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Frontiers in Life Science

Volume VII

Editors

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PREFACE

Life Sciences have always been a fundamental area of science. The exponential increase in the quantity of scientific information and the rate, at which new discoveries are made, require very elaborate, interdisciplinary and up-to-date information and their understanding. Enhanced understanding of biological phenomenon incorporated with interdisciplinary approaches has resulted in major breakthrough products for betterment of society. To keep the view in mind we are delighted to publish our book entitled ***"Frontiers in Life Science Volume VII"****. This book is the compilation of esteemed articles of acknowledged experts in the fields of basic and applied life science.*

This book is published in the hopes of sharing the new research and findings in the field of life science subjects. Life science can help us unlock the mysteries of our universe, but beyond that, conquering it can be personally satisfying. We developed this digital book with the goal of helping people achieve that feeling of accomplishment.

The articles in the book have been contributed by eminent scientists, academicians. Our special thanks and appreciation goes to experts and research workers whose contributions have enriched this book. We thank our publisher Bhumi Publishing, India for taking pains in bringing out the book.

Finally, we will always remain a debtor to all our well-wishers for their blessings, without which this book would not have come into existence.

Editors

CONTENT

USE OF WASTE MARBLE POWDER TO ENRICH SOIL PRODUCTIVITY AND TO REDUCE ENVIRONMENTAL HAZARDS

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Abstract:

India is the world's third-largest producer of natural stones, with an 11 percent share of the global market. Rajasthan contributes significantly to the country's mining output. Rajasthan is known for having an endless supply of many sorts of dimensional stones, such as marble, granite, lime stone, kota stone, etc. The marble industry is one of Rajasthan's most well-known industries. Approximately 30% to 40% of the material is left as waste in the form of powder or slurry during mechanical processing. Nearly half of all precious mineral resources are squandered due to a lack of technological advancements in mining, processing, and polishing, with gang saws accounting for more than half of the waste generated.

Due to a lack of adequate quarrying technology, a large amount of trash is generated. Improper waste disposal has resulted in land degradation, loss of aesthetics, pollution, health and safety risks. As a result, environmental regulations are making recycling of industrial waste and byproducts a mandatory requirement in accordance with the notion of sustainable development. Marble waste is high in Calcium and contains a variety of vital elements that can be converted into a variety of useful, valuable, and more accessible forms that can be used as a soil amendment and may be advantageous to plant growth. This will aid in the reduction of pollution in the environment.

Keywords: marble industry, waste, environment pollution, soil fertility, etc.

Introduction:

India possesses enormous deposits of all types of natural stone with a variety of excellent properties. These are granites, marbles, slates, sandstones, lime stones, quartzite, etc. In this modern world, the marble industry is the fastest growing industry. Iran, Italy, China, Turkey, India, Egypt, Spain, Brazil, Algeria, Sweden, and France are the main marble-producing countries (Rana *et al.*, 2016; Rana *et al.*, 2017; Munir *et al.*, 2018; Seghir *et al.*, 2018). India is the third-largest marble-producing country in the world, and almost 10% of the world's marble powder is quarried here (Ashish, 2018).

In addition, the import and processing of stone are majorly done in countries such as Pakistan, United States, Egypt, Saudi Arabia, Portugal, Germany, France, Norway, and Greece.

In the country, Rajasthan state has more than 95% of marble processors. There are around 4000 marble mines and 1100 marble processing units spread over 16 districts of Rajasthan. It is an important source of employment and income in the state, often providing entry points to low-skilled, rural, seasonal migrants, despite the influx of new industries in the state in recent years. The state has the potential to develop into a global mining hub. Marble was used for building tombs, temples, and palaces.

Marble is a rock resulting from the metamorphism of sedimentary carbonate rocks, most commonly limestone rock. Metamorphism causes variable recrystallization of the original carbonate mineral grains. The purest calcite ($CaCO_3$) marble is white in color. Marble containing hematite (Fe_2O_3) is reddish in color, whereas marble containing limonite ($FeO(OH)nH_2O$) is yellow in color. The green colour of marble is due to the presence of serpentine $(Mg, Fe)_3Si_2O_5(OH)_4$.

Marble deposit centres in Rajasthan

The important regions of marble deposits in Rajasthan are Udaipur-Rajsamand-Chittorgarh region, Makrana-Kishangarh region, Banswara-Dungarpur region, Andhi (Jaipur)-Jhiri (Alwar) region, and the Jaisalmer region.

- **Makrana:** Makrana is the source of the marble used in the Taj Mahal. It is situated at a distance of 60 km from Kishangarh and falls in the Nagaur district of Rajasthan. The region has various mining ranges, mainly Doongri, Devi, Ulodi, Saabwali, Gulabi, Kumari, Neharkhan, Matabhar, Matabhar kumari, Chuck Doongri, Chosira, and Pahar Kua, amongst others.
- **Rajnagar Marble:** World's largest marble producing area, with over 2,000 gang saw units located in the nearby town of Udaipur to process the material produced. Agaria is a variety of this area, with numerous other varieties and patterns, primarily in white base. The marble is dolomitic and often has quartz intrusions.
- **Andhi Marble:** Located near the capital city of the state of Jaipur (also known as the 'Pink City'), it is dolomitic marble with intrusions of tremolite, and is commonly known by the name of pista (pistachio) marble, because of the green coloured tremolite against an off-white background. One of the famous varieties of this area was known as Indo-Italian, owing to its resemblance with Satvario Marble. Most of the mining of this famous field is now banned by the Supreme Court of India because of the vicinity of the area to the Sariska Tiger Reserve.
- **Salumber Marble:** Also known as Onyx Marble, it has thick bands of green and pink hints. A resemblance to Onyx Marble from Pakistan gives it this name. This is also highly dolomitic.

- **Jaisalmer Stone:** Jaisalmer stone is found in the Jaisalmer district of Rajasthan. It has not been metamorphosed and hence is still a limestone. It is known as Yellow Marble in trade circles. It is mined in the Jaisalmer District.
- These are ultra-basic rocks in shades of brown and green found in Rajasthan. The criss-cross linear pattern gives it a remarkable resemblance to a photograph of a dense forest. These are also known as forest green and brown or fancy green and brown.
- **Rajasthan-Abu Black:** This is one of the rare black-textured marbles available. This decorative marble with a black textured surface is used in temples and sculptures.
- **Indian Green Marble:** The most quarried Indian Green Marble is found in the towns of Kesariyaji and Rishabhdev in the Udaipur District of Rajasthan, India. This Indian green marble is known by name all over the world. In Europe, people know Indian green marble as Verde Guatemala. Many varieties are available in Indian green marble. Indian Green Marble is exported to Africa, Europe, Australia, the Middle East, and many Asian countries.

Marble industry waste

The marble stone industry generates waste on a large scale. During different stages of stone mining and processing procedures, a large quantity of marble waste is generated. Of that, up to 60% is generated as a result of marble quarrying alone. During mechanical processing, about 30% to 40% is left as waste. Nearly 50% of the precious mineral resources are wasted due to non-upgradation of technology in mining, processing, and polishing, with gang saws contributing to more than 50% of the waste generation. Lack of proper quarrying technology leads to a huge generation of waste. Additional waste is generated from fractured blocks, the sawing and polishing processes, and the rejection of broken or damaged slabs.

Types of marble industry waste

Marble industry waste can be broadly categorised in two ways:

- **Solid waste:** This type of waste is produced during quarrying and mining activities. The levelling of the unshaped blocks and slabs with huge amounts of stone fragments has a variable dimension.
- **Slurry waste:**The stone slurry waste is a viscous material resulting from the sawing, shaping, and polishing processes. It contains approximately 65% water. The stone slurry is usually disposed in open areas, causing many health problems, land contamination, and sewerage network blockages.

Adverse effects of marble industry waste on environment

There are many adverse effects of marble industry waste on the environment. The existing disposal practices of marble slurry are causing adverse impacts on ecology, human health, water and air quality due to very fine particles. Marble dust in finer form that is produced

as a result of its sawing and cutting can cause harmful health issues. Furthermore, the dumping of this marble dust can result in poor soil properties and a fertility reduction of the respective land (Sarkar *et al.,* 2006). Almost 30% of marble waste is produced during the working of marble stone (Aliabdo, 2014).

Some effects of the marble industry's waste on the environment are listed below:

- **Ecological impact:** Reduced porosity and permeability of the top soil along with the increasing alkalinity have tremendously affected the soil fertility. The percolation rate of rain water due to clogging of pores of top soil has also increased surface run-off, which reduced recharging of ground water.
- **Air pollution:** deposition of particulate/fugitive dust on roads up to 2 cm causes emission of particulate matter due to vehicular activities and strong wind currents. Translocation of slurry dust affects the flora and fauna of surrounding areas.
- **Water Pollution:** Disposing the slurry waste near to water bodies or roadside areas can deteriorate the surface and ground water quality by increasing turbidity, suspended solids, and Calcium and Magnesium hardness.
- **Effect on plants:** As air blown, fine particles settle on the epidermal layer of crops and vegetation and block the stomata (responsible for plant respiration).
- Continuous exposure to marble dust can cause severe respiratory disorders in labourers such as bronchitis, asthma, and chronic obstructive pulmonary disease (COPD). Dermal and eye irritation are the most common problems in the nearby population.
- The workers working near/on the processing gang saw machine are exposed to a continuous noise level of 90-120 dB. This noise level can damage physiological and psychological human health. Increased noise levels can cause annoyance, aggression, hypertension, high stress levels, hearing loss, and sleep disturbances.

After considering the aforementioned issues, it becomes clear that repurposing this type of trash in other industrial processes or converting it into value-added goods is critical. Proper waste management is a must to address the challenges caused by waste created in the stone industry. The high cost of water as well as the environmental issues involved with slurry disposal have prompted studies and research to reduce both economic and environmental losses. Because its chemical analysis reveals the existence of various macro, micro, and ultra-micronutrients, this mineral waste can be used as a soil nutrient after being reclaimed into an appropriate form.

Materials and Methods:

The raw material used in the present study has been taken from different mining units of Rajsamand and Nagaur (Raj.), which is an exceedingly fine-grained, uniformly crystalline calcareous rock of sedimentary nature composed of lime. Despite using the best mining

technology, a substantial amount of waste is generated, mostly in the form of powder, and some waste is formed while cutting and sizing marble stone slabs/tiles at the quarry floor.

The X-Ray Fluorescence spectroscopy technique (XRF) is used to analyse the major and trace elements in this garbage at the Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi. XRF (X-ray fluorescence) is a non-destructive analytical technique used to determine the elemental composition of materials. XRF analyzers determine the chemistry of a sample by measuring the fluorescent (or secondary) X-ray emitted from a sample when it is excited by a primary X-ray source. Each of the elements present in a sample produces a set of characteristic fluorescent X-rays ("a fingerprint") that is unique to that specific element.

This energy release is in the form of the emission of characteristic X-rays, indicating the type of atom present. The intensity of the energy measured by the different detectors is proportional to the abundance of the element in the sample. The energy is released in the form of distinctive X-rays that reveal the sort of atom present. The intensity of the energy detected by the various detectors is related to the element's abundance in the sample.

Results and Discussion:

A lot of waste produced during quarrying and other activities. XRF analysis of this waste reveals the presence of certain macronutrients and micronutrients which can be utilized as soil amendments to improve the growth of the plants (Table 1).

Calcium, Sulfur (S), and Magnesium are known as macro-nutrients (required in comparatively larger amounts). Iron, Zinc, Copper, Boron, Manganese, Molybdenum, Chloride, and others are the micro-nutrients (required in a smaller quantity) for growth and development. They constitute in total less than 1% of the dry weight of most plants.

In wasted marble powder, a variety of nutrients have been found (Table 1).

Table 1: XRF analysis of waste marble powder

Compound	Percentage	Compound	Percentage
CaO	55.2	TiO_2	< 0.8
LOI	41.75	K_2O	< 0.1
MgO	0.9	MnO	<0.1
SiO_2	0.9	Na_2O	<0.1
Al_2O_3	0.2	P_2O_5	<0.1
Fe_2O_3	0.5	$CaCO_3$	96.25

Marble powder's primary ingredient is Calcium. Plants require calcium as a component. By rupturing seed walls, it promotes seed germination by releasing seeds from their dormant

state. In addition to acting as a counter cation for inorganic and organic anions in the vacuole and as an intracellular messenger in the cytoplasm, Ca^{2+} is crucial for structural activities in the cell wall and membranes. Additionally, potassium is necessary for a number of plant enzyme processes. It also controls the pattern of metabolites in higher plants, changing the amounts of metabolites. By encouraging a robust root and stem system, it increases crop disease resistance.

In addition to enhancing chlorophyll production and content, Titanium may aid in Nitrogen fixation and photosynthesis. Plants can absorb iron, a micronutrient that is crucial for photosynthesis, respiration, and energy transfer. Magnesium helps in phosphate metabolism, plant respiration, and enzyme activation, all of which are essential for plant growth. It has numerous functions in the plant. However, it is a part of chlorophyll, the pigment that gives plants their green colour and facilitates photosynthesis. Manganese has a direct role in photosynthesis by assisting in the formation of chlorophyll.

This study shows that, following the proper chemical transformation of the waste, the nutrients present in Marble waste may be utilised to enhance soil fertility and plant growth.

Conclusions:

The marble industry's waste is a useful commodity since it contains many plant nutrients. Manufacturing value-added goods from marble waste will definitely be helpful in the economic health of the country and also useful in the degradation of environmental pollution. The elements present in the marble waste can works best as plant nutrient in their converted value added form. This research not only marks a turning point in sustainable and comprehensive growth, but it also serves as a beacon of hope against the long shadows of the bleak darkness that surrounds the world's hunger. It is clear from the preceding discussion that marble waste can be used as a soil nutrient enhancer.

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METHODS FOR IDENTIFYING KEY BACTERIA FOR AGRICULTURE

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Abstract:

A revolutionary approach is needed to address food and energy needs in a way that is socially conscious and eco-friendly. Plant production has always faced fast-growing food and energy demands, but now a new approach is needed to find the right solution. Our plants benefit from bacteria in this manner, making them extremely powerful tools. It is therefore important to follow certain aspects to exploit that resource, such as isolating strains for their direct use (when possible) and accurately identifying the strain used, not only through morphological but also by molecular techniques, to ensure the biosafety of the person who will use the technology developed. Biological fertilizers, phytostimulators, and biocontrol agents are potential uses of strains based on their characterization. Following the identification of the main characteristics of bacteria, there are various options in regards to the interaction between a plant and bacteria. These options include signaling recognition, penetration, and establishment, as well as whether the bacteria are rhizospheric, epiphytic, or endophytic. Our objectives, before diving into the complexity of the subject, were to lead to a better understanding of the agricultural interests of bacteria. This included how they might function as fertilizers, phytostimulators, or biocontrol agents, and how they might work.

Keywords: Biological fertilizers, phytostimulators, bacteria, bio control agents

Introduction:

The switch to microorganisms has been driven by the need to reduce chemical products (chemical fertilizers, pesticides, and supplementation), as well as the need to enact sustainable agriculture and protect the environment (Vale *et al.*, 2010). This happens as a result of the ongoing presence of bacteria in the soil, rhizosphere, and interior plant tissues (Hallmann *et al*., 1997). All growth-promoting bacteria, including those in the rhizosphere, help the host plant by absorbing and degrading organic chemicals present in root exudates. The preferred location of bacterial colonisation may change across plants or between various growth-promoting microorganisms (Barraquio *et al.,* 2000). Endophytic bacteria benefit from the fact that they develop inside plant tissues and have less population losses as a result of environmental

interactions (Sharma and Nowak, 1998). Indeed, it is critical to understand how bacteria features operate in plants and assist impacts; normally, each bacterium expresses a large number of traits to plants. Once it is established how the consortium of microbial inoculants interacts with plant systems, it will be feasible to harness additional benefits from microbial inoculants for increasing plant development and yield.

Bioproducts, Biofertilizer, and Biopesticides

Biofertilizers are a type of bioproducts characterised by their bioactivity and potential to improve biological processes. Biopesticides and biofertilizers have the potential to increase agricultural productivity when used in a sustainable manner. Biofertilizers, which are induced by a pool of bioactive chemicals derived from a wide range of ecologically friendly sources, are commonly related with the stimulation of plant growth and responses to abiotic challenges. Beneficial bacteria can produce phytohormones and other compounds**.** (Borriss, 2011), Algae, yeast, and mycorrhizal fungus are only a few examples of biomasses and their extracts that participate in fermentation and create amino acids. A biofertilizer is a substance that contains an active ingredient or organic agent that is free of agrochemicals and is capable of acting directly or indirectly on all or a portion of cultivated plants to increase productivity, without taking into account their hormonal or stimulating value. The regulatory definition of biofertilizers does not specifically specify the sources, but, for instance, Brazilian regulation identifies bioactivity as the primary effect (Brasil, 2004). Both standards need bioactivity and/or certain active components to define a biofertilizer. Despite the fact that hundreds of bacteria and fungi have been identified to enhance plant development, only a limited number have been commercially exploited as bio fertilizers, according to (Balachandar, 2012). Several naturally occurring compounds, such as fulvic acid, amino acids, and kelp extracts, might be classified as biofertilizers with known bioactivities. These compounds are not bioactive, however they are commonly sold as fertiliser blends with additional mineral nutrients. Plant growth promotion (PGP) classification and differentiation from biopesticides, bio-inoculants, mineral fertilisers, and bio stimulants are desirable in order to encourage researchers and businesses to find new biofertilizer sources and deliver them to the market in accordance with regulations as a sustainable tool for growers. Before conducting field studies on biofertilizers, researchers may benefit from developing simple bioassays to identify and explain the PGP impact. Bioassays developed from the 1960s to the 1990s, after the development of plant hormones and plant growth regulators knowledge, may be particularly useful in screens to discover PGP bioactivity on potential biofertilizer sources, such as the classic bioassay described by (Zhao *et al.,* 1992). This environmentally friendly technology could be incorporated into the new agriculture with the clear characterization of biofertilizers in relation to their bioactivity and the consolidation of nomenclature of biofertilizers in both scientific and regulatory literature as a class of natural source bioactive

products. Focused on finding and characterising microorganisms with potential for application in agriculture as biofertilizers, bio-inoculants, or even biopesticides, several forward-looking tactics are explored.

Recognizing Strain

The development of control measures, the prevention of disease transmission, or the identification of biotechnologically relevant reference strains all rely largely on precise identification of microorganisms. However, in order to enhance procedures and diversify research strategies, it is critical to apply rapid and sensitive approaches that offer reliable data for microbe identification (Atkins and Clark, 2004). While visual, biochemical, and serological tests, as well as fatty acid and exopolysaccharides (EPSs) profiles and enzymatic standards, can assist identify bacteria, they are limited and insufficient for reliably distinguishing between species and strains (Oliveira *et al.,* 1999). However, when combined with molecular biology, these established techniques can serve as effective tools for characterising and identifying microbial genotypes (Oliveira *et al.,* 1999). A bacterial species, according to Rosselló-Mora and Amann (2001), is a group of genetically linked strains with a high degree of similarity in a number of independent properties. Microbiologists can quickly identify novel species since prokaryotic organisms must share more than 97 percent of the original strain's 16S ribosomal gene sequence to be classified species.

