solution (2% sodium carbonate) was placed inside the lid of the plate. Plates were sealed with parafilm and incubated at 37°C for 4-5 days.

2.4. DETECTION OF BIO CONTROL POTENTIAL OF HBYM-1

Acquire freshly grown bacterial culture HBYM-1 in nutrient broth. Spread the freshly grown fungus culture on potato dextrose agar (PDA) plate. Punch a hole in PDA plate. Inoculate the freshly grown bacterial culture in the hole. Incubate it at 28 °C for 2-4 days. Positive result for bio-control activity was confirmed by clear zone formation around the hole inoculated with bacterial culture.

3. RESULTS AND DISCUSSION:

3.1. ISOLATION, SCREENING, AND BIOCHEMICAL CHARACTERIZATION OF BACTERIAL STRAIN

Strain HBYM-1 is rod shaped bacteria with undulate, circular margin, white colour with smooth surface and raised elevation (Fig. 1A). Screening on pikovskaya's agar plates represents the activity of phosphate solubilisation (Fig. 1B). Furthermore, the strain was analyzed for metabolic properties by observing their response to diverse biochemical reactions using specific HiMedia test-kits for carbohydrate and by performing different other test (Table 1). This performance put forward their unique metabolic potential and categorized them conditionally into the group of Bacillus sp.

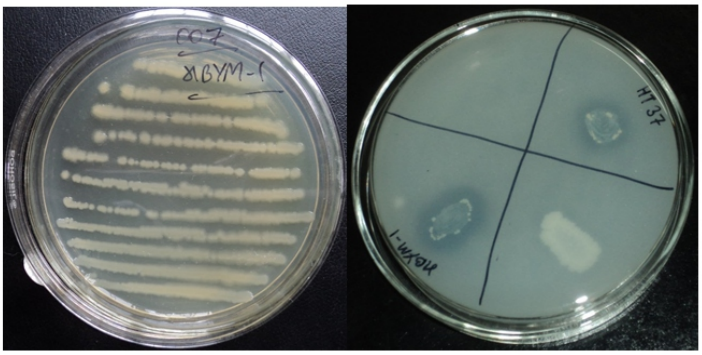

Fig 1: Morphological characterization of HBYM-1 (A); Phosphate solubilization potential (B)

S. No.	Biochemical Tests	Response
1	Gram's staining	-
2	Morphology	Rod shaped
3	VP test	+
4	Indole test	+
5	MR test	-
6	Citrate test	+
7	Glucose test	+
8	Adonitol test	-
9	Arabinose test	-
10	Lactose test	+
11	Sorbitol test	-
12	Mannitol test	+
13	Rhamnose test	-
14	Sucrose test	+
15	Nitrate reductase test	+
16	Gelatinase test	+
17	Starch hydrolysis test	-
18	Catalase test	+
19	Oxidase test	+
20	H_2S test	-
21	Gluconate test	-
22	Casein hydrolysis test	-
23	Deamination of A.A.	+
24	Urease test	-

3.2. QUANTIFICATION OF PHOSPHATE SOLUBILIZATION AND ITS OPTIMIZATION

HBYM-1 strain has the potential to solubilize phosphate which are unavailable for plants. The phosphate solubilisation potential was checked and optimized on pikovskaya's agar medium by clear halo zone and quantified by use of measuring their phosphate solubilisation index (PSI) 2.87 in

Pikovskaya's agar plates. On the other hand quantitative estimation was done by a method in which phosphate solubilisation liberated in broth from tri-calcium phosphate solubilisation was deliberate by means of KH2PO4 curve at 600 nm to be (240-190 µg mL-1) on 6th day of growth. The statistics also support results (Fig. 2A), while examining of pH there is pattern of pH reduction from pH 7.0 to 3.19. Thus, the results suggest the improved phosphate solubilisation with the screened bacterial strain could be due to result of higher acid production which honestly dissolves the rock phosphate, production of organic acids by PSB have been reported earlier (Vikram et al., 2007).

Optimum pH and temperature for screened strain HBYM-1 at which strain solubilized maximum phosphate was found at pH 4 followed by pH 5.0 (Fig. 2B) and optimum temperature was 30°C followed by 35°C and 40°C respectively. The result suggests that isolated strain capable in solubilizing phosphate in acidic conditions and it will be persuasive acidic phosphate solubilizer and for temperature optimization, results advises that screened isolate are capable to solubilised phosphate in a wide range of temperature 25°C to 40°C. (Fig. 2C).