Characteristics of Biofertilizers

One of the most well-known properties of biofertilizers is their ability to supply nutrients to plant roots such as nitrogen, phosphorus, and iron.

Fixation of biological nitrogen

Nitrogen accounts for approximately 78 percent of the gases in the atmosphere (N2). Plants cannot absorb nitrogen in this form since it can only be absorbed as nitrate (NO 3) and ammonium (NH4 +). 2009 (Taiz and Zeiger). Nitrogen becomes the "initial" limiting factor of vegetal development in plants with low nitrogen levels, which lowers production (Dures *et al.*, 2004). Biological nitrogen fixation (BNF) is the process by which some prokaryotic organisms take N_2 from the environment and transform it into the absorbed form of NH3 (Reis *et al.,* 2006). These organisms (N2-fixing forms) include symbiotic bacteria such as Rhizobium, an obligatory symbiont in leguminous plants, Frankia in nonleguminous plants, and nonsymbiotic bacteria such as *Azospirillum, Azotobacter*, and *Acetobacter (*Saharan and Nehra, 2011). A sequence of chemical signals is used by symbiotic bacteria like rhizobia to encourage the production of nodules in legume plants, which is what is known as a "nitrogen machine." Legumes begin this interaction by secreting substances from their roots, such as flavonoids. In response to the binding of the flavonoids to receptors in the plasma membranes of suitable soil rhizobia, the rhizobia produce Nod factors (nodulation factors), which allow bacteria to penetrate roots

through root hairs. The root hair plasma membrane then permits an inflow of calcium ions, and this calcium alters by making the root hairs inflate at their tips and coil around the rhizobia. As a result of the bacteria injecting infection proteins into the root hairs, the cell wall breaks, and the plasma membrane forms a tubular thread through which the rhizobia may enter. The infection thread's tip connects with the plasma membrane of a cortical cell as the bacteria migrate through it into the root cortex. The rhizobia are then ejected into the cytoplasm of the cortical cell while the host membrane remains intact (Taiz and Zeiger, 2009). The organic forms that are transmitted to plants are determined by the amide exporters or ureide exporters found in xylem sap. Temperate zone legumes, such as the pea (Pisum), clover (Trifolium), broad bean (Vicia), and lentil, transport nitrogen via amides, chiefly asparagine and glutamine (Lens). In tropical climates, legumes such as soybean *(Glycine*), kidney bean (Phaseolus), peanut (*Arachis),* and southern pea *(Vigna)* are preferred ureide exporters (Taiz and Zeiger, 2009). Soybean (*Glycine max*), which is grown in Brazil utilising an efficient method, is one example of BNF's contribution to crops. The choice of a strain compatible with Brazilian cultivars, as well as BNF's high efficiency and resilience to Brazilian conditions, contributed to success. A Brady rhizobium colony that has been formed can provide up to 92 percent of the total N accumulated in plants (Hungria *et al.,* 2006). Rhizobium spp. strains may also fix 66-78 percent of the total nitrogen required by plants in common bean (Phaseolus vulgaris), another important legume (Franzini *et al*., 2013). Microscopic study under sterile conditions was utilised to demonstrate how free-living, associative, or endophytic bacteria interact with plants (Roncato-Maccari *et al.,* 2003).

Phosphate Solubilization and Phytase Production

Phosphorus (P), which makes up around 0.2 percent of a plant's dry weight and is the second most significant element for plants after nitrogen (Kucey, 1988), is essential. It is found in a variety of essential substances such as phospholipids, ATP, and nucleic acids. P is also involved in the control of important enzyme activities and metabolic pathways (Taiz and Zeiger, 2009). In general, there is a lot of phosphorus in the soil (usually 400 to 1200 mg/kg of soil) (Khan et al. 2007), however due to its reactivity, the bulk of this phosphorus is insoluble and not accessible to help plant development (López- Bucio *et al.,* 2002). Organic substances such as inositol phosphate (soil phytate), phosphomonoesters, and phosphotriesters, as well as inorganic minerals such as apatite, contain insoluble phosphorus (Khan *et al.,* 2007). In chemical fertilisation, soluble inorganic phosphorus is typically utilised, but a considerable percentage of that material immobilises rapidly after application and is lost since it is no longer available to plants (Feng *et al.,* 2004). It is possible to increase phosphorus nutrition by "mobilising" phosphorus as insoluble inorganic polyphosphates and phytate, which account for 20-50 percent of total soil organic phosphorus (Richardson *et al.,* 2001). Phosphate-solubilizing bacteria (PSB) transform insoluble forms of inorganic P in the rhizosphere into plant-accessible forms by

releasing phosphates predominantly through organic acids such as gluconic and citric acid, both of which are produced by various soil bacteria (Rodriguez *et al.,* 2004). However, phytases such as myo-inositol hexakisphosphate phosphohydrolases may mineralize the abundant organic P form known as phytates, which accounts for 10 to 50% of all P in most soils, so that the P may be used in plant nutrition. The ability of the same bacteria strains to synthesise phytase and organic acids makes them desirable for agricultural use (Richardson *et al., 2001*).

Siderophores

Iron is the fourth most common metal on earth, however neither bacteria nor plants can easily digest iron in aerobic soils. There are two possible states of this element in an aqueous solution: Fe 2+ and Fe 3+. Because the Fe 3+ forms frequently form soluble oxides or hydroxides that reduce their bioavailability, plants and microbes cannot use them (Zuo and Zhang, 2011). Because iron functions as a cofactor in several enzymes crucial to physiological activities including respiration, photosynthesis, and nitrogen fixation, iron is an important nutrient for plants, and deficiencies result in severe metabolic changes (Taiz and Zeiger, 2009). Because bacteria, fungi, and plants compete for nutrients in the rhizosphere, microorganisms and plants have a far more difficult time collecting adequate iron. As a result, siderophores can either encourage growth directly or indirectly exercise biological regulation (Hu and Xu 2011). There are two ways for plants to obtain iron: I acidification of the rhizosphere, which is followed by Fe 3+ ion reduction by membrane-bound Fe (III)-chelate reductase and Fe 2+ uptake into root cells; and (ii) secretion of low-molecular-weight phytosiderophores, which solubilize and bind iron before being transported into root cells by membrane proteins (Altomare and Tringovska, 2011). These approaches, however, typically fall short of what plants require to survive, particularly in calcareous or alkaline soils. As a result, it is critical in this scenario to feed plants with iron in a form that they can utilise (Zuo and Zhang, 2011).

Microorganisms create siderophores due to the low availability of Fe +3 in the solution. Ammonium sulphate (NH 4) 2 SO 4 and amino acids promote bacterial growth and siderophore synthesis, whereas urea creates the most siderophores (Sayyed *et al.,* 2005). Several siderophores may form complexes with particular elements such as copper, aluminium, and molybdenum (Benite *et al.* 2002). These chemicals might bind iron molecules in solution to a specific membrane receptor on the cell membrane's exterior, where they are absorbed, making iron available for plant growth promotion (Taiz and Zeiger, 2009). Glick (2012) illustrated the benefits of bacterial siderophores to plants using images from many studies. The research cited reveal benefits for plants such as Arabidopsis thaliana, mung bean (*Vigna radiata*), and peanut (*Arachis hypogaea*). When grown on mung bean plants in iron-limited conditions, the pseudomonas produced a siderophore that enhanced the plant's chlorophyll content while decreasing the chlorotic look of the leaves (Sharma *et al*., 2003). Additionally, Pseudomonas

fluorescence improved performance in Arabidopsis thaliana by increasing iron levels in plant tissues through the Fe-pyoverdine complex in plants (Vansuyt *et al.,* 2007). When plants are stressed by their environment, bacteria's distribution of iron to the plants becomes even more crucial (e.g., heavy metal pollution). As a result of the high soil levels of heavy metals, siderophores aid in reducing the stress placed on plants (Braud *et al.,* 2006).

Characteristics of phytostimulants

Plant hormones are a family of naturally occurring chemical substances that impact physiological activities in response to environmental stimuli at low concentrations (Davies, 2004). When these plant responses are inadequate, rhizosphere microorganisms may create or alter phytohormones (Salamone *et al.,* 2005), allowing a range of bacteria to affect phytohormone levels and influence the plant's hormonal balance and response to the environment (Glick *et al.,* 2007).

Auxins

The principal auxin found naturally in plants is indoleacetic acid (IAA) (Taiz and Zeiger, 2009). IAA may be involved in a number of physiological processes in plants, including photosynthesis, pigment formation, responses to light, gravity, and fluorescence, biosynthesis of various metabolites, stress resistance modulation, control of vegetative growth processes, and more specifically, cell division and differentiation, stimulation of seed and tuber germination, accelerated xylem and root development, and initiation of lateral and adaxial growth (Spaepen and Vanderleyden, 2011). In general, the plant and the bacteria collaborate, as demonstrated when the bacterial IAA increases the length and surface area of the roots, allowing the plant to have greater access to the nutrients in the soil. Furthermore, bacterial IAA weakens plant cell walls, increasing root exudation and providing more nutrients for rhizosphere bacteria to grow (Glick, 2012). However, bacterial IAA absorption may alter the endogenous pool of plant IAA. The quantity of IAA produced by the plant determines whether bacterial IAA supports or hinders plant growth (Glick 2012). Pseudomonas putida inoculated canola *(Brassica campestris)* seeds, for example, extended roots compared to an IAA-deficient mutant and an uninoculated reference (Xie *et al.,* 1996). IAA influences root nodulation and is generated by the majority of rhizobia strains investigated (Badenoch-Jones *et al.,* 1984).

ACC Deaminase and Ethylene

The plant hormone ethylene not only promotes root initiation, fruit ripening, flower wilting, and leaf abscission, but it also stimulates seed germination, activates the synthesis of other plant hormones, inhibits root elongation, nodule formation, and mycorrhizae-plant interaction, and responds to both biotic and abiotic stresses (Taiz and Zeiger. 2009). Methionine amino acid acts as a biological precursor in two stages of ethylene synthesis in higher plants. S-adenosylmethionine (SAM) undergoes the first reaction when it is transformed into 1-

aminocyclopropane-1-carboxylic acid (ACC) by the ACC synthetase enzyme (ACCS). Then, the ACC is broken down by ACC oxidase (ACCO), a process that requires oxygen (O 2) and iron. Ethylene is then produced by this reaction once it is triggered by CO2 (Yang and Hoffman, 1984). Rhizobium spp. infection of legumes can cause an increase in plant ethylene levels; this higher concentration of ethylene can prevent further rhizobia infection and nodulation (Ma *et al., 2002*). Some rhizobia restrict the growth of ethylene and increase the number of nodules by creating a compound called rhizobitoxine (Yuhashi *et al.,* 2000), which chemically inhibits the activity of ACC synthase, one of the enzymes responsible for the biosynthesis of ethylene. The ACC deaminase enzyme, which is produced by other rhizobia strains, eliminates part of the ACC (the immediate precursor to ethylene in plants) before it can be converted to ethylene (Ma *et al.,* 2002). When the bacteria colonised the seeds or roots, they produced additional tiny molecules in reaction to tryptophan, which allowed the bacteria to synthesis and secrete IAA. This allowed the plant to develop more quickly (Patten and Glick 1996, 2002). Together with natural plant IAA, this bacterial IAA can promote plant growth or activate the enzyme ACC synthase, which converts the chemical S-adenosyl methionine into ACC, the direct precursor of ethylene in higher plants. Excluded from seeds or plant roots, such as in canola (Brassica napus) plants inoculated with Enterobacter cloacae (Penrose and Glick, 2001), a portion of the newly synthesised ACC is then ingested by the bacteria and converted to ammonia and -ketobutyrate by the enzyme ACC deaminase, both of which are easily assimilated. As a result of the degradation of ACC from the direct precursor of ethylene, which results in a gradient in ACC concentration between the interior and outside of the plant, ACC exudation is encouraged and the quantity of ethylene inside the plant is reduced (Glick *et al.,* 1998).

Cytokinins

Cytokinins are plant hormones that govern a number of physiological functions, including cell division, shoot growth, and senescence (Mok and Mok, 1994). Cytokinins are created and transported to the shoot from developing seeds and root tips. The zeatin is the most prominent illustration (Taiz and Zeiger 2009). Although there are significant variations in cytokinin production in bacteria and plants, they all begin with the isopentenyl group being transferred from dimethylallyl diphosphate (DMAPP) to the N6-amino group of adenine by either adenylate isopentenyl transferase (AIPT) or tRNA-IPT. While bacterial AIPTs prefer AMP and plant AIPTs use ATP/ADP as an isopentenyl acceptor, tRNA-IPTs target specific tRNA sites (Sakakibara *et al.,* 2005). Bacillus licheniformis, Bacillus subtilis, and Pseudomonas aeruginosa all emit zeatin and zeatin riboside, which are cytokinins identical to those produced by plants (Hussain and Hasnain, 2009) According to certain studies, bacterially produced cytokinin combines with plant cytokinin and influences plant growth and development. Inoculating lettuce *(Lactuca sativa*) plants with Bacillus subtilis increased the cytokinin content

of both the shoots and the roots. When zeatin and its riboside concentration was 10 times higher than in the control, they accumulated most readily in roots. According to (Arkhipova *et al.*, 2005), cytokinin accumulation in inoculated lettuce plants resulted in a 30% increase in plant shoot and root weight; cytokinin levels were also significantly correlated with shoot length, fresh weight, and dry weight in plants inoculated with Pseudomonas, Bacillus, and Azo spirillum, resulting in the production of zeatin, zeatin rib (Hussain and Hasnain, 2011).

Gibberellins

Although active GAs only have 19 carbon atoms, gibberellins (GAs) are a class of hormone made up of a group of terpenoids with 20 carbon atoms. GAs have a significant role in internode elongation, seed germination, pollen tube development, and blooming in rosette plants because they are primarily engaged in cell division and elongation inside the subapical meristem (Stowe and Yamaki, 1957; Taiz and Zeiger, 2009). Although there isn't much evidence for bacterial biosynthesis, some studies suggest that, generally speaking, much like in higher plants, the earliest steps of the gibberellin biosynthetic pathway in bacteria may be controlled by membrane-related cytochrome P450 monooxygenases (Tully *et al.*, 1998). Gibberellins produced by bacteria can alter the hormonal equilibrium in plants, resulting in structural alterations. *Bradyrhizobium* sp. strain infected Phaseolus lunatus plants improved internode elongation, as shown by (Dobert *et al.*, 1992). Increased levels of GA 1, GA 19, GA 20, and GA 44 were found in the nodules produced by the bacterial strain that promoted internode elongation when gibberellin content was measured using deuterated internal standards and gas chromatography and mass spectrometry (GC-MS) analyses. By applying extracts from medium incubated with bacteria *Bacillus pumilus* and *Bacillus licheniformis* as well as exogenous GA3, it was possible to effectively reverse the dwarf phenotype in Alnus *glutinosa* seedlings that was brought on by paclobutrazol, an inhibitor of gibberellin biosynthesis (Gutiérrez-Manero *et al.*, 2001). Red pepper plug seedling development is stimulated by Bacillus cereus, B. macroides, and B. pumilus. In the culture broth of their microorganisms, gibberellins (GAs) were found. The physiologically active GAs GAs 1, 3, 4, and 7 had contents that were significantly greater than those of the other GAs, indicating that the GAs were the source of the growth-promoting action (Joo *et al.*, 2004).

Biocontrol Characteristics

In addition to being an effective and feasible replacement for biocontrol, microbial agents are also thought to be safe for the environment and human health (Zucchi and Melo, 2009). They replace chemicals because chemicals can have negative environmental effects that might have an impact on soil and food, as well as because chemicals encourage the evolution of resistant infections and reduce the number of beneficial species (Silva *et al.* 2004). Microorganisms'

capacity for biocontrol can rely on a variety of methods, including the creation of poisonous chemicals (M'Piga *et al.*, 1997).

Antibiosis

According to Cook and Baker (1989), antibiosis is the most frequent phenomena in which a bacterium prevents the growth of other organisms by creating harmful substances (antibiotics). These substances can have either a volatile or non-volatile composition. When employing in vitro techniques to combat microorganisms, this inhibitory property is frequently employed to assess the potential activity of the bacterium, mostly on harmful fungi. The dual culture approach on agar medium is the most used technique (Rana *et al.*, 2011), The purification and chemical identification of the antibiotic compound, the detection and quantification of secondary metabolites in the rhizosphere, and the identification of the regulatory genes that regulate the antibiotic compound's expression are some steps that can be used to demonstrate how bacterial antibiotics contribute to the biological control of the disease (Haas and Keel, 2003). The expression of secondary metabolites can be influenced by fungal, bacterial, and plant metabolites as well as oxygen tension, osmotic conditions, carbon sources, and soil mineral content (Haas and Keel, 2003). Phenazines and biosurfactants generated by Pseudomonas aeruginosa against Pythium splendens on bean (*Phaseolus vulgaris*) and *Pythium myriotylum* on cocoyam (*Xanthosoma sagittifolium)* are two examples of well-known antibiotics with biocontrol capabilities produced by bacteria (Perneel *et al*., 2008).

Hydrogen Cyanide

Hydrogen cyanide (HCN), a secondary chemical often produced by rhizosphere pseudomonads, has been proven to be harmful to root metabolism and development (Schippers *et al.*, 1990). HCN, on the other hand, may potentially directly boost plant development by increasing the quantity of root hairs (Luz, 1996). This mechanism, however, varies dramatically from cyanogenesis in other bacteria because I amino acids other than glycine enhance HCN formation, and (ii) both carbons of glycine are employed as sources of cyanide carbon. Glycine is an HCN precursor for Pseudomonas aeruginosa, according to Castric (1977). When cells reach a density that encourages quorum-sensing activation—cyanogenesis is not always governed by quorum sensing—cyanide is frequently created with a decrease in oxygen content, completing the exponential phase in the cells. Some bacteria create HCN, which inhibits the cytochrome oxidase of several species. This loss of quorum sensing was found to eliminate cyanogenesis in Chromobacterium violaceum CV0 (Throup *et al.*, 1995). The producer strains are largely insensitive to HCN and have a different cyanide-resistant cytochrome oxidase.

Competition for Nutrients and Niches

A microorganism must successfully compete for the available nutrients and niches in order to successfully colonise the plant. Reducing illness occurrence and severity requires

limiting competition between infections and beneficial organisms for resources and habitats (CNN) (Kamilova *et al.,* 2005). While direct evidence of successful competition between viruses and nonpathogens is difficult to find, some indirect evidence does (Glick, 2012). For instance, several nonpathogenic soil microorganisms may quickly colonise plants and consume the majority of the nutrients present, which makes it difficult for pathogens to flourish. The bacterial pathogen Pseudomonas syringae PV. Tomato was prevented from generating pathogenic symptoms by treating plants with the leaf bacterium *Sphingomonas spp.* (Innerebner *et al.,* 2011). The effective colonisation of the avocado root tip by the Pseudomonas strains AVO110 and AVO73 led to the selection of these two strains. However, only AVO110 showed a considerable level of resistance against Rosellinia necatrix-induced white root rot of the avocado (*Persea americana*). The two strains are different in that they colonise different areas of the root: AVO73 was primarily found forming dispersed microcolonies over the root surface and in close proximity to lateral roots, areas not colonised by this pathogen. Biocontrol strain AVO110 was observed to colonise the root at preferential penetration sites for R. necatrix infection (intercellular crevices between neighbouring plant root epidermal cells and root wounds) (Pliego *et al.,* 2007). These findings clearly imply that biocontrol microorganisms working through CNN must successfully colonise the same microenvironment as the disease.

Lytic Enzymes

To enter the inside of the host, the plant pathogens need entrance points. Therefore, in order to effectively compete for these infection sites, the biological control organisms must accumulate certain traits. Utilizing the available nutrients and successfully preventing phytopathogens from growing vegetatively or germination can achieve this (Punja and Utkhede, 2003). Some bacteria can manufacture extracellular enzymes such chitinases, 1,3-glucanases, lipases, cellulases, and proteases. These techniques allow them to gain ecological benefits. These enzymes occasionally work in concert with antibiotics, which is significant for the antagonistic effect they have on phytopathogenic fungi. Through the purified toxins and chitinolytic and glucanolytic enzymes obtained from the same bacterial strain, Pseudomonas syringae pv. syringae demonstrated biocontrol effect against Trichoderma atroviride (Fogliano *et al.,* 2002). The beginning of the interactions in the antagonism process involving fungi is thought to be the cell wall, which serves as a barrier to safeguard the acts carried out by microorganisms. Chitin, 1,6-glucans, and other polysaccharides make up the majority of the cell wall of fungi (Bartinicki-Garcia, 1968). Chitinases break down the cell walls of fungi to produce oligomers that activate additional hydrolytic enzyme genes, intensifying the host attack (Viterbo *et al.,* 2002). These oligomers can then be broken down to be utilised as food. Due to the formation of a mucilage that encases the hyphae, they can also shield cells from dehydration (Pitson *et al.,* 1993) Proteases are enzymes that break down peptide bonds; they can be categorised based on their

ability to function best in an acidic, neutral, or alkaline environment, their specificity for a given substrate (collagenase, elastase, etc.), or their similarity to other enzymes (pepsin, trypsin, casein, etc). (Kubicek, 1992).

Induced Systemic Resistance

It is believed that immunity is the norm and exception susceptibility in plant pathology. Otherwise, every plant may become infected by any virus, and in the short period of evolutionary terms, the earth's vegetable population would vanish (Romeiro, 1985). The term "induction of resistance" can refer to either localised resistance in tissues treated with the inducing chemical or systemic resistance that is seen far from the site of induction (Moraes, 1992). This reliance reveals particular modifications in plant metabolism that involved the production and/or accumulation of chemicals, which is a crucial feature in the phenomena of induced resistance (Taiz and Zeiger, 2009). The terms ISR (induced systemic resistance) and SAR (systemic acquired resistance) are used to describe the circumstance in which plants activate their defence mechanisms after being exposed to an inducing agent. A hypersensitive response (HR) is induced during SAR induction, which is characterised by the planned death of cells adjacent the infection, operates against biotrophic bacteria, and hence inhibits access to water and nutrients. This HR is triggered not only at the induction site, but also at other distant locations (Sticher *et al.*, 1997). The HR becomes activated as a result of a signal from salicylic acid (AS) (Glazebrook, 2005) The plant that must endure ISR exhibits no changes; the inducing agent is typically a nonpathogen, and its induction is independent of salicylate; another signalling pathway appears, which is further linked to jasmonates and ethylene; and finally, the plant that must endure ISR does not accumulate PRPs (Pieterse *et al.*, 2005). Bacteria can carry out the IRS process, and several studies have demonstrated that different bacteria can handle different phytopathogen types. The disease severity of Erwinia carotovora subsp. carotovora in Arabidopsis seedlings was less severe when compared to seedlings that were not exposed to bacterial volatile blends from Bacillus subtilis and B. amyloliquefaciens before pathogen injection. This bacterial volatile was enough to drive Arabidopsis seedlings to enter ISR (Ryu *et al.*, 2004).

Conclusions:

We provided methods in this chapter to assist with the characterization of fascinating bacterial strains for use in sustainable agriculture since it is crucial to choose the right bacteria with the appropriate properties when employing bioproducts, biofertilizers, or biopesticides. We discussed strain identification, biological nitrogen fixation, phosphate solubilization, phytase production, siderophores, phytostimulants, and biocontrol characteristics. However, the ideas, explanations, and directions for using these environmentally favourable sources were first provided.

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ANTIBACTERIAL POTENTIAL OF MARINE *STREPTOMYCES* STRAIN RTMI-3 AGAINST *KLEBSIELLA PNEUMONIAE*

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Abstract:

Actionomycetes is potential source of bioactive metabolites. The screening of actinomycetes from varied environment needed for to get novel metabolites. The marine environment has unexplored for bioactive metabolites and it has chemically diverse with wide range of biological and pharmaceutical applications. The rapid emerging of multidrug resistant, biofilm forming pathogens such as *Klebsiella pneumoniae* causing the various the types of opportunistic infections. In the present study nine marine actinomycetes strain isolated from sea water sample, of Ratanagiri, M.S. India, were screened for their antibacterial potential against *K. pneumoniae* using agar well diffusion assay. The strain RTMI-3 was selected as potent strain based on size of inhibition zone (1.5 cm). The strain was identified as *streptomyces* sp. based on morphological features. Different media on growth and antibacterial activity of RTMI-3 was studied and yeast extract malt extract medium was found to be of suitable composition to increase growth and inhibition effect of RTMI-3. Antibacterial metabolite of RTMI-3 was extracted and purified fraction using silica gel column chromatography. The purified fraction was partially characterized by thin layer chromatography (0.95 Rf), UV spectroscopy (290 and 370 nm) and FTIR spectroscopy. The current study predicted that the *streptomyces* strain RTMI-3 has potency to inhibit *K. pneumoniae* and can be used to control related infection.

Keywords: *Streptomyces sp*, *Klebsiella pneumoniae*, FTIR, TLC, U.V spectrophotometer

Introduction:

The wide spread use of antibiotics in hospitals lead to causing emergence of multi drug resistant organisms of low virulence like *Klebsiella* causing serious opportunistic infections. During the past decade extended-spectrum-beta-lactamase (ESBL) producing *K. pneumoniae* have emerged as one of the major multi drug resistant organisms Lucet *et al*. (1999). The incidence of ESBL producing *Klebsiella* isolates in central India has reached to 76.5% (Jacoby and Han, 1996) it indicates that the critical infections caused by ESBLs producing organism are resistant to particular antibiotics.

K. pneumoniae is notable opportunistic nosocomial pathogen causing a variety of infections including urinary tract infections (6-17%), pneumonia (7-14%), septicaemia (4-15%), wound infections (2-4%), neonatal septicaemia (3-20%) and infections in the intensive care units (4-17 %). It has been estimated that *K. pneumoniae* cause 5-7% of the total bacterial nosocomial infections (Podschun *et al*., 1998; Manasa *et al*., 2014). Self medication, overuse of drugs and mutation in specific genes leads to causes antibiotics resistance against specific pathogen.

Antibiotics are antimicrobial compounds produced by living microorganisms. These compounds were used therapeutically and prophylactically in the control of infectious diseases. Over 4,000 antibiotics have been isolated before, but only 50 have achieved wide usage. The other antibiotic compounds failed to achieve commercial importance for some reasons such as toxicity to human and animal ineffectiveness or high production costs. The actinomycetes comprise an extensive and diverse group of gram-positive, aerobic, mycelial prokaryotes with high G+C content (> 55%). The majority of actinomycetes are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants (Goodfellow, 1989). The genus *Streptomyces* was proposed by Waksman and Henrici (1943) were classified in the family Streptomycetaceae on the basis of morphology and subsequently cell wall chemotype (Williams *et al*., 1989).

The species of *Streptomyces* are considered as biotechnological valuable prokaryotes because 70-80% of isolated metabolites have been produced such as antibiotics, immune modulators, anticancer drugs, antiviral drugs, antioxidants, herbicides, enzymes and insecticides (Bull *et al*., 1992; El-Naggar *et al*., 2006; Bhosale *et al*., 2011). The investigation of actinomycetes from terrestrial sources are nearly exhausted and therefore the focus of industrial screening has moved to various unexplored habitats with unusual environment including saline lakes, forests, deserts and marine sediments have been studied (Hozzein *et al*., 2011).

Marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites. Recently, the study recognized that actinomycetes originated from marine source have become a source of novel antibiotic and anticancer agent with unusual structure and properties (Jensen *et al*., 2005; Ahmad *et al*., 2016).

The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Although, considerable progress is being made within the fields of chemical synthesis and engineered biosynthesis of antibacterial compounds, nature still remains the richest and the most versatile source for new antibiotics (Baltz *et al*., 2006). The current study aimed at the isolation and extraction of marine

actinomycetes from Ratnagiri beach, (Maharashtra, India) for antimicrobial activity against *K. pneumoniae* sample collected from Shankarrao Chavan Government Medical College, Nanded, M.S., India. The current investigation of antibacterial activity of marine actinomycetes strains yields some potentially useful compounds to control the infection of *K. pneumoniae* causing various diseases.

Materials and Methods:

Chemicals

All the chemicals and solvents of analytical grade were procured from Qualigens, S. D. Fine, Hi Media and Merck, Promega Corp, Medison, WI 53711, USA.

Media

All dehydrated culture media and media ingredients were procured from Hi media laboratories Pvt. Ltd, Mumbai, India.

Sampling area and sample collection

The water sample were collected from Ratnagiri sea shore of Maharashtra state India, which lies between north to south along the western coast of India. It 17° 58' 40.1736" to 16° 33' 17.01" North latitude and 73° 2' 24.144" to 73° 21' 2.487" East longitude. Altitude ranges about 11m having coastline of 237 Km. The water sample were collected in 500 ml sterile screw capped bottle and adequate space was provided for aeration and through mixing. The sample was labeled, placed in ice box and transported to Microbiology Laboratory, School of Life Sciences, SRTM University, Nanded, M. S., India. The samples were stored in refrigerator at 4°C for further study.

Isolation of actinomycetes

The water samples were collected from Ratnagiri sea shore were treated for the enrichment of actinomycetes. 10ml of seawater sample were added in 90ml of starch casein broth and incubated for 7 days at 30°C at 120 rpm. After incubation the serial dilutions of sample were prepared and dilutions of 10^{-3}-10^{-7} were spreaded on the starch casein agar plates supplemented with 50µg/ml of nystatin, streptomycin and penicillin. The plates were incubated at 30°C for 7 days. After incubation discrete, well isolated colonies selected and maintained on starch casein agar slants at 4°C for subsequent studies Williams and Cross (1971).

Bacterial strain

Pathogenic bacterial strain *Klebsiella pneumonia*e were obtained from Shankarrao Chavan Government Medical, College Nanded, M.S., India.

Procedures for preparation of 0.5 McFarland standards

A pure colony of test bacteria was taken by using a sterile wire loop and transferred into test tubes containing a sterile nutrient broth. The test tubes were incubated at 37°C for 24 h until the visible turbidity and density equal to that of 0.5 McFarland standards. After adjusting the turbidity, the broths were added in soft agar.

Screening for antibacterial activity against *K. pneumoniae*

The 9 isolate of actinomycetes were separately inoculated in 100ml of Erlenmeyer flasks containing 50ml of soybean casein digest broth. These flasks were incubated at 30ºC for 7 days under static condition. After completion of incubation period the broth were centrifuged at 10,000 rpm for 20 min at 4ºC. The biomass of each isolates was separated by centrifugation and the supernatant of each were used for screening against *K. pneumoniae* by agar well diffusion method. Seed agar plates were prepared by using soft agar and hard agar. In soft agar, the amount of agar agar powder was taken half than hard agar. The media were sterilized by 15 lbs pressure that is 121ºC for 15 min. After sterilization firstly hard agar were poured in sterile petri plates and allowed for to solidify. Soft agar was seeded with culture of *K. pneumoniae* (generated by overnight incubation and adjusted to a turbidity equivalent to Mcfarland standard of 0.5 in sterile nutrient broth). The 10% of bacterial inoculums immediately added in to soft agar, mixed and poured on hard agar plates. After solidification of soft agar medium wells of about 6 mm diameter were punched with sterile cork boror and volume of 100µl of each supernatant was inserted into respected wells. The plates were kept at 4ºC for 30 min for diffusion of bioactive metabolite in the agar medium and plates were incubated at 30 ºC for 24h. After incubation, observed inhibition zone around the wells. The isolates showing highest diameter of inhibition zone was selected for further studies.

Identification of actinomycetes

The actinomycetes colonies were subculture and maintained on starch casein agar (SCA) slants. The cover slip method were used for the identification (Cross, 1989). The sterilized cover slip is carefully inserted at an angle of about 45° into starch casein agar in a petri plate until about half of the cover slip is in the medium. Actinomyctes are then inoculated along the line where the medium meets the upper surface of the cover slip. After the suitable incubation, cover slip in the medium facilitates the distinction between substrate mycelium and aerial mycelium. Then the cover slip was stained and observed under microscope and identified.

Effect of media composition

The effect of 5 different media namely yeast extract malt broth, soybean casein digest broth, starch casein broth, glycerol L-aspergine broth, inorganic salt starch broth were used for

the media optimization for the strain RTMI-3. The Erlenmeyer flasks containing 50ml of respective medium were inoculated with selected strain and incubated at 30ºC for 7 days. After incubation period, the broths were centrifuged at 10,000 rpm for 20 min at 4ºC. The supernatant was collected and screened for antibacterial activity against *K. pneumoniae* by agar well diffusion method (Rabah *et al.*, 2007). The plates were incubated at 30ºC for 24h. After incubation, the bioactive metabolite shows zone of inhibition. The highest zone of inhibition shown by strain were selected for optimization of YEME media for larger production.

Production of Bioactive Metabolite

For the large scale production of bioactive metabolite from RTMI-3 strain were used. The 10% of active culture was inoculated into the 2 L of yeast extract malt extract medium for fermentation and it was carried out for 7 days at 30°C. After fermentation, the broths were subjected for extraction and purification of metabolites.

Extraction and Purification

The bioactive metabolite from the fermented broth were extracted by using centrifugation at 10,000 rpm for 20min at 4°C. The extracted media was separated by separating funnel with equal volume of ethyl acetate. The mixture of media and ethyl acetate were mixed for 30 min and after shaking, the broth were allowed to settle and separate in two layers i.e. upper was of organic and lower was of aqueous. Organic layer were collected in evaporation bowl because of lower density of ethyl acetate than water, and allowed to evaporate. After evaporation bioactive metabolite were collected.

Purification of the bioactive metabolite was carried out by column chromatography using silica gel (60 to 200 mesh) size of chromatography grade. The column (20 X 1cm) was cleaned using water and rinsed with acetone. After drying, a small piece of cotton was placed at the bottom of the column. Silica gel was then packed in the column by using chloroform. The small piece of cotton again placed on surface of the silica after settling of silica gel and allowed to set for 1 to 2 h. After that crude bioactive metabolite were loaded on the top of the column and eluted using gradients of chloroform and methanol. 20 fractions were collected from 10 gradients of chloroform and methanol such as (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10). Each fraction contains 5ml of elutant. The collected fractions were evaporated and check for antimicrobial activity of each fraction against *K. pneumoniae*. The fractions showing zone of inhibition around wells, such all fractions were mixed together and performed the TLC of mixed fractions by using precoated TLC plates. The mixture of fractions were loaded on TLC plates and allowed to run by using chloroform:methanol (3:7) solvent system Attimarad et al. (2012). After running 1/3rd distance of plates, were permitted to dry in hot air oven for 10 min at 110ºC.

Then the plates were kept in iodine vapour chamber and observed for formation of single or multiple spot through which the purity of compound were determined Busti *et al*. (2006). The visual spot were scraped and tested for antimicrobial activity against *K. pneumoniae* by using agar well diffusion method.

Characterization of Bioactive Metabolite

The characterization bioactive metabolite by Ultraviolet (UV) and Fourier transform infrared (FTIR) spectrum were recorded as shown in (fig. 3 and fig. 4). For UV spectrums used Shimadzu UV-170 spectrophotometer, carried by 1 mg of sample was dissolved in 10 ml of chloroform and the spectra were recorded at 200-400 nm range. While the infrared spectra recorded on Shimadzu IR-470 model. The spectra were scanned in the 400-4000 cm^{-1} range. The spectra were obtained using potassium bromide pellet technique. Potassium bromide was dried under vacuum at 100°C for 48 h and 100 mg of KBr with 1 mg of sample was taken to prepare KBr pellet. The spectra plotted as intensity versus wave number.

Results and Discussion:

The bioactive molecule produced actinomycetes were surveyed from Ratnagi beach, M.S., India. The screening of marine *streptomyces* sp. against *K. pneumonia*e have studied previously by Mohseni *et al*. (2013) and Thirumalairaj *et al*. (2015). The current study isolates 9 strain from seawater sample of Ratnagiri seashore, among those 4 isolates were able to shows inhibition activity against *K. pneumoniae* as shown in (Fig.1). The strain RTMI- 1, 2, 4, 8, 9 have been not shown antibacterial activity while the strain RTMI- 3, 5, 6, 7 initial shows the percentage zone of inhibition i.e 75%, 66.66%, 70.58%, 70.58% respectively as shown in (table 1). The highest zone of inhibition shown by strain RTMI-3 was subjected to further media optimization. The percentages zone of inhibition by strain RTMI-3 after media optimization using yeast extract malt extract media it was shows 80%, due to rich source and yield of bioactive metabolites as shown in (table 2). The potential strain RTMI-3 were identified up to genus level as *Streptomyces* sp. based on cover slip method, it was observed under 100x objectives of light microscope and showed branching, filamentous bacteria and showed spiral shape spore chain. When compared to study of Maleki and Mashinchian (2011), the screening of *streptomyces* sp. from soil sample shows 37% inhibitory activity against *K. pneumoniae* which is half less than marine *streptomyces* strain isolated in current study. The crude bioactive metabolites from 2 L of yeast extract malt extract broth containing *streptomyces* was yields 0.24 gm. The appearance of metabolites shows greenish in color, sticky in nature and readily dissolved in chloroform. The purification of bioactive metabolite was done by column chromatography. The fractions of compound were collected by 5 ml of 20 fractions from 10

gradient by eluting chloroform:methanol as solvents, each fraction were collected in separate tube, evaporated and tested for antibacterial activity. The 16 fractions showed zone of inhibition against *K. pneumonia*e. The fractions showing zone of inhibition mix together and performed Thin Layer Chromatography (TLC) by using chloroform:methanol (30:70) as solvent system. The plate incubates in Iodine vapour chamber for visualization, and it observes single blackish spot on TLC plates. The Rf value of purified compound was recorded as 0.95. While the previous study done by Ilic *et al*. (2005), shows the two Rf value of bioactive compound i.e 0.70 and 0.88. The antibacterial activity of purified compound and mixture of fractions were showed same results as tested against the *K. pneumoniae*. The characteristics of absorption peaks shown by UV and FTIR spectroscopy determines the nature of bioactive compounds. The maximum UV absorbance peaks of bioactive compound was recorded 290 and 370 nm in chloroform solvant. The FTIR spectrum data shows absorption at 2924 to 2852 cm^{-1} which indicates aldehyde (CHO group), absorption at 1714-1743 cm^{-1} shows carbonyl groups, absorption at 1602 cm^{-1} showed the amide group (CO-NH) means the presence of peptide bond, absorption at 970-1261 cm^{-1} for ether group. The FTIR spectrum for bioactive metabolites shows the absorption band between 1456-2950 cm^{-1}. The similar trends studied by Maleki and Mashinchian (2011) at absorption peak at 1643 cm^{-1} which shows the polygenic nature of compound having the antibacterial activity.