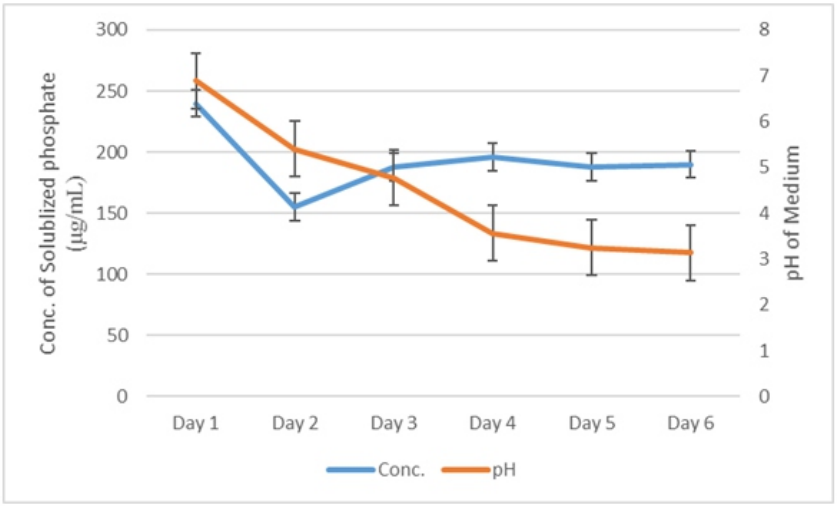

Fig 2A: Quantification of solubilized phosphate

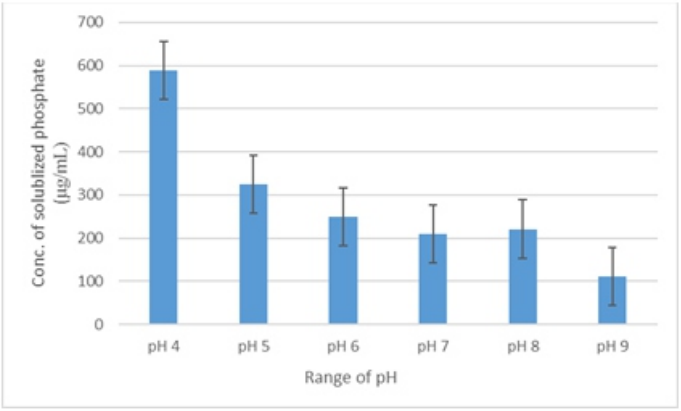

Fig 2B: Effect of pH on phosphate solubilisation

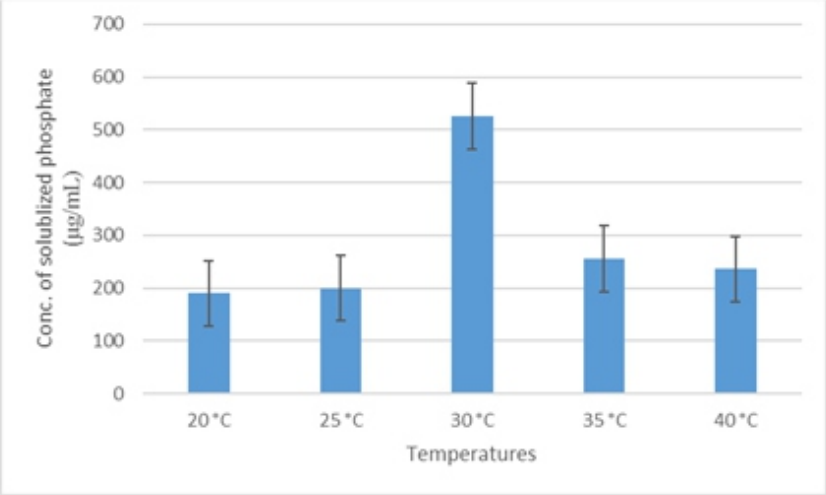

Fig 2C: Effect of temperature on phosphate solubilisation

3.3. SIDEROPHORE AND HCN PRODUCTION

Siderophore production was confirmed by CAS assay, appearance of a reddish-brown zone on CAS plates suggests the positive result for siderophore production (Fig. 3A). In aerobic conditions at physiological pH, the reduced ferrous (Fe2+) form is unstable and is readily oxidized to the oxidized ferric (Fe3+) form, which normally occurs as a poorly soluble iron hydroxide basically unavailable to biological systems (Krewu-lak and Vogel, 2008; Osório et al., 2008).