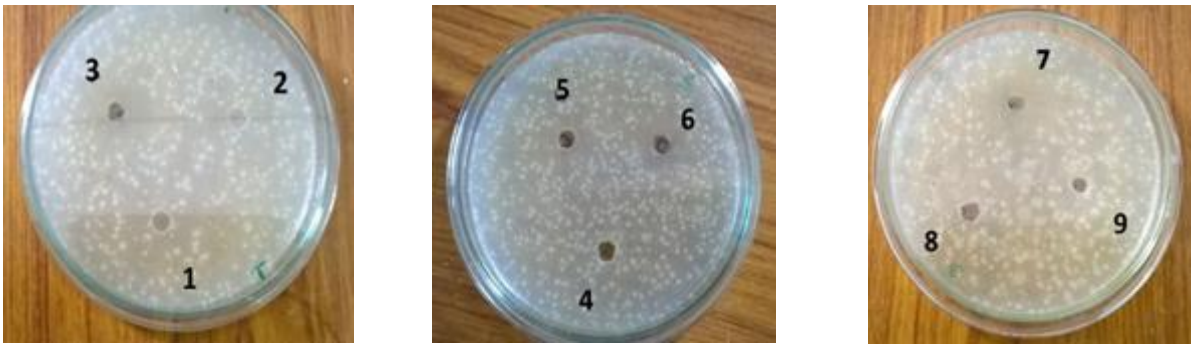

Figure 1: Primary screening of actinomycetes against *K. pneumoniae*

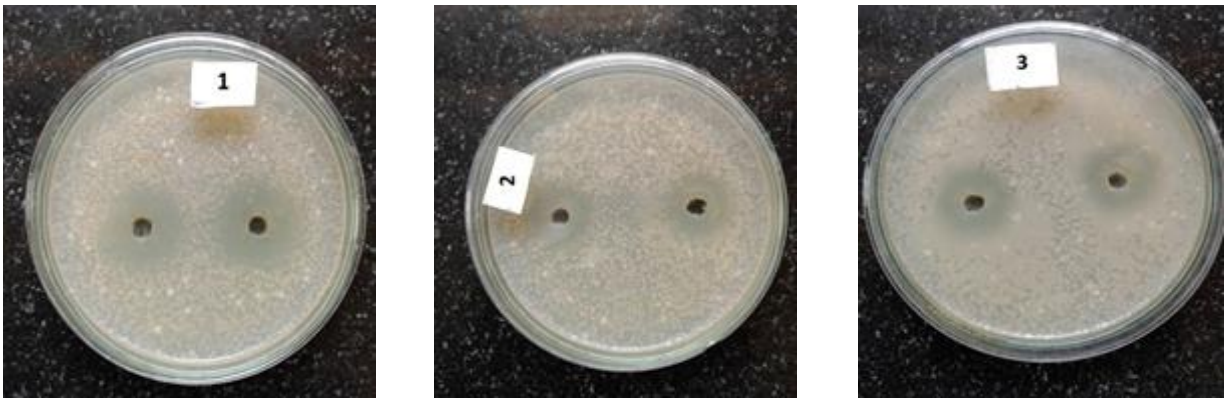

Figure 2: Effect of different media composition for the activity of actinomycetes

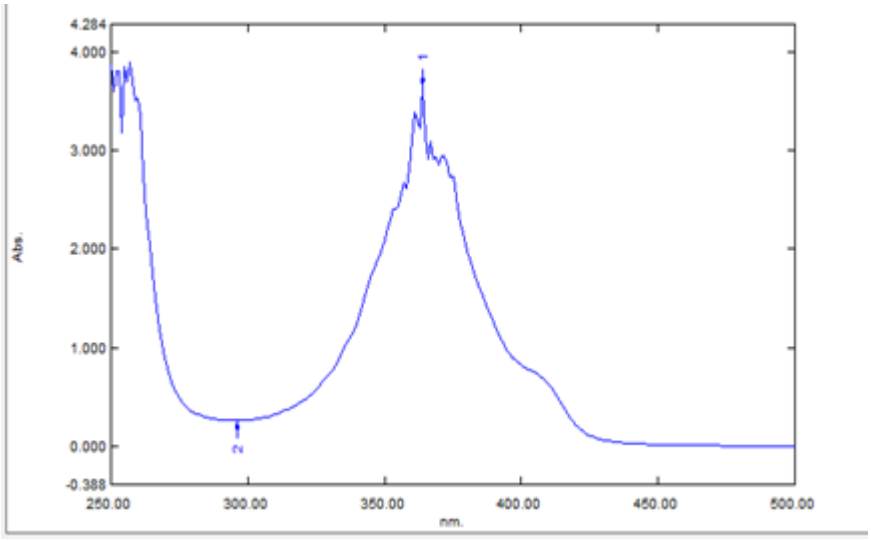

Figure 3: UV spectrophotometer graph

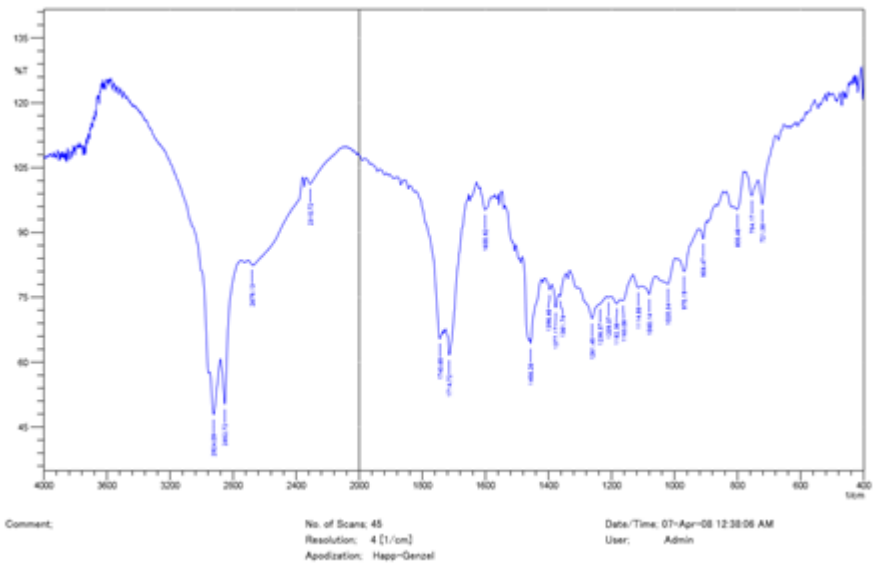

Figure 4: FTIR spectrum graph

Table 1: The isolates showing zone of inhibition and percentages of inhibition

Sr. No.	Isolated actinomycetes strain	Zone of inhibition (cm)	Percentage of inhibition
1	RTMI 1	-	-
2	RTMI 2	-	-
3	RTMI 3	1.5	75
4	RTMI 4	-	-
5	RTMI 5	1.0	66.66
6	RTMI 6	1.2	70.58
7	RTMI 7	1.2	70.58
8	RTMI 8	-	-
9	RTMI 9	-	-

Table 2: The effect of different media composition on zone of inhibition and percentages of inhibition

Sr. No	Media name	Zone of inhibition (cm)	Percentage of inhibition
1	Yeast extract malt extract	2 cm	80
2	Soybean casein digest broth	1.5 cm	75
3	Starch casein broth	1.4 cm	73.68
4	Glycerol L- Asparagine broth	1.6 cm	76.19
5	Inorganic salt starch broth	1.6 cm	76.19

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A CRITICAL REVIEW ON MICROBIAL FUEL CELL (MFC) TECHNOLOGY IN WASTEWATER TREATMENT FOR THE GENERATION OF CLEAN ENERGY

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Abstract:

Nowadays the world is encountering a serious peril from the consumption of unsustainable resources, fresh water shortage, and food scarcity due to the spontaneous use of fossil fuels. Because of the increasing population, the need for freshwater, energy, and food will increase. And the necessity for treating and recycling waste water will increase. Microbial Fuel Cells (MFC) can be used as a great alternative energy conservation system for producing bioenergy. It is a bio-electrochemical hybrid system that involves electricity generation and wastewaters treatment along with nutrient recovery. MFC has many benefits like energy saving, reduced sludge generation, and energy conservation. Many disciplines contributed to enhance the efficiency of MFCs. In this review, we will discuss the performance of MFC in different wastewater treatments along with energy generation.

Keywords: Microbial fuel cell, Substrate, Anode and cathode material, Wastewater treatment.

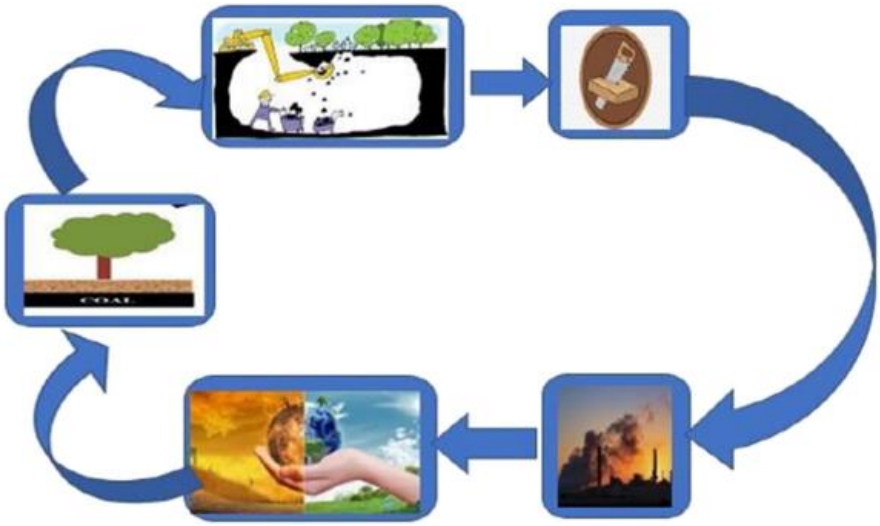
Introduction:

Water, food, and energy are considered indispensable for all living forms to survive and grow and they are interlinked with each other. One of the most crucial dangers the world is now facing is the consumption of non-renewable energy sources and environmental pollution. As a result, alternative renewable energy sources that are environmentally and economically imperishable catch great interest. Besides the growing energy crisis, rapid urbanization, increasing population, and industrialization have a huge effect on environmental pollution. Water pollution is another great threat that should be taken care of. However, wastewater could be a very good source of generating energy and nutrients for plants.

Wastewater treatment methods include conventional aerobic activated sludge treatment, anaerobic digester, membrane technology, ion exchange, adsorption, chemical precipitation coagulation, and electrolytic reduction. However, these technologies are associated with any one of the drawbacks such as energy demand, a large amount of residual generation, and ineffective in catching energy potential from wastewater [1].

Electrochemical energy generation from wastewater:

Atmospheric pollution is increasing day by day due to the release of sulfur and nitrogen oxides during the combustion of fossil fuels. To control this energy and environmental crisis, a new energy alternative based on renewable sources is developing with electricity as the backbone of energy. Electrochemical processes cause a very little amount of energy loss. As a result, they produce minimal waste heat (heat pollution) and greenhouse gases (air pollution). Fuel cells, metal/air batteries, and redox flow batteries are a good set of energy generators. They can convert chemical energy to electrical energy, modular construction is possible and they can easily be transported. But the main drawback is that the catalyst is very expensive for oxygen cathode of metal/air batteries. In some cases, redox flow batteries and fuel cells require an ion exchange membrane with a working temperature above the room temperature. On the other hand, Microbial Fuel Cell (MFCs) are the bioelectrochemical system that uses wastewater as the energy source by generating electric power. This also helps to improve the quality of the water environment.



Schematic diagram showing reasons for the increase in demand for energy requirement, resulting in depletion of non-renewable energy resources [2]

Role of microorganisms in MFC operation:

From the definition, we know MFC involves microorganisms. Here, microorganisms act as biocatalysts for oxidizing substrates and transferring electrons to the anode by substrate oxidation for the generation of bioelectricity. Microorganisms can also transfer electrons without an exogenous electron mediator. *Clostridium, Geobacter, Shewanella, and Pseudomonas* are involved in this category. The extracellular electron transfer mechanism is also responsible for the transfer of electrons to electrodes.

They are described as follows: (i). electron transfer mechanisms associated with either direct electron transfer involving the transfer of electrons by pili outgrowth, (ii). By redox-active proteins, which are present on the outer membrane of microorganisms, namely c-type cytochrome membrane-bound proteins, and (iii). Indirect electron transfer (MET) using mediated electron shuttles, such as oxidized and reduced shuttle molecules [1].

Microbial Fuel Cell (MFC):

Microbial fuel cell (MFC) is an eco-friendly process that is very helpful for generating bioenergy and wastewater treatment process. Here, electrogenic or electroactive bacteria (EAB) convert chemical energy to electricity. Thus, MFCs have the ability to provide feasible wastewater treatment with a low carbon footprint.

An MFC consists of an anode, cathode, microorganism, substrate (anolyte), and conductive wire (external circuit). A conventional dual-chamber (or two-chamber) MFC consists of an anode and cathode chambers (or compartments) that are separated by a cation exchange membrane (CEM), also known as a proton exchange membrane (PEM). Various types of separators have been used for MFCs such as canvas; microporous filtration membranes, nylon-infused membrane, carbon paper, NafionTM, UltrexTM, and ceramics. In the anode chamber, the EAB is responsible for the generation of electrons and protons. The reaction is typically represented by the oxidation of acetate:

$$CH3COO- + 4H2O \rightarrow 2HCO3 - + 9H+ + 8e-$$

The EAB acts as a biocatalyst for the oxidation of substrate and transferring electrons to the anode. Microscopic observations have revealed that EAB proliferates over the anode surface to form a multi-layered biofilm. The EAB in the monolayer biofilm that is in direct contact with the anode typically utilizes outer-membrane redox proteins and cytochrome cascades to transfer electrons directly to the anode.

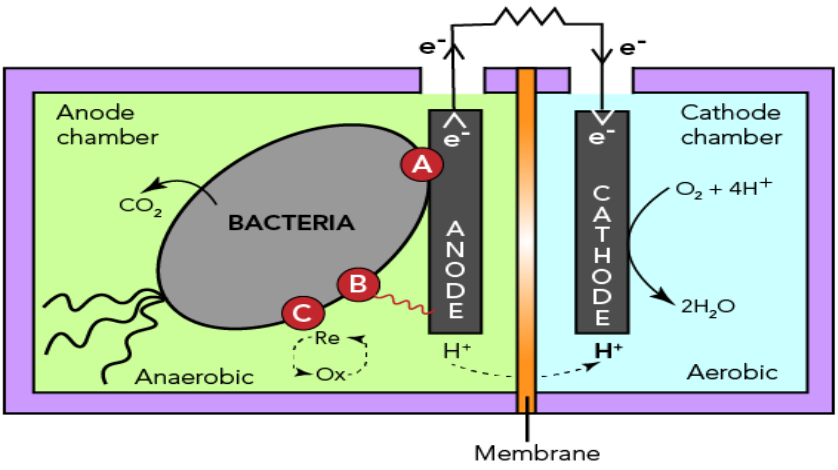


Illustration of Microbial Fuel Cell

On the other hand, the microbes in the outer layers develop nanowire structures to connect with the anode surface or use other microbes via an extracellular conductive matrix to transfer electrons, known as interspecies electron transfer. In addition to the direct electron transfer, the indirect transfer can also occur via soluble electron shuttles or mediators that transfer extracellular electrons to the anode. Based on electron transfer mediators, MFCs can be divided into mediator and non-mediator (or mediator-less) microbial fuel cells [3].

Energy generation by MFCs:

The two important dangers that the world is facing are scarcity of water and water pollution. MFC technology may overcome these challenges. The main criteria for MFCs are types, numbers, and the catalytic activity of the microorganisms. The electrochemical activity of microorganisms can be lost due to the loss of energy at the anode.

The physicochemical and electrochemical factors include, but are not limited to, the types and effective surface area of the electrode, electrolytic resistance, rate of the proton transport through the PEM, and rate of the reduction reaction at the cathode, and external resistance applied across the electrodes. The organic loading rates and type and concentration of the substrate are the operational parameters. The intricate interdependence of these factors and parameters makes the optimization of the MFC difficult. For instance, the rate of substrate conversion can be affected by the total amount of electroactive bacterial cells, a phenomenon of mixing mass transfer, bacterial growth kinetics, organic loading rate per biomass (grams of substrate per gram of biomass per day), transmembrane efficiency for the proton transport, and total potential of the MFC [3].

Role of MFC in wastewater treatment:

MFCs in wastewater treatment have played a major role in both water treatment and the production of bioenergy. The sustainable credentials of wastewater treatment in MFCs may include: (i) generation of electricity from substrate energy, (ii) operation in the absence of gas, (iii) reduced energy loss during aeration, (iv) operational capability even at low temperatures, (v) low activated sludge associated to anaerobic digestion operation, and (vi) use when electrical power is insufficient [1].

Nutrient removal and recovery:

Wastewater treatment plant effluent contains many nutrients. Eutrophication potential and the quality of receiving water can be improved by the removal of nutrients. MFCs have advantages in wastewater treatment plants because it has an electricity generating capacity. PO_4^{2-} and NH_4^+ can be removed. According to MFC is connected by an algal bioreactor externally and it can be used to treat domestic wastewater. This system improved the removal of total P from

58% to 92% and removed 81.9% of organics (as COD), as high as 95.5% of total N, and 96.4% of total P.

Nutrient recovery can also be done by MFCs. P and N recovery by MFC is mainly linked with the formation of struvite, $NH_4MgPO_4.6H_2O$. Struvite is a slow-release fertilizer and has a great commercial value. It is formed by similar molar ratios of ammonium, magnesium, phosphate, and six molecules of water. By using MFC we can also recover nutrients from urine.

Concluding features and future prospective:

MFC research keeps both clean energy production and wastewater treatment. Constant energy production and efficient wastewater treatment processes are very essential and it requires an efficient and cost-effective MFC system. An external circuit is required to set up a circuit for the extraction of energy. Recent studies revealed that for prolonged generation of large-scale MFC process small or no energy is needed. Recovery and reduction of excess sludge from wastewater is the main interest of MFC application.