This low molecular weight iron binding protein is well known to exhibit antagonistic activity against phytopathogenic fungi. Microbes release siderophores to chelate iron from the insoluble mineral form by formation of soluble Fe3+ complexes that can be taken up by active transport mechanisms. Some previously report proved that Bacillus sp. produce maximum siderophore after 48 hrs. (Pahari et al., 2016).

Moreover, hydrogen cyanide production was checked for isolated strain by inoculating on King's B medium and positive response was observed by remarkable change in colour from yellow to red against the control and thus supposed to efficient bio-control agents (Fig. 3B) and thus concluded that the isolated strain HBYM-1 was the most effective. These results substantiate with the earlier studies on HCN production (Ahmad et al., 2008). It has been concluded that this positive strain will be beneficial for growth of crop production along with suppressing the growth of pathogen and help in enhancing the growth.

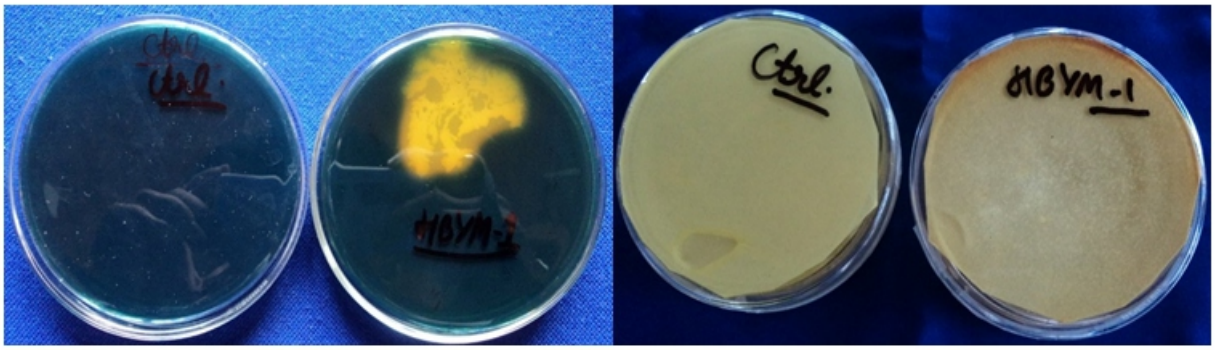

Fig 3: Production of Siderophore with Control (A) and HCN production with control (B)

3.4. BIO-CONTROLACTIVITY

Subsequently the confirmation of antagonistic activity against pathogenic fungus Trichoderma longibrahaitum was observed with a zone of inhibition in PDA plate point out the inhibition of fungal culture by bacterium HBYM-1 (Fig. 4). Many rhizosphere-inhabiting fluorescent pseudomonads exhibit antagonistic effects toward fungal pathogens of plant roots, thereby protecting the plant from disease like Maize ear rot, Head blight, Panama disease in Banana and tomato root rot were reported (Meera and Balabaskar, 2012, Noori and saud, 2012).

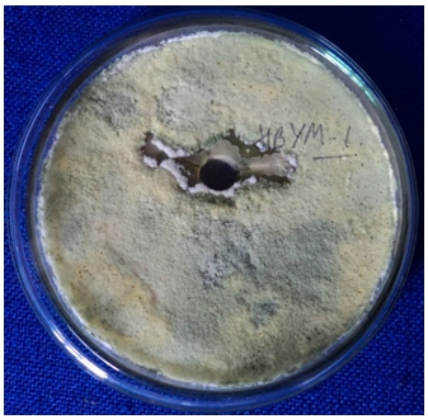

Fig 4: Bio-control activity

CONCLUSION:

Microorganisms with close connotation with plant roots and uphold growth of plants by augmenting nutrient accessibility are termed as PGPRs. According to Anwar et al., 2014, a bacterial culture can be entitled under PGPB category if it has some characteristics like phosphate solubilisation, phytohormone production and bio-control potential. In the present study, isolated strain HBYM-1 fulfil all the parameters of an ideal bio-fertilizer as it shows positive response against all PGP activities. HBYM-1 have the potential to endorse PGP traits even in stressed conditions such as acidic medium or temperature and pH variations also.

For future, combinatorial settings should be explored with some other PGP cultures so that it can be commercialized for field trials.

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