Microorganism selection is a fundamental criterion in MFC technology for the wastewater treatment process with electricity generation. The development of a dynamic, conductive, and penetrable biofilm will be the main assignment for electrode microbiology manipulation. Improving the electrogenicity by altering microorganisms using genetic manipulation and synthetic biology develops a handful of power generation from MFC during wastewater treatment. Thus, engineered organisms replaced the native microbes over time. Therefore, selection of appropriate engineering strain for long-term MFC operation [1].

So, we come across that MFC is a great and emerging technology for generating clean energy and electricity from wastewater effluent. Membrane technology improves the sewage water and enhances the treatment efficiency. In the coming days, more advanced MFC technology will help to develop an eco-friendly and more reliable source of energy generation from the wastewater system (recover and reuse of nutrients).

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MOLECULAR APPROACHES FOR IDENTIFICATION OF FUNGI AND BACTERIA

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Abstract:

Modern taxonomy has been influenced by genetic methods and indeed, much of the classification and identification is based on specific gene sequences, primarly 16 S rDNA gene for bacteria, and 18 S rDNA and ITS 1 for fungi, with a variety of other housekeeping and virulence genes based on discriminatory power added on top. The most crucial element in the classification of viruses is how their genomes are organised. Genotypic approaches include any procedures involving DNA or RNA. Other effective nucleic acid and protein based approaches for classification include DNA-DNA hybridization, markers like RFLP and RAPD, and Multilocus sequence analysis (MLSA) etc. are also very useful. An extremely vast array of work has been done in the taxonomy of these plant pathogens and their systematic position is now more or less, settled. However, further work is constantly being carried out mainly on the basis of genotyping data based on protein and nucleic acids due to their accuracy to resolve contentious species complexes.

Why use Molecular techniques?

Phenotypic traits are prone to alter as a result of environmental changes

Genotypic based identification techniques would be useful and possibility of being more precise, repeatable, easy and quick.

Molecular approaches for identification of Fungi

Universal fungal primers for: Multi-copy Genes→ rDNA gene cluster 18S, ITS1/2, 5.8S, 28S, 5S, IGS

Actin, Alkaline Protease (*ALP*), Beta glucan synthetase (*FKS*), Chitin Synthase,*GP43*, Histone, Lanosterol - α - demethylase (*LIA1*), *URA5*, Secreted Aspartic Protease (*SAP*), etc. are species or genus specific.

Genus- or species-specific primers → 18S, ITS1/2, 28S rDNA, Mitochondrial DNA, Histone. e.g. *Candida, Cryptococcus, Aspergillus.*

PCR-RFLP

(Restriction Fragment Length Polymorphism Analysis) → Species Level

- Direct PCR of a single colony form a primary isolation plate, tissue sample, or clean culture
- Agarose gel electrophoresis
- Digestion with restriction enzymes
- Agarose gel electrophoresis
- ID via comparison with the data base

Real Time PCR

- **ABI 7700 System (TaqMan)**
- Reporter Dye and Quencher Probe Detection

  → Quantitative DNA and Species Detection

  e.g. *Candida sp.* detection Guiver et al. J. Clin. Pathol. 2001 54:362-366
- **Roche LightCycler**
- SYBR Green Detection

  → Quantitative DNA Detection

  e.g. *Pneumocystis carinii* detection Kaiser et al. (2001) J. Microbiol. Meth. 45:113-118
- Hybridization Probes (Donor Fluor and Acceptor Fluor) Detection

  → Quantitative DNA and Species Detection

  e.g. *C. albicans* and *A. fumigatus* detection Loeffler et al. (2000) JCM 38:586-590

Multiplex PCR

- Multiplex PCR is based on the use of several PCR primers in the same reaction allowing the simultaneous and sensitive detection of different DNA targets, reducing time and cost.
- Annealing temperatures for each of the primer sets must be optimized to work correctly within a single reaction.
- This method is useful in Plant Pathology since plants are usually infected by more than one pathogen.

DNA barcoding

- The idea of a standardized molecular identification system emerged progressively during 1990s with the development of PCR-based approaches for species identification.

- PCR-based methods have also been frequently used in fields related to taxonomy, food and forensic molecular identification (Teletchea et al. 2008) and for identification of eukaryotic pathogens and vectors.
- DNA barcoding uses short standardized genomic sequences to identify species chiefly through PCR amplification by using primers that are applicable for the broadest possible target taxonomic group.
- **ITS (Internal Transcribed Spacer) Sequencing is caccepted as Universal Barcode for fungi.**

Molecular approaches for identification of Bacteria

Genetic techniques have influenced modern taxonomy and a significant portion of classification and identification is based on specific gene sequences. Genotypic approaches encompass any procedures requiring DNA or RNA.

Analyses using 16S rDNA

The technique, which is currently very close to gold standard for taxonomic purposes today, is sequencing of the 16S rRNA gene of bacteria. Additionally, 23S rRNA gene sequence is also considered in many studies but lack of extensive databases for comparison is a drawback. All bacteria contain the 16S rRNA, which is functionally constant and is consistently worked well as a combination of conserved and changeable regions. While it has proved to be the foundation for modern taxonomy, there are certain caveats and it has to be considered along with other techniques for formal identification purposes, especially at the species level. Generally universal sets of primers are used to amplify the 16S rRNA gene product gene (~1.5 kB).

DNA base content

Determination of moles percent guanosine and cytosine constitutes a classical method of establishment of genomic content. This is now being used along with other genotyping methods to establish taxonomic position of an organism. The G+C concentration varies between 3% and 10% within species and between 10% and 15% within genera. G+C concentration ranges from 24-76 percent overall.

DNA-DNA hybridization

This technique assesses the degree of sequence similarity between genomes in an indirect manner. For the establishment of species, a cut-off value of 70% similarity is taken into account. However, the approach must be standardized and repeatable among laboratories, which is frequently a disadvantage. Therefore, it is only used in cases where the similarity between two 16S rRNA gene sequences is greater than 98 per cent. There have been reports where 16S rRNA gene sequence has shown 99% similarity and yet DNA-DNA hybridization values have been

60% or less. Hence, this method has to be used with caution and performed under highly standardized conditions.

Various genotyping methods

Sub-typing techniques are currently used due to their reliability, simplicity and performance and a high degree of strain differentiation. Genotypic techniques like Restriction Fragment Length Polymorphism (RFLP), Randomly Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Repetitive Element-Polymerase Chain Reaction (REP-PCR) and some more recent techniques include Ribotyping and Multi Locus Sequence Analyses (MLSA). RFLP. One of the earliest technique was RFLP, and include whole genome DNA extraction, restriction digestion employing particular utilising gel electrophoresis and restriction enzymes to see the DNA bands.

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PHYSIO-CHEMICAL STUDY OF SOIL OF VARIOUS LANDS AREA IN DHOLPUR (RAJASTHAN)

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Abstract:

The soil contamination is increasing at a very rapid rate due to influx of pollutants in the agriculture ecosystems. This has changed the physical, chemical and nutritive value of the soil and has caused damage not only to the productivity of soil but has also caused damage to the human health as pollutants get accumulated in the tropic levels. The physical and chemical properties of the soil and the impact of the pollutants were investigated in the different soil.

Keywords: Soil, physical properties, chemical properties, dholpur

Introduction:

The upper layer of the earth in which plants grow, called soil.Soil is a mixture of organic matter, minerals, gases, liquids and organisms that together support life.Earths body of soil is the edosphere which has four important functions.It is a medium for plant growth. It is a means of water storage, supply and purification.It is a modifier of earth's atmosphere.It is a habitat for organisms.A gram of soil can contain billion of organisms, belonging to thousands of species, mostlymicrobial and in the main still unexplored.Soil is a major component of the earth's ecosystem. The world's ecosystem are impacted in far reaching ways by the processes carried out in the soil from ozone depletion and global warming to rain forest destruction and water pollution with respect to earth's carbon cycle . Soil is an important carbon agent and degrades. contaminants, the property being called natural attenuation. Typically Soil maintain a net absorption of oxygen and methane and undergoes a net release of carbon dioxide and nitrous oxide.Soil offer plants physical support, air, water, temperature, nutrients and protection from toxins. Soil provide readily available nutrients to plants and animals by converting dead organic matter into various nutrient forms.Soil can effectively remove impurities, kill disease agents and degrade contaminants the property being called natural attenuation.A typical soil is about 50% solid (45% minerals + 5% organic matter), 25% water and 25% air.

Study sites:

Soil analysis is a set of various chemical processes that determine the amount of available plants nutrients in the soil, but the chemical, physical and biological soil
properties are also important for plant nutrition or soil health. Chemical soil analysis determines the content of basic plant nutrients; nitrogen, phosphorus, potassium, pH, organic matter, sulphur, total alkanity, total hardness and other physical characteristics.

Sampling sites:

Soil samples were collected from six different areas which are as follows:

1.Farm soil, 2. Barren land soil, 3. Roadside soil,
4. Garden soil, 5. Riverside soil, 6. Industrial area soil

Material and Methods:

Experimental

Alkalinity	**Neutralization method**
Chloride ion	Mohr's method
Conductivity	Electrical conductivity meter
D.O.	Winkler method
Hardness	Complex method
pH	pH strip method
Sulphate	Gravimetric method
TDS	Evaporation method
Texture	Feel method

Colour:

Colour of soil varies widely. It is an easily observable characteristic.

Barren land: Black and dark grey colour. This is due to the organic matter contents.

Farm soil: Black and dark grey, the variations from this soil are mainly due to organic matter.

Roadside: Brown colour, this is the most common soil colour and it is due to the organic matter and iron oxides.

Garden soil: Red-Yellow, red colour is associated with unhydrated ferric oxides where as yellow colour indicates some degree of hydration.

River side: Grey-yellow colour, grey colour is due to organic matters where yellow colour indicates some degree of hydration.

Industrial soil: Dark grey and black colour , due to presence of organic matters,ferric oxides and sulphates ion.

pH:

The most significant property of soil is pH level. It effect on all other parameters of soil. If pH is less than 6 then it is said to be an acidic soil, the pH range from 6 to 8.5 is a normal soil and greater than 8.5 is called to be alkaline soil.

Electrical conductivity:

Conductivity is also a very important property of the soil. It is used to check the quality of the soil. It is measurement of the presence of ions in the solution. Alkaline soil is always a good conductor.

Requirement: Beaker, conductivity meter, funnel, filter paper etc.

Procedure: Take 5 ml of soil sample and dissolved it into 20 ml of distilled water. Then filtered it and dip the electrode of conductivity meter into the soil solution & note down the readings.

Total alkalinity (TA):

Requirement

Reagents: Methyl orange indicator, 0.01 N HCl solution.

Apparatus: Burette, conical flask, measuring cylinder, beaker, funnel etc.

Procedure: Took 20-20 ml of water in 5 beakers and dissolved 1 gm of soil in every beaker then filtered this solution. Filled up the burette with 0.01N HCl solution. Take 10 ml of filtered solution in a conical flask by using pipette. Add 2 drops of methyl orange indicator if no colour is produced then alkalinity is zero and if pink colour appears then titrate it with HClsolution until the pink colour disappears. Note down the readings and express the results in mg/L.

Calculation: $N_1V_1 = N_2V_2$

$N_1 = N_2V_2 / V_1$

Strength of alkalinity: $S = N_1$ x equivalent weight of $CaCO_3$

Total hardness (TH):

Requirement:

Reagents: EDTA Solution, EBT indicator, buffer solution (pH =10)

Apparatus: funnel, burette, pipette, conical flask, beaker, filter paper etc.

Procedure: Take 20-20 ml distilled water in 5 beakers then dissolved 1gm of soil sample in every beaker and filtered the solution. Filled up burette with EDTA solution. Take 10 ml of soil sample in a conical flask then add 2ml of buffer solution and 2-3 drops of EBT indicator, wine red colour appears. Then titrate it with EDTA solution blue-green colour appears. Then note down the readinds and express the result in mg/L.

Calculation: $M_1V_1 = M_2V_2$

$M_1 = M_2V_2 / V_1$

Strength of hardness: S = M_1 x molecular weight of $CaCO_3$

Total dissolved solid (TDS):

Requirement: Porcelain dish, measuring cylinder, beaker, funnel, filter paper etc.

Procedure: Take 100 ml distilled water in a beaker using measuring cylinder and dissolve 20gm of soil in this beaker then filtered it. A washed and dried porcelain dish was taken and weighed before use. 100 ml filtered soil solution is transferred in dried porcelain dish and warm it until the water disappears then cooled and weighed it and note down the readings.

Calculations:

TDS = (A-B) x 1000

Here ; A = final weight of china dish

B = initial weight of china dish

Dissolved oxygen (D.O.):

Requirement:

Reagents: Megnus sulphate, thio sulphate, alkaline KI, H_2SO_4, starch indicator

Apparatus: Burette, pipette, conical flask, measuring cylinder, funnel, dropper, beaker etc.

Procedure: Take 20 gm of soil sample and dissolve it into 100 ml distilled water and filtered it. Mix 2 ml of megnus sulphate and alkaline KI in soil solution, White precipitate form. Now add 1 ml of H_2SO_4 in white ppt then white ppt disappears and brown colour occurs. Take 10 ml of this solution and add KI then dark brown colour appears. Now add 2-3 drops of starch indicator dark brown colour converted into blueish colour. Titrate this solution with sodium thiosulphate when solution become colourless note down that readings.

Calculation: $N_1V_1 = N_2V_2$

$N_1 = N_2V_2 / V_1$

D.O. = 8 x 1000 x N_1

Sulphate (so_4^{-2}):

Requirement:

Reagent: Barium chloride solution , hydrochloric acid

Apparatus: Silica crucible, beaker ,funnel, whatmann filter paper etc.

Procedure: Take 2o gm of soil sample and dissolve it into 100 ml distilled water and filteed it. Add 2-3 drops of barium chloride solution and heat it, after some time mix 2 ml of HCl solution and boil it. Cooled this solution and filtered it. Take a silica crucible washed and dried it & weighed it. Heat the silica crucible and again weighed it. Note down both readings. Put the filter

paper in silica crucible and burn it untill filter paper converted into white ashes then weighed it and note down the readings.

Calculation: $S = \frac{233.38 \times W \times 100}{96 \times 20}$

We know that 233.38 gm of $BaSO_4$ contains 96 gm of sulphate.

W = difference between silica crucible

Texture:

Soil texture is mostly defined by feel method which is as follow-

First of all take a table spoon of soil in palm. Add water dropwise and knead the soil, if soil remain in ball shape then place the ball of soil between thumb and forefinger gently pushing the soil with thumb squeezing it upward into a ribbon.If soil form a ribbon then excessively wet a small pinch of soil in palm and rub with forefinger.

Chloride (Cl^-):

Requirement:

Reagent: Silver nitrate, Potassium chromate indicator

Apparatus: Burette, Pipette, conical flask, dropper, funnel, filter paper etc.

Procedure: Take 20 gm of soil sample and dissolved it into 100ml of distilled water and filtered it. Filled up the burette with N/20 silver nitrate solution. Take 10ml of soil sample and add 2-3 drops of potassium chromate indicator then yellow colour appears. Now titrate it with N/20 silver nitrate solution, wine red colour occurs. Then note down the readings and express the result in gm/mL.

Calculation:

$$N_1V_1 = N_2V_2$$

$$N_1 = N_2V_2/V_1$$

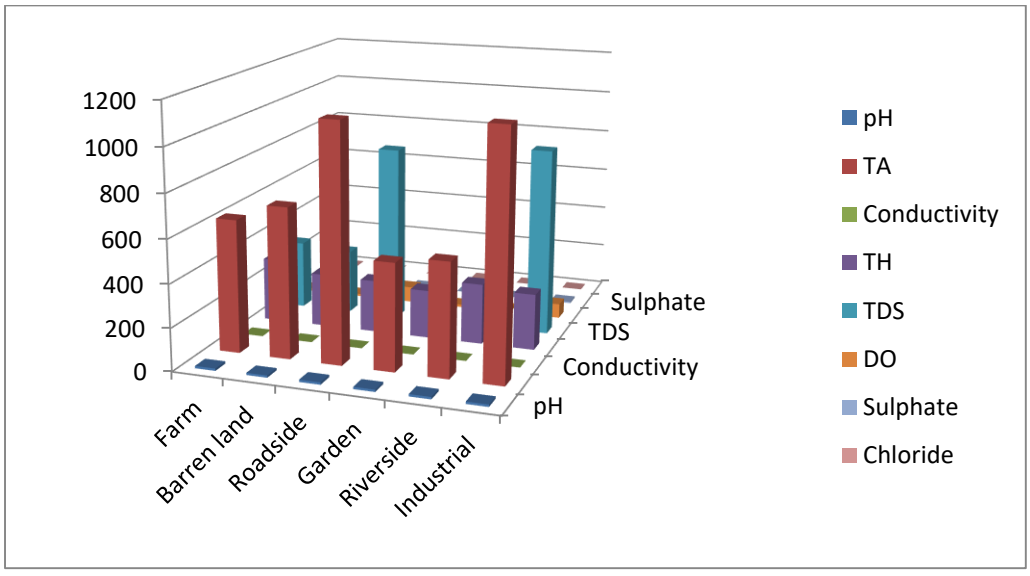
Strength of chloride S = N_1 x molecular weight of chloride

Results:

Soil analysis of the effluents from six sampling sites has been carried out for Ph, colour, conductivity, TDS, DO, hardness, alkalinity, sulphet, chloride, texture. The results are as follows:

Results of the study:

Methods	Farm	Barren land	Roadside	Garden	Riverside	Industrial
Colour	Black & Dark grey	Black & Dark grey	Brown	Red-Yellow	Grey-Yellow	Black & Dark grey
pH	10.9	10.3	10.8	10.4	10.8	10.9
Conductivity	0.7	0.4	0.1	0.6	0.5	0.3
Texture	Sandy loam	Loamy	Sandy loam	Loamy	Loamy	Sandy loam
TA	620 mg/L	700 mg/L	1100 mg/L	500 mg/L	530 mg/L	1130 mg/L
TH	300 mg/L	252 mg/L	248 mg/L	228 mg/L	285 mg/L	264 mg/L
TDS	320 mg/L	300 mg/L	820 mg/L	150 mg/L	180 mg/L	870 mg/L
DO	14 mg/L	10.64 mg/L	74.4 mg/L	12.48 mg/L	16.58 mg/L	68.6 mg/L
Sulphate	2.80 gm/ml	6.08 gm/ml	4.5 gm/ml	1.46 gm/ml	1.06 gm/ml	6.24 gm/ml
Chloride	0.18 gm/L	0.17 gm/L	0.02 gm/L	0.48 gm/L	0.24 gm/L	0.14 gm/L



Discussion:

pH: We found that the value ofsoil pH in this area indicates an alkaline nature of soil because of some alkali salts like OH^- ions.

Conductivity: All samples are good conductor. Maximum conductivity was found in farm soil because of higher contration of ions and minimum conductivity was found in roadside soil because of lower concentration of ions.

Total Alkalinity: Maximum alkalinity was measured in industrial soil because quantity of calcium carbonate and other compounds were high and minimum alkalinity was measured in garden soil because many plant nutrients including iron,zinc,copper is reduced at high pH values.

Total Hardness: Maximum hardness was found in farm soil because of Ca^{+2} and Mg^{+2} ions. Minimum hardness was found in garden soil because Zn, Fe, Co, Mn is reduced at high pH values.

TDS: Maximum TDS was found in Industrial soil because soil particles may contain soluble components that can dissolve, this will increase the TDS. Minimum TDS was found in garden soil because fewer components were dissolved.

DO: Maximum DO was found in roadside soil because loss organic matter and Zn deficiency and presence of nitrogen, magnesium and phosphates. Minimum DO was found in barren land soil because of loss of soil structure, poor internal drainage and soil acidity are other problems.

Sulphate: Maximum sulphate was found in industrial soil because of concentration of ions was high and minimum sulphate was found in riverside soil because of low concentration of ions.

Chloride: Maximum chloride was found in garden soil because concentration of ions was high and minimum chloride was found in roadside soil because of lower concentration of ions.

Conclusion:

It was observed that different areas of soil had influences on the physiochemical characteristics of the soils. If sulphate and chloride was up to the mark then it is harmfull for soil. Soil texture for all soil samples are different.

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A BRIEF OVERVIEW ON ORIGIN, MECHANISM AND THERAPEUTICAL APPLICATIONS OF CRISPR-CAS9 TECHNOLOGY

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Abstract:

Clustered Regularly Interspaced Short Palindromic Repeat or CRISPR-Cas9 technology has redefined our approach to gene editing techniques and gene therapy. Apart from being expeditious, highly efficient, and categorical, it has emerged as a potentially potent implement in the treatment of lethal diseases such as cancer and AIDS. Cas9 technology with CRISPR enables precise and effective cleavage of a desired target DNA sequence, and given the relative facileness and simplicity of building sgRNAs, it has greatly facilitated genome editing. Leaving chemotherapy and radiotherapy for cancer behind, CRISPR has played a crucial role in the development of immunotherapy techniques for cancer. CRISPR-engineered oncolytic viruses additionally show optimized tumor selectivity and enhanced immune stimulation to cure cancer. It has also provided an adaptable gene-editing approach that has been effectively applied to AIDS prevention and reduction.

Keywords: CRISPR-Cas9, Gene editing, Gene therapy, Cas9 Technology, AIDS, Cancer, Medical Therapeutics.

Introduction:

Since its discovery in 2012, CRISPR-Cas9 technology has been responsible for a revolution in gene-editing techniques. This applied science has fueled the targeted gene editing methods that involve the application of engineered nucleases. Clustered Regularly Interspaced Short Palindromic Repeat technology, or the technology better hailed as CRISPR by genome editors and geneticists is a new important platform for generating RNA-guided nucleases (RGNs) such as Cas9, along with customizable specificities. Not only can CRISPR/Cas9 technology effectively mediate gene editing, but it can also confer biological activities. Inactive Cas9 (dCas9) is produced by mutations in two nuclease domains of Cas9, which is used as a locus-specific DNA-binding protein (Gilbert *et al.*, 2013; Qi *et al.*, 2013).

Apart from being rapid, RGN-mediated genome editing is facile and has enabled us to efficiently manipulate and modify endogenous genes in a wide variety of important cell types and novel organisms. CRISPR technology has redefined our approach to gene editing techniques

and gene therapy. This review focuses on the study of the mechanism of CRISPR-Cas9 and the revolutionary changes brought about by it in gene therapy for cancer and AIDS. CRISPR-Cas9, as a consequence of its high efficiency and accuracy, has emerged as a potentially powerful tool in the treatment of cancer. It has shown an unparalleled clinical potential to detect novel targets for cancer therapy and to dissect chemical-genetic interactions. CRISPR has provided insight into how tumors respond to drug treatment. Furthermore, CRISPR-Cas9 has the potential to rapidly engineer immune cells as well as oncolytic viruses for cancer immunotherapeutic applications. The (Cas9) system is also being developed as a powerful gene-editing tool to treat HIV/AIDS. It can be utilized to treat HIV-1 infection and clear the provirus by targeting cellular co-factors or the HIV-1 genome, as well as to induce transcriptional activation of latent virus in latent viral reservoirs for elimination. Also, in human cells and animal models, this adaptable gene-editing method has been effectively applied to HIV/AIDS prevention and treatment.

Mechanism:

CRISPR-Cas9 technology has originated from an immune defense mechanism that is found in archaea and bacteria, which provides immunity to the organism against invading viruses and plasmids. This adaptive immune system relies on ribonucleoprotein effector complexes. By storing the memory of encounters with foreign DNA in distinct spacer sequences, it allows the bacteria to eradicate invading phages, conjugative plasmids, and mobile genetic elements into CRISPR arrays. CRISPR systems naturally integrate foreign DNA molecules into CRISPR arrays. These subsequently produce crRNAs containing protospacer regions that are antigenic to the invading DNA molecules. This process is followed by the hybridization of each crRNA molecule with non-coding tracrRNA. This hybridization forms a crucial hybrid of crRNA-tracrRNA which forms a complex with Cas nucleases that tend to cleave target-DNA sequences nearby to short sequences called protospacer adjacent motifs (PAMs). This CRISPR/Cas system can be efficiently manipulated to target genes of interest to regulate their functions effectively in any eukaryote. Further studies in the molecular biology of the CRISPR/Cas reveal the way it can be used while using synthetic guide RNAs (gRNAs) and other components to the target gene of interest in DNA molecule for the desired application and discoveries of disease-causing genetic variations.

There are three types of CRISPR/Cas systems according to popular classification out of which the type II CRISPR/Cas system is commonly used. It consists of three components: an endonuclease (Cas9), a CRISPR RNA (crRNA), and a transactivating crRNA (tracrRNA). . The crRNA and tracrRNA molecules comprise the guide RNA (gRNA), which can be replaced by a synthetic fused chimeric single gRNA (sgRNA). The sgRNA possesses a unique 20-base-pair

(bp) sequence that is meant to complement the target DNA site, and it must be followed by a short DNA sequence known as the "protospacer-adjacent motif" (PAM), which is required for Cas9 protein compatibility. When the sgRNA and Cas9 nuclease are both produced in the cell, they create a ribonucleoprotein (RNP) complex, which is guided to a target DNA site by the sgRNA. Cas9 accurately cleaves the DNA to induce a DSB after the sgRNA attaches to the target sequence using Watson-Crick base-pairing. The cleavage takes place within the protospacer, three nucleotides upstream of the PAM, resulting in blunt ends. Cas9's RuvC and HNH active-site motifs act on the (-) and (+) strands, respectively, and are responsible for the cleavage of opposing DNA strands. The cell machinery heals the DSB using one of two basic processes, depending on the cell state and the presence of a repair template: homology-directed repair (HDR) or non-homologous end joining (NHEJ). The HDR pathway works by recombining a donor DNA template at the DSB location, resulting in precise repair. Specific sequences or mutations can be introduced into a target section of the genome via homology-directed repair. The more common NHEJ mechanism is an error-prone system that generates frameshift mutations by randomly inserting or deleting nucleotides at the DSB site (indels). It can thus be utilized to induce specific gene knockouts (KO). Cas9 technology with clustered regularly interspaced short palindromic repeats enables accurate and effective cleavage of a desired target DNA sequence, and given the relative ease and simplicity of building sgRNAs, it has greatly facilitated genome editing. The use of distinct sgRNAs allows this method to be multiplexed, which is an extra benefit. Only the CRISPR/Cas9 system, among genome editing nucleases, can edit several loci at the same time by introducing sgRNAs to distinct sites. When two sgRNAs are used in the same cell, minor deletions, complicated rearrangements, and even full chromosomal suppression can occur.

CRISPR-cas9 in cancer therapeutics

Cancer is considered a complicated disease. Dysregulation of cancer can be done by acquiring effective immunity against cancer cells. This immunity solely depends upon the interactions that occur between the host, tumor, and the environment that surrounds them. Enhancing the immune response to cancer cells by applying immunotherapy has emerged as a hopeful option in the treatment of cancer. Such immune responses are enhanced either by applying synthetic chimeric antigen receptor (CAR) therapy or by targeting the programmed death receptor 1 (PD-1).

Using CRISPR/Cas9, catalytically inactive dCas9 can be recruited by gRNAs to specific target DNA sites and can be exploited to activate or repress specific target genes by fusing them to transcriptional activation or inhibition domains. This advantage is used in cancer therapy for

the regulation of endogenous gene expression. On the other hand, targeted epigenome editing can also be a boon. Multiple kinds of cancer involve epigenetic factors. In these cases, if we target the epigenetic regulatory machinery such as histone modifiers as well as proteins involved in altering DNA methylation, cancer dysregulation can be carried out efficiently. Another approach in CRISPR/Cas9-based cancer therapeutics may involve the exploitation of oncolytic viruses. Genetic modifications can help viruses lose their virulence against normal cells but their capability to attack and lyse cancer cells possessing deficiency of antiviral defenses can be maintained. Direct cellular lysis, one of the various mechanisms concerned with the viral-induced destruction of cancer cells also directly triggers further immune stimulation through tumor antigens released from dying cancer cells. CRISPR-engineered oncolytic viruses also show optimized tumor selectivity and enhanced immune stimulation.

Along with these and many more CRISPR-Cas9 mediated genome editing applications, cancer therapeutics appear to have a promising future.

CRISPR-Cas9 in HIV-1/AIDS therapeutics

AIDS remains a severe threat to worldwide human health, despite the tremendous efforts of researchers in the prevention and treatment of HIV-1 infection. The CRISPR-Cas9 system has recently been developed as a promising gene-editing method that could be used to cure AIDS. To minimize the infection caused by HIV-1, CRISPR can be used to target cellular co-factors or viral DNA. It's being tinkered with to eliminate the provirus and stimulate transcriptional activation of latent virus in latent viral reservoirs. In human cells and animal models, this adaptable gene-editing approach has been effectively applied to AIDS prevention and reduction.

In 2013, CRISPR/Cas9 was used for the first time to prevent HIV-1 infection by disrupting latent HIV-1 provirus (Ebina *et al.,* 2013). By targeting HIV-1 LTR using CRISPR/Cas9, researchers were able to decrease the production of HIV-1 genes in Jurkat cell lines (Ebina *et al.,* 2013). Soon after, researchers used Cas9/gRNA to target conserved sites in the HIV-1 LTR U3 region, which resulted in inactivating viral gene expression and restricting virus replication in an HIV-1 latently infected T cell line, pro-monocytic cell line, and a microglial cell line with little genotoxicity and no detectable off-target editing (Hu *et al.,* 2014). Furthermore, combining two successful sgRNAs that target separate sections of the HIV genome had become able to stop the virus from replicating and escaping (Lebbink *et al.,* 2017). A single sgRNA-driven CRISPR/Cas9 editing triggered mutational inactivation of HIV-1 provirus has also been described (Wang *et al.,* 2018). In this way, CRISPR-Cas9 has brought about revolutionary changes in the therapeutics of HIV-1/AIDS.

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ELECTROCHEMICAL IMPEDANCE SPECTROSCOPY (EIS): OVERVIEW

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Introduction:

Electrochemical biosensing is based on the reactions that either consume or produce electricity. It studies charge transfer, mass transfer and diffusion process; the processes influencing conductivity, resistivity, and capacitivity of an electrochemical system. Electrical changes occurring can be measured by three methods-

- Potentiometry- measuring potential difference between electrodes
- Coulometry- measuring current in a cell over time
- Voltammetry- measuring current by altering the potential

Biosensor is a device used to measure chemical or biological reactions by generating signal proportional to analyte concentration in a reaction. Impedimetric biosensors consist of electrode, transducer surface, bio-receptor and analyte. EIS measurements are based on two domains- time and frequency. It measures impedance of a system over a range of frequencies. EIS gives insight to the characteristics of electrode surface properties and thus it gives electrical fingerprint of the sample. Different redox couples/ redox mediators can be used to study the intrinsic properties of the electrode surface and interaction of analyte with the modified electrode surface.

What are soluble redox mediators?

Redox mediators diffuse to electrode surface, transfer electrons to/from electrode and undergo oxidation and reduction respectively. The electron transfer occurs between lowest unoccupied molecular orbital of redox couple and electrode surface. Thus, the energy of electrode surface can be modulated by applying voltage through external source.

Examples of redox couple are: ferrocyanide and ferricyanide redox couple, leucomethylene blue and methylene blue dye.

Electrochemical methods:

1. Cyclic voltammetry: It gives insight about the features of modified electrode surface. Amount of material present on the electrode surface has direct relation to the area under cyclic

voltammetry curve. Shape of the CV curve also give indication about the ionic interaction and charge transfer at the electrode surface.

2. EIS: data is commonly represented by Nyquist plot in which every point is characteristic of impedance at a given frequency. Kinetically controlled high frequency region is depicted as a semicircle and low frequency region denoting diffusion limited process is depicted as a Warburg line. Charge transfer resistance R_{ct} is depicted as a diameter of the semicircle which has inverse relation to charge transfer. For frequency information Bode plot can be used.

3. Microscopic and spectral technique: X-ray photoelectron microscopy can be used to characterize electrode surface. A graph is obtained by plotting binding energy and intensity where peaks are obtained for different values of binding energy which give indication of groups attached to the surface.

4. Attenuated total reflectance: Fourier transform infrared spectroscopy, ATR-FTIR spectroscopy can also be used to characterize functional groups of the monolayer. Comparative study can be done by taking spectra of the single compound and compound adsorbed on the electrode.

5. Scanning electron microscope, SEM: It gives indication about morphology and chemical composition of the surface.

6. Atomic force microscope, AFM: It gives insight about the surface modification at microscopic level.

AC voltage is applied to the electrodes and in return response current is measured. Impedance thus can be measured as ratio of excitation voltage and response current. EIS and IFC impedance flowcytometry are used for analysis of single cell. Heterogeneity among cells can be analysed by measuring different electrical parameters of a cell like cytoplasm conductivity, cellular resistance, membrane capacitance etc. First microfluidic IFC device used to measure single cell impedance at different frequencies were coplanar electrodes (Zhang *et al*, 2021)

Impedance sensor types:

1. Faradaic impedimetric sensors: It requires electrode with conductive surface and redox active molecules in the solution. Electrochemical process at electrodes surface is characterised by charge transfer resistance R_{ct}. Binding of conductive molecules allows the transfer of electrons and thus result in decrease of R_{ct} values. Similarly increase in R_{ct} is observed with attachment of non-conductive molecules to the electrode surface.

2. Non-Faradic/ Capacitive sensors: It requires an insulating layer on the sensing surface. Reaction occurring at the surface is determined by the C_{dl} value i.e., double layer capacitance.

e.g., Impedance spectroscopy performed in PBS solution alone is of non-faradic type and in presence of ferrocyanide/ferricyanide is of Faradic type.

Important terms related to impedance spectroscopy:

1 Impedance: Quality of a circuit that retards electron flow across the electrical circuit. There are circuit elements with more complex behaviour i.e., why we use impedance which unlike resistance is not limited by simplifying properties. Impedance is a complex value, comprises of real (electrical resistance) and imaginary (electrical reactance, admittance) part.

Complex numbers contain both real and imaginary part represented by equation b+ic where b, c = real numbers and i= imaginary number i.e., $\sqrt{-1}$

Z = R+jX

R= system's resistance or static property of electrical circuit, independent of voltage, current, frequency

J= imaginary component

X= opposition of circuit element to current change which arises due to change in electrical and magnetic field, its frequency dependent.

Impedance spectrum obtained is used to characterize membrane, diffusion process. For this equivalent circuit is prepared using four elements: ohmic resistance, capacitance, constant phase element and Warburg impedance. In order to compare electrode electrolyte interaction Randle's circuit is used which gives information about solution resistance, charge transfer resistance, Warburg impedance and double layer capacitance. Impedance can be measured by Bodes plot, between $\log|Z|$ and phase shift ϕ as function of $\log f$ or by using Nyquist plot between real and imaginary values of Z. Each point measured on Nyquist plot gives impedance at single frequency (Lisdat and Schafer 2008). Resistance has only magnitude and no phase. In AC circuit two impeding mechanisms other than resistance are – inductance and capacitance which have similar function as resistance but have both magnitude and phase.

2. Admittance: Frequency dependent component arises in inductor, capacitor after applying Alternating current (AC).

3. Inductance: Opposition offered by the magnetic field to the flow of AC current is called inductance (L). The opposition offered by the magnetic effect in AC circuit is called inductive reactance. Inductive device opposition to current flow depends on the resistance of the wire and magnetic properties of the circuit.

4. Electrical double layer Capacitance: It refers to the polarization of ionic charge developed at the surface of electrodes. The amount of charge developed depends upon the surface area and

ion size. When voltage is applied, ions will start moving to oppositely charged electrode. The interface between electrode and electrolyte is called electrochemical double layer.

Capacitors can pass AC current because of their charging and discharging action. It can be used to pass AC current and block DC current because on applying DC voltage capacitor will charge to the value of source voltage and dielectric between the plates will stop current flow (Dale R Patrick and Stephen W Fardo 1999).

Electrochemical system is pseudolinear:

It possesses superimposition property, if input consist of the weighted sum of several signals, then output will be the weighted sum/ superimposition of response to each signal. Therefore, electrochemical system is pseudolinear, if we double the voltage, it will not necessarily double the current.

Normally, a small AC potential of 1-10mV is applied to the cell, and we can observe the nonlinear response to such small voltage change. Usually, small excitation signal is used so that cell's response is pseudolinear. We apply sinusoidal potential excitation and response is the AC current signal which can be analyzed as sum of sinusoidal functions (Fourier series). Non-linear response is not seen in case of DC potential because here we measure only cell's current at excitation frequency.

In linear/ pseudolinear systems response to sinusoidal voltage will be sinusoidal current at same frequency but shifted in phase (ϕ).

$E_t = E_0 \, sin(\omega t)$

$\omega t \; (radian/second)$

$\omega = 2\pi f$

$I_t = I_0 \, sin(\omega t + \phi)$

Expression analogous to Ohm's law-

$Z = E_t/I_t = E_0 \, sin(\omega t)/I_0 \, sin(\omega t + \phi) = z_0 \cdot \frac{sin(\omega t)}{sin(\omega t + \phi)}$

Potentiostat measures impedance by applying potential wave to working electrode and measuring the current wave. Complex impedance i.e., sum of real and imaginary components can be calculated as a function of angular frequency ω ($2\pi f$) by varying excitation frequency of applied potential over a range of frequencies (Min *et al.*, 2015). Real component is called resistance and imaginary as reactance which is frequency dependent. It's an opposition of circuit elements to change in current flow which arises due to build-up of electric and magnetic fields. Elements impedance and capacitance results in lag in response to potential change and thus shift

in the current by $\pi/2$ radius relative to the applied voltage. In case of inductive circuit voltage leads current by ϕ in phase which is opposite i.e., current leads voltage in capacitive circuit.

EIS role in biosensing:

Surface can be modified in various ways to increase the detection limit and performance of the biosensor. Sensor surface attached to a bioreceptor acts as a physicochemical detector which transforms biological signal to analytical signal. Modified screen-printed electrodes of graphene, gold, silver etc. are commonly used for the detection purpose. Bioreceptor can be a protein, nucleic acid, cell, organic tissue etc. For diagnostic purpose detection of protein biomarkers or genetic biomarkers like mRNA, miRNA, DNA can be done. Gold modified electrodes are used for detection of analyte by developing a self-assembled monolayer (SAM) which can be modified accordingly. In case of Faradic EIS detection long term use of gold modified electrodes should be avoided if we are using ferro/ferricyanide redox couple as cyanide result in etching of gold atom. Quantitative analysis of the analyte can be done by determining the impedance. EIS biosensors are able to achieve real time detection but require theoretical simulation of data analysis (Zamfir *et al*, 2020). EIS doesn't require labels as in fluorescence techniques. EIS biosensors allow low limit of detection and thus low volume of clinical samples are required for detection. Such devices can be used for detection of protein onco-markers approved by FDA (Bertok *et al*, 2019). Portable machines are used in food industry, clinical diagnosis- determination of hematological parameters, environmental monitoring, detection of blood glucose using glucometer has brought revolution in medical world.

Biomodification techniques:

1. Thiol based metal surface interaction: self assembled monolayers can be formed with thiol compounds like mercaptan, sulfhydryl on the gold and silver electrodes. Cleaning of the surface can be achieved by methanol, ethanol, piranha solution etc. Adsorption of compounds with hydroxyl or carboxyl groups result in formation of hydrophilic surface whereas adsorption of alkyl chains results in formation of hydrophobic surface.

2. EDC-NHS interaction: It is mainly used to further activate the active group like carboxyl present on the compound adsorbed on the electrode surface so that it can bind with the biological material like antibodies. EDC acts as a catalyst to increase the coupling efficiency. NHS reacts with carboxyl group to form stable amine reactive ester intermediate more stable than O-acylisourea formed by EDC reaction. At physiological conditions intermediate formed by the NHS can be coupled with the primary amines. EDC NHS carbodiimide chemistry provide strong covalent interaction and can be frequently used with the compounds with carboxyl and amine groups.

3. Pi-pi stacking interaction: Non-covalent interaction achieved mainly by using graphene or carbon nanotubes. Here carbon is sp^2 hybridized with empty p_z orbital which participate in pi-pi interaction and form nano-bio conjugates. Therefore, compounds having aromatic rings e.g., amino acid tryptophan can bind to carbon surface by hydrophobic, electrostatic and pi-pi interactions.

4. Avidin-biotin interaction: It's a non-covalent interaction which is known to be the strongest bond in nature. Interaction can occur with streptavidin, a tetrameric protein has 4 binding sites/ neutravidin, de-glycosylated form of avidin. Streptavidin coated nano-particle surface can be used to capture biotinylated DNA, biotinylated fluorophore etc. (Yuce and Kurt 2017)

In potentiostatic method electroactivity is monitored by measuring potential difference between working, counter and reference electrodes.

In amperometric method change is oxidation state of electroactive species has direct relation to analyte concentration.

Circuit modelling:

John Edward Brough Randle's proposed a Randle's circuit. Non-ideal Nyquist plots may be obtained in case of interface defects or non-homogenous surface modifications. In most of the cases pre separation of analyte is required for efficient detection and the biosensor must show selectivity e.g., for prostate cancer antigen PSA, a prostate cancer biomarker detection biosensor should not bind to human serum albumin non-specifically (Akbari jonous *et al.*, 2019).

In case of portable devices like Palm Sens there's problem with shielding. Some conditions must be met for impedimetric measurements: AC amplitude must be small enough to keep a linear response and large enough to increase signal-to-noise ratio, stability of the system during measurement, causality AC response should correlate with AC stimulus only. Nyquist plot and its corresponding Randle's circuit are shown in Fig.1 and Fig.2 respectively.

Two other plots which describe real time EIS measurements are:

- Lissajou plot (AC current vs AC potential)
- Resolution plot (AC current and AC potential vs time)

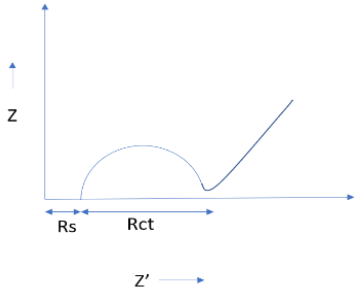

Fig.1

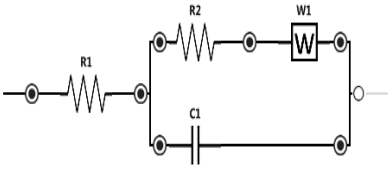

Fig.2

Commercial software packages available for data evaluation are –EIS spectrum analyser and LEVM or ZSimpWin and ZView. Different data evaluation methods are incorporated within software that comes with potentiostat/galvanostat, NOVA software (Metrohm) and PS Trace (Palm Sens). EIS spectra can also be interpreted by Poisson Nernst Plank PNP equation (Bertok *et al*., 2019).

Why it's a sensitive technique in comparison to others?

- In electrochemical experiment the number of electrons and diffusion coefficient will be known; scan rate and electrode surface area can also be fixed.
- EIS can be used to analyse signal over wide range of frequency range i.e., 1mHz to 1MHz using potentiostat.

According to standard equation the current produced has direct relation with the analyte concentration. This amplification result in increased observed sensitivity. Working electrode surface determine the performance of the electrochemical sensor.

Characterization technique of chemically modified electrode- Measuring the contact angle- hydrophobic surfaces have large and hydrophilic ones have small contact angle. Thus, hydrophobicity or hydrophilicity can be introduced and monitored by developing monolayers over the electrode surface.

Applications:

1. Detection of pathogenic bacteria: For the first-time binding kinetics of glycosyltransferase in real time was evaluated using EIS proves beneficial for detection of pathogenic bacteria and cancer cells (Heine *et al.*, 2021).

- EIS has been used to detect E. coli O157:H7 bacteria by immobilizing polyclonal E. coli antibodies on gold electrodes modifies with SAM (dos Santos MB *et al.*, 2009).
- Polymer functionalised electrodes are used to detect staphylococcus in just 20 minutes and also utilized to discriminate between gram positive staphylococcus and gram-negative E. coli (Schulze H *et al.*, 2021)
- Label free biosensor is used to detect *Brucella melitensis* in milk and pure culture at 4×10^5 and 1×10^4 respectively (Wu H *et al.*, 2013).
- S. Typhi specific single strand capture probe on the surface of gold nanoparticle was detected by the modified screen-printed electrode (Bacchu *et al*, 2022).

2. Assessment of food quality parameters:

- Detection of adulterants like water, formaldehyde, hydrogen peroxide, sodium hydroxide in bovine milk through EIS (Durante *et al.*, 2015)

- Early detection of mastitis in cow was done by measuring milk electrical conductivity (Ferrero *et al.*, 2014)
- Fish quality was assessed through EIS by measuring phase angle and admittance, using three electrode system (Niu and Lee 2000)

3. Monitoring corrosion of metal surfaces: Metal surfaces are protected from corrosion by coating with organic polymers. EIS can be used to detect the degradation rate and determining the quality of metal (McIntyre and Pham 1996).

4. Detection of diseases:

- Detection of different diseases can be done by exploiting the principle of antigen-antibody interaction through EIS. Rapid label free detection of SARS-CoV-2 antibodies was done using impedance sensing platform (Rashed *et al*, 2021).
- Detection of glycated haemoglobin by immobilizing it on self-assembled monolayer on gold electrode by selective chemical reaction with boric acid (Park *et al*, 2008)

Summary:

EIS plays role both in biological and non-biological fields**.** It provides an electrical fingerprint of the sample. Analyte can be detected by the biological component of the sensor, this binding event generate electrical impulses proportionate to analyte concentration, monitored by the transducer. First scientifically proposed and commercialised biosensors for analyte detection were electrochemical sensors. Detection of various epidemic diseases can be done using this technology. EIS is quick, non-invasive and a sensitive technique and thus it has wide applications.

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SUSTAINABLE CONSERVATION OF HONEY BEES: A GLOBAL PHENOMENON

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Abstract:

Pollinators are an essential part of the world's biodiversity because they provide crops and wild plants with essential ecological services. Since bees are important Angiosperm pollinators, both humans and biodiversity should be concerned about their apparent decline. There are at least 9 native honey bee species in East Asia. These bees are immensely important since they are major pollinators of nearly one-third of crop species, give some of the world's poorest people a sizable source of income, and serve as prey for several endemic animals. Invasive species, developing diseases, the use of pesticides, and climate change also have the potential to have an influence on bee populations. Habitat loss is the main threat to bee variety. We argue that future conservation plans must include; reducing habitat loss, improving agricultural habitats for bees, teaching the general public and experts about bee taxonomy, basic autecological and population genetic studies to support conservation strategies, DNA barcoding's value for bee conservation, the effects of invasive plants, animals, parasites, and pathogens, and the inclusion of this data to comprehend the risk of climate change on the current diversity of bees.

Keywords: Conservation, Deforestation, Colonies, Honey bee population, *Apis spp.*

Introduction:

An essential ecological service is the pollination of agricultural crops by insects. Insect pollination is necessary for 87 of the top 115 global food crops to produce fruit, vegetables, or seed (Klein *et al.*, 2007). Estimates place the value of insect pollination for global agricultural

production at 153 billion, or 9.5% of the value of global agricultural output used for human consumption. In terrestrial habitats, bees are the primary pollinators of wild plants and agricultural crops (Buchmann and Nabhan, 1996). As a result, they play a crucial role in providing the ecological service of pollination (Costanza *et al.,* 1997). In context of predictions that global population of humans would reach 9 billion by 2050 (United Nations, 2004) and the concomitant rise in the usage of agricultural landscapes (Tilman *et al.,* 2001). In the years to come, bees will become even more crucial to human existence and the preservation of a large portion of terrestrial biodiversity.

The South and Southeast Asian countries deforestation has persisted relentlessly over the past 25 years (Sodhi *et al.,* 2004). Rising incomes and per capita consumption, as well as an expanding human population, unavoidably put more strain on natural ecosystems. The widespread conversion of primary forest to short-cycle forestry, rubber and oil palm plantations, agriculture, and urban areas is particularly concerning for honey bee sustainability (Kevan and Viana, 2003; Sodhi *et al.,* 2004). All of these efforts entail destroying mature trees that are good for nesting, and they frequently involve reducing the amount of food available and using pesticides. Direct interactions with people can occasionally lead to the destruction of colonies (Underwood, 1992). The economic motivation for hunting and gathering in the remaining forests may increase substantially of growing population and opulence combined with a desire for natural products gathered from the wild (Nath *et al.,* 1994; Chen *et al.,* 1998; Wilkie and Carpenter, 1999; Nath and Sharma, 2007). Since many crops depend on animal pollination for the quantity and quality of their yield, the present global reduction of insect pollinators (Potts *et al.,* 2010) could have a severe impact on human health and food production (Potts *et al.,* 2016). It is common practise to introduce managed pollinators, particularly colonies of the western honey bee (*Apis mellifera*), to meet the need for pollination services in crops (Garibaldi *et al.,* 2017) Contrarily, beekeepers frequently keep managed colonies, at least for a while, away from agriculture, even in regulated regions (Henry *et al.,* 2012) because agricultural landscapes negatively affect honey bee survival through, for example, chemical exposure and narrow flower supply (Potts *et al.,* 2010; Potts *et al.,* 2016; Henry *et al.,* 2012; Requier *et al.,* 2017). The latest research has demonstrated that the introduction of numerous managed colonies into places that are protected may have a negative impact on wild bee colonies (Danner *et al.,* 2016; Thomson, 2016; Geslin *et al.,* 2017; Magrach *et al.,* 2017; Malinger *et al.,* 2017; Norfolt *et al.,* 2018).

Our need for bees is accompanied by mounting evidence that their population is declining (Williams P.H., 1982; Fitzpatrick *et al.,* 2007). This drop seems to be primarily caused by human

impacts, with a number of things having significant causative roles. The main contributing cause to the fall of bee populations, as well as the collapse of biodiversity in general, appears to be habitat loss (habitat deterioration and explicit destruction) (Foley *et al.,* 2005). Through genetic isolation and eventual inbreeding (Zayed, 2009) or merely the inadequacy of isolated habitat islands to support sustainable bee populations, habitat degradation, a direct effect of habitat loss—will have an influence on surviving populations (e.g., Ellis *et al.,* 2006). Bee populations in surviving ecosystems can be greatly impacted by invasive and emergent species, whether they are plants, other free-living animals, or parasites and pathogens (Stout and Morales, 2009). Future climate change is likely to have a significant influence on the biodiversity of bees, just as it has on other insects today (Parmesan *et al.,* 1999). The most pervasive and well-documented dangers to bee populations, after habitat loss, seem to be exotic species, parasites, and pathogens (Stout and Morales, 2009). However, climate change may pose the biggest concern in the future. Agri-environment plans or reserves with limited space are the main tenets of conservation. Climate change will cause species ranges to alter (Parmesan *et al.,* 1999) and make current farming practises geographically nonviable. Despite the aforementioned, native bees are still widespread across a large portion of their natural territory. In fact, the Eastern hive bee *A. cerana* and the red dwarf honey bee *Apis florea* are both increasing their ranges into New Guinea and the Middle East, respectively (Mossagegh, 1993, Anderson, 1994). *A. cerana* is still widespread in Hong Kong, one of the worlds most urbanised and changed landscapes, and it is crucial for pollinating the remaining flora. However, there are clear indications that dangerous processes are at action on some species in some locations, and we believe that these processes have already caused or are about to cause local extinctions. On the island of Hong Kong, where they are ostensibly absent, this may have already happened to dwarf bees (Corlett, 2001). On peninsular Malaysia and the southern part of Thailand, the red honey bee *A. koschevnikovi* is currently quite rare (Otis, 1996). The likelihood or possibility of a given species going extinct entirely is unknown, but the threat exists and the ramifications of such an event could be grave.

In order to determine what is known about bee decline on a global basis, what is unknown, and how we could move ahead to a world where bee diversity is managed and maintained sustainably for future generations, our goal in this work is to synthesise information.

Principal Menace to Bee population

Denuding of trees

The gloomy fact of devastation of forest in Southeast Asia is described by Sodhi et al. (2004). In the next hundred years, this region, which now has the fastest rate of tropical deforestation in the whole world, is expected to lose 42 percent of its biodiversity and 75 percent

of its original forest cover. According to Brown et al. (2001), locations with more forest cover typically have higher *Melipona spp.* richness. Given the relative simplicity of their collection and identification, his findings suggest that *Melipona spp.* could serve as helpful markers of the effects of deforestation. The same result can be drawn from *M. melanoventer* and *M. ruventris brachychaeta* whose occurrence implies their greater sensitivity to deforestation. In disturbed and largely pristine sites in Singapore and Jahor in peninsular Malaysia, Liow et al. (2001) utilised honey baits to trap bees along selected sites. It is clear from this that honey bees are not favoured in oil-palm fields. This makes sense given that nesting sites are uncommon within the palm plantations and that nectar is exclusively produced by ground flora. There are no hollows available for cavity nesting bees, and *A. dorsata* cannot build a nest on the palm fronds due to their dense leaves. Dwarf bees could be able to build nests in palm leaves.

Honey harvesting

Honey bee hunting has been going on for more than 40,000 years, and it's still very common in the area (Crane, 1999). *A. florea* or *A. andreniformis* colonies can be taken by simply shaking the bees off, cutting the branch carrying the colony, and taking the honeycomb away. If there is plenty of food available, we assume that the colony recovers from the theft of its comb the majority of the time. If the hunt is conducted during the day, some destroyed colonies might be able to reassemble. Nevertheless, the hunting is frequently done at night. The hunter strikes his torch against the branch holding up the colony, throwing smoke billowing. The perplexed bees wander around on the forest floor, frequently with burned wings, as they follow the sparks (Tsing, 2003; Oldroyd and Wongsiri, 2006). These harvests must result in the loss of numerous queens, and their colonies go off along with them. Many hunters prefer night time hunting because it results in fewer bee stings. Most colonies are exterminated by this kind of hunting. For fact, BPO saw a tree being cut down in the Nilgiri Hills of Tamil Nadu, India, where hunters killed more than 100 colonies in a given night. Recently, there have even been reports of hunters killing bees with chemicals before collecting honey. It's likely that hunting demand is rising in many places. The motivation to produce a high-value, easily-transported good like honey rises when a trading goods economy is transformed into a cash-based economy (Nath *et al.,* 1994; Tsing, 2003; Nath and Sharma, 2007). The need for wild honey, which some people believe to be more natural, pesticide-free, healing, and tastier than honey made from domestic colonies, may rise as urban and rural areas become more prosperous.

Disappearing Colony

Bees that build their nests in cavities need those spaces. We believe it is likely that spaces are rarely a limited supply because *A. cerana* can nest in man-made structures or in the crevices

of coconut palms (*Cocus nucifera*). However, Inoue et al. (1990) discovered that *A. cerana's* growth is constrained when it makes its nests in the tiny hollow of coconut palms, which may prevent them from producing reproductive swarms of a reasonable size. The destruction of *A. dorsata*, a large honey bee, nesting trees, is of more concern. Colonies of *A. dorsata* frequently migrate over great distances while returning year after year to the same breeding location (Paar *et al.,* 2004). Furthermore, *A. dorsata* often nests in huge groups, with as many as 100 colonies on a single tree (Oldroyd *et al.,* 2000; Paar *et al.*, 2004).

Pests and pathogens

Various fungal, viral, and bacterial illnesses as well as parasitic insect and mite infestations can harm honey bee colonies (Bailey and Ball, 1991; Oldroyd and Wongsiri, 2006). when wild populations are under stress due to environmental deterioration, pests and infections may start to have negative consequences. For instance, Allen et al. (1990) discovered an *A. laboriosa* population in Nepal that was badly infested with European foulbrood (*Mellisococcus pluton*), which they connected to the environmental stress caused by deforestation. The artificial migration of honey bee populations between countries, which may expose wild populations to novel parasites and pathogens to which they are not resistant, is perhaps more important than environmental stress. At some point, *A. mellifera* was introduced into the majority of Asian nations, probably definitely exposing wild Asian *Apis* to new infections. Thus, the *A. mellifera* colonies that well-intentioned but ineffective assistance organisations imported into Kathmandu may have been the source of the European Foulbrood identified by Allen et al. (1990). The so-called Thai Sac Brood virus, which kills early pupal stages and frequently proves fatal to colonies, has been severely infecting *A. cerana* populations since the 1980s (Nath *et al.,* 1994; Chinh, 1998; Abrol, 2000). This may result through human-caused translocations of temperate *A. cerana* strains into tropical regions or from the introduction of *A. mellifera*. *A. cerana* is also known to cause European foul brood (Bailey, 1974). The *A. cerana*, *A. koschevnikovi*, and *A. dorsata* are parasitized in Borneo by the Conopid fly (*Physocephala parralleliventris Krober*) (Diptera: Conopidae) (Tingek *et al.,* 2004). It captures flying bees during flight and lays an extremely small larva on the integument. The larva pierces the bee's cuticle and begins to eat the bee from within. If this fly spreads to populations that haven't already been exposed to it, it could have disastrous effects. The little hive beetle *Aethina tumida* is a new danger to Asian species. it can cause major harm, particularly in warm, moist climates. The pest typically subsists properly on honey bee colony material that falls to the ground. Most of the time, the bees keep the adult beetles into niches (Ellis and Hepburn, 2006). The host colony's defences are occasionally

overcome by the beetles, though. The colony will either flee or be wiped out within a day or so after the larvae invade the brood comb.

Global climate warming and fires in forests

According to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change (2007), rainfall in some parts of south east Asia has decreased as a result of the Earth's average temperature rising by 0.74°C over the past 100 years, which is thought to be the result of a 70% increase in greenhouse gas emissions. Global temperatures will likely rise by an additional 0.4°C during the next two decades as a result of anticipated increases in greenhouse gas emissions. According to Duncan et al. (2003), the peak years for wildfires in Southeast Asia correlate with severe ENSO-induced droughts, which are predicted to happen more frequently with climate change. The structure of plant communities is changing throughout the Asian region as a result of drought, extreme wildfire events, and human influences, such as purposeful fire setting related to jhuming agriculture (Brown, 1998; Nath and Sharma, 2007). In some aspects, oligolectic, migratory species like Asian honey bees will be better equipped to adjust to environmental issues and variations in habitat types than most other insects. *A. andreniformis* (Wongsiri *et al.,* 1997) and *A. koschevnikovi* (Otis, 1996) are two species that are known to be obligate forest dwellers, and as the rainforest retreats, their ranges will become more limited. The actual reason for the fall of *A. koschevnikovi* in Malaysia and the rare of *A. andreniformis* in much of Thailand, however, is unknown because both of these species live in disturbed regions, including urban areas in Borneo.

Use of toxic chemicals

Individual foragers and entire colonies can kill from exposure to the majority of pesticides (Desneux *et al.,* 2007). Longan (*Dimocarpus longan*), litchi (*Litchi chinensis*), and citrus are three commercial fruit crops that yield significant amounts of honey and are therefore very enticing to bees (Crane *et al.,* 1984). Dwarf bees can nest in other orchard trees including the mangostane (*Garcinia mangostana*) and the rambutan (*Nephphelium lappaceum*) (Oldroyd and Wongsiri, 2006). Insecticides are frequently sprayed on these orchards, killing any colonies that are nested in the tree canopy (personal observations). Colonies that nest outside the crop but forage inside it may be harmed by spraying during flowering. Some nations have low regulations for pesticide use, which increases the risk of bee exposure to pesticides, for instance through stream contamination.

Light poles

Foragers are drawn to the lights attracted by the presence of open nesting species like *A. dorsata* and *A. andreniformis* that nest close to sources of light at night (personal observations).

In this process, a lot of bees are destroyed. If *A. dorsata* queens are drawn to lights during mating flights, then queens may also get lost in this manner.

Competition of introduced species

There have been some worries expressed occasionally over the potential for invasive *A. mellifera* to outcompete and displace native honey bees in Asia (Verma, 1991). *A. mellifera* wild populations are unknown in Asia. First, European honey bees struggle to regulate their rates of brood production in tropical environments with little fluctuation in day duration, and as a result, they struggle to reach swarming strength (Rinderer, 1988). Second, the parasitic mite *Tropilaelaps clareae*, which is also found in areas where *A. dorsata* is endemic, is likely to infest and eradicate any invasive *A. mellifera* colonies. Feral colonies are likely to get killed from Varroa destructor even in the absence of *T. clareae*. According to Anderson and Trueman (2000) and Solignac et al. (2005), host changes from Varroa destructor to *A. mellifera* are uncommon, hence native Varroa are often unlikely to infest *A. mellifera* colonies introduced to Asia. However, *V. destructor* is already present in the majority of *A. mellifera* populations around the world, including introduced Asian populations. Therefore, it is improbable that a domesticated population would become wild after becoming infected with Varroa (Anderson, 1994; Anderson and Sukarsih, 1996; Oldroyd and Wongsiri, 2006).

Despite the aforementioned, it is evident that *A. mellifera* beekeeping has largely overtaken *A. cerana* beekeeping in India, Japan, Pakistan, China, and Thailand, hence decreasing *A. cerana* population sizes in these countries. Although at least in Japan, the periods of mating flights do not coincide, there is some indication that very high densities of *A. mellifera* drones may interfere with *A. cerana* matings (Ruttner and Maul, 1983).

Human interference

A result of the current phase of global warming's rising sea levels, hundreds of islands, some huge and others little, were formed, dividing even before populations of honey bees into isolated populations (Smith *et al.,* 2000; Smith, 2002; Oldroyd and Wongsiri, 2006). This isolation, especially in *A. cerana* and its allied species, has contributed to the wide diversity of honey bee phenotypic traits we observe today (Hepburn *et al.,* 2001; Radloff *et al.,* 2005). The homogenization of the gene pool caused by anthropogenic honey bee migration between geographic regions may reduce biodiversity.

Honey hunting tourism

Although tourism is occasionally seen as a cause for conservation (Wynberg, 2002), it is more frequently seen as a negative force (Noss *et al.,* 1996; Pickering and Hill, 2007), particularly when it involves hunting (Anon, 1991). A Google search for "Honey hunting tour"

turns up dozens of businesses that provide guided tours of honey hunting locations, including operations in Nepal, Thailand, Bangladesh, Tibet, and Bhutan. These behaviours are more likely to lead to more colony deaths and to hunting during inappropriate seasons, when colonies are under stress and are less likely to sustain.

Ideas to save bee diversity: Operation honey bee

It is apparent that forest clearing adds to the reduction of honey bees, and the reason for this can only add to the ensemble of other plants and animals that are also suffering from this problem. However, in order for conservation policies to be effective, they must be grounded in both excellent science and matter of fact. For this reason, we should also concentrate on problems that can be solved in the short term.There is no doubt that action is required if we are to stop and reverse the current reductions in bee populations and protect their future diversity. Minimizing heavy agricultural growth and preserving natural habitats within the agricultural panorama should help to promote bee variety, while considerable study has to be done to evaluate the efficacy of such programmes.

Prioritizing the education and assistance of scientists in bee alpha taxonomy is a second crucial step. It is well known that there are fewer taxonomists in general (Batley and Hogendoorn, 2009; Eardley *et al.,* 2009; Patiny *et al.,* 2009). Although DNA barcoding may help us assess and comprehend bee species richness, it is neither a universal fix or a substitute for conventional taxonomy (Zayed, 2009).

Thirdly, studies on basic autecology (Murray *et al.,* 2009) and pollination that are not restricted to very well or model crops, blooming plant species, or bee taxa are urgently needed. To prioritise conservation based on the provision of ecosystem services, these studies will be essential. Additionally, they will offer the information required to integrate with climate change models in order to ascertain the effects of this unavoidable alteration to our planet's systems on the diversity and abundance of pollinators.

Fourthly, and illustrated by recent dramatic declines in American honey bee populations (Cox-Foster *et al.,* 2007), we need to understand how both native and invasive parasites and pathogens impact individual bees, and how this impact ramifies into population-level effects international to local scales.

In *A. dorsata* and *A. laboriosa* harvesting is frequently a damaging procedure, although this should not be. Bee hunters frequently also practise conservation, making them open to suggestions that could aid in bee conservation. They typically speak out strongly in favour of preserving forests (Nualsanong, 2000) in Kalamantan and some other Indonesian provinces, as well as in Vietnam, Cambodia, and Cambodia, efforts are being undertaken to promote the non-

destructive collection of honey from *A. dorsata* colonies (Tan and Ha, 2002; Waring and Jump, 2004). In order to capture bees during the day rather than at night, this includes utilizing bee smokers and protective clothes to protect hunters from stings. Furthermore, to draw migratory *A. dorsata* swarms, bee hunters can build "contilever" in the forest.

Conclusion:

We make a strong case that the neglected conservation of wild honey bees should receive more attention than managed honey bee management. Our study has indicated that overhunting, habitat degradation, and maybe climate change collectively pose a serious threat to some Asian honey bee species. Due to significant habitat loss, wild *A. mellifera* and other bee populations are both threatened and extinct in their natural habitats. Species like *A. andreniformis* and *A. koschevnikovi*, which appear to be restricted to densely forested environments, at least in mainland South East Asia, are of special concern. Overhunting, removing land for agriculture, and exotic diseases could pose threats to *Apis laboriosa*, a resident of hilly areas. Another component, human-mediated hybridization linked to manage colonies, poses a threat to *A. mellifera* wild populations. We accentuate the critical need of conducting research on the population vital statistics of wild honey bees. Wherever it is possible legally, we urge a ban on the destructive harvesting of large bee nests. Although there are many studies being done right now, it is up to the bee research community to convince governments and other supporters of study and conservation that bees are necessary for a healthy earth and a healthy human population. If we don't make this swiftly and strongly, we're only responsible for ourselves.

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IMPACT OF UNDERLYING STRESSORS ON BEES

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Abstract:

Bees are excellent friend of nature as well as of human beings because bees are not only providing pollination services to wild flora but also playing pivotal role in pollination of domesticated crops which is increasing chances of reproduction and helps in maintaining floral diversity as well as crop productivity. But various biotic and abiotic stressors are acting on these tiny creatures and are associated with the decline of their population day by day. Various factors like industrialization, urbanization and conversion of wild habitat into agricultural lands led to the destruction of their natural habitat. Similarly, Intensive agricultural practices like, monoculture affecting their nutritional quality and use of pesticides disrupting their cognitive memory and causing reproductive impairment. Changing climatic condition is also one of the major stressors which is affecting bees both phenologically and spatially by shifting them towards more elevated regions and by disrupting synchrony between bee emergence with flower blooming. other factors like introduction of alien species intentionally or unintentionally increased the competition for limited resources, domestication increased the pathogen load and electromagnetic rays emitted from cellphones hamper their navigational skills and negatively affect their foraging behavior. All these biotic and abiotic stressors individually or in interaction not only affecting bees individually but showing some additive effect on colony level. So it has become important to come out with necessary steps to support their population before we lose them completely.

Key word: Bees, habitat destruction, monoculture, climate change, competition, decline

Introduction:

Pollination is the transfer of pollen from an anther of a plant to the stigma of a plant, which helps in pollination and seed setting. Agent which helps in transfer of pollen from male anther to female stigma are known as pollinators and they can be biotic or abiotic. Pollination which depends on non-living agents like wind and water is classified under abiotic pollination. Pollination which depends on living agents to transfer the pollen from one flower to another like animals, birds and insects is termed as biotic pollination. About 80% of angiosperms rely on biotic pollination where between 100,000 and 200,000 species of animal act as pollinators of the world's 250,000 species of flowering plant around 1,500 species of birds and bats visit flowers and contribute in pollination and majority of these pollinators belongs to insect group (Abrol., 2012). About 80% of all plant pollination is done by animals. The remaining 20% belongs to abiotic pollinated species where, 98% pollination is done by wind and 2% by water (USDA).

Insects as pollinator

When Pollination is carried by insects it is termed as Entomophily**.** The major insects which contribute as a pollinators are bees, flies, moths, butterfly, beetles and wasp where bees individually contribute 73% of pollination followed by flies 19%, wasp 5%, beetles 5% and 4% by butterflies and moth.

Plant- pollinator interaction

Nearly 99% of wild flowering plants need pollinators like bees to transfer pollen for successful sexual reproduction. In turn, Plants provide food (Nectar + Pollen) form habitats and provide a wide range of other resources to pollinators. Pollinators consequently play a key role in regulating ecosystem services by supporting food production, habitats and natural resources (promotepollinators.org)

Importance of pollinators

The annual economic value of the crops pollinated by biotic agents worldwide is estimated to be between $235 billion and $577 billion. Worldwide, an estimated 20,000 species of wild and managed bee pollinate flowers, aiding plant reproduction (Vanbergen., 2021). Biotic pollination is necessary for production of approximately 85% of flowering plant species (Klein *et al*., 2007).

Without pollinators, what would happen?

- The spread of pollen would not occur which will decrease the chance of reproduction occurring in many plants.
- Crops such as vegetables, fruits and nuts would reduce as these horticultural crops are majorly dependent on bees for pollination.

- Diversity would be limited.
- Food would become costlier.
- It would result in poor health; as highly nutritive food would become harder to obtain.
- With poor nutrition, comes a higher risk of infectious diseases.

Major stressors

Factors contributing in decline of bees include mainly habitat destruction, monoculture, use of pesticides, diseases/parasitism, climate change, domestication and electromagnetic rays emitted by mobile phones. The more destructive forms of human disturbances are land use changes such as fragmentation and conversion to secondary forest habitat to agricultural farmland.

Habitat destruction and fragmentation

Habitat destruction is the process by which natural habitat of native species is destructed by anthropogenic activities like harvesting of natural resources, industrialization and urbanization which make it incapable to support its native population whereas, habitat fragmentation is the process where main habitat gets divided into smaller parts which causes biodiversity loss. Disrupted landscape lacks the diverse resources which is essential for adequate bee nutrition. proper nutrition is required by pollinators for provisioning of the offspring's and directly contribute in the success of colony. Habitat destruction is the leading cause of biodiversity loss and pressure from the agriculture is the principle human cause of habitat destruction. In agriculture, a major impact of habitat manipulation on honey bees is its, key influence on overall reduction of available floral nutrient resources. When seasonal assessment regarding disruption to pollen availability in farmland system consisting of rapeseed and sunflower was studied by (Requier *et al*., 2016) they resulted in a decline in pollen harvest in May & June between the mass flowering of rapeseed (*Brassica napus*) and sunflower, (*Helianthus annuus*) blossom and the decline was negatively related to colony brood size and subsequent colony adult population, as well as decline in pollen harvest resulted in increase of varroa mite load in the colony. A severe pollen decline between mass flowering of rapeseed and sunflower reduced seasonal and overwinter survival of bees by 50%. Therefore, the effect of food resource availability and intensive agriculture is directly related to outcomes in bee health, fitness, and overall survival (Requier *et al.*, 2016).

Monoculture

Monoculture in terms of bee ecosystem it is defined as short massive gluts of the same species flower which is dominating an agricultural landscape. Agro-ecosystems have become increasingly intensified in recent years with large monocultures, exposure to monoculture negatively impacted bees by depriving their approach to a wide range of floral resources that are necessary to fulfill their nutritional requirements. Pollinators require a variety of nutrients in their diets like carbohydrates (in the form of sugar) from nectar or honey, Amino acids (in the form of protein) from pollen, lipids, vitamins, minerals, and water in the correct ratios for proper growth and development. Monoculture can provide bees with only part their necessary nutrition requirement. Since the pollen and nectar of many plant species are not rich in the complete spectrum of above mentioned elements, large-scale monoculture exposure may prevent honey bees from obtaining all of these elements. Therefore, it is advisable to incorporate a wide variety of flowering plant species into a cropping system that are placed within the realistic foraging distance of honey bee hives. When (Knapp *et al*., 2019) studied the mutualistic relationship *Bombus terrestris* with European farmland, mass flowering crop corguette flowers to see how effective *B. terrestris* is at pollinating courgette and in return how courgette may affect *B. terrestris* colony dynamics resulted that courgette flowers provided vast quantities of nectar to ensure a high visitation rate, which combined with abundant pollen grains, enables bee to have a high pollination potential. While *B. terrestris* showed a strong fidelity to courgette flowers for nectar than the pollen. In the morning, *B. terrestris* was reported to visit significantly more abundant in the crop than in the margin because of flowers of courgette opens in morning hour. However, in the afternoon *B. terrestris* were significantly more abundant in the margin than in the crop when courgette flowers are closed (Figure 1). and no longer providing nectar but when the incoming pollens were collected and assessed from the returning foragers, they found that incoming workers brought pollen from hedgegrow flowers and none of the pollen belonged to courgette flower (Table 1.), which suggest that monoculture crops provide one part of the nutrition to pollinator so mixed cropping is necessary to provide pollinators a proper amount of nutrition.

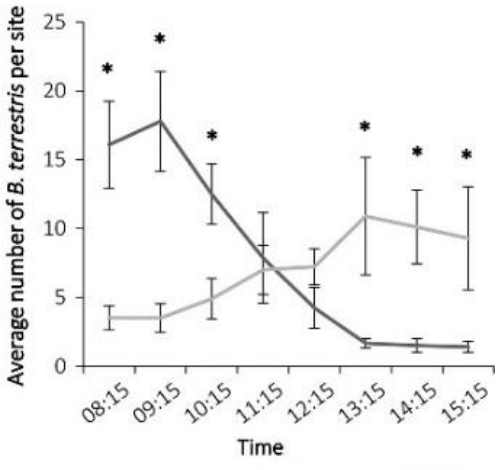

Figure 1. Visitation of *B. terrestris* on corguette flowers (Knapp *et al*., 2019)

Table 1: List of pollens collected by *B. terrestris* returning foragers (Knapp *et al*., 2019)

Species name	Common name	Number of pollen loads
Brassica spp.	Brassica spp.	15
Rubus fruticosus	Bramble	11
Papaver rhoeas	Common poppy	7
Veronica filiformis	Speedwell	4
Helianthemum chamaecistus	Common rockrose	3
Linaria vulgaris	Common toadflax	3
Verbascum thapsus	Great mullein	3
Echium vulgare	Viper's bugloss	2
Hedera helix	Common ivy	2
Ribes sanguineum	Flowering currant	2
Calystegia sepium	Hedge bindweed	1
Centaurea cyanus	Cornflower	1
Centranthus ruber	Red valerian	1
Heracleum sphondylium	Hogweed	1

Pathogen/Parasites

Bees are naturally affected by a different parasite, pathogens, and insect pests, which is considered as one of the major reason in their declining. Among parasites varroa mite is one of the important ectoparasites causing harm to individual honey bees and as well to entire colony by transmitting viruses such as DWV (Deformed wing virus). Whereas in case of bacterial diseases globally American foulbrood (AFB) (*Paenibacillus* larvae) is considered as the most devastating disease of honey bee brood, which is having ability to spread quickly. In case of fungal diseases *Nosema* species which is an obligate intracellular parasite is reported to affect both individual honey bee and entire colony of *A*pis *ceranae* and *A. mellifera* and have ability to transmit from one generation to other. The study conducted by (Chu *et al*., 2016) on *Nosema bombi* infection intensity among different castes of *Bombus auricomus* resulted that *N. bombi* infection intensity was caste-dependent, males had greater *N. bombi* infection levels than gyne, queen and workers. The queen had relatively low *N. bombi* load, similar to that of the gynes and it is reported that *Nosema* infection was highest in *bombus* male and males has ability to transmit *Nosema* during

mating and male susceptibility to *Nosema bombi* reduced available male's population to fertilize queen this may impact colony fitness of bumble bee colony.

Use of Pesticides:

Increased pesticide application over the last decade has contributed to recent large-scale bee declines. Their acute and sub-lethal toxicity is harmful to apis and non-apis bees Rinkevich *et al*. (2015). Bee encounter multiple pesticides on crops which may affect individual behavior or have ability to affect whole colony function. Therefore, pesticides are considered as a major bee stressor, pesticide either kill pollinators or show some sub-lethal effect like impairment of learning, impairment of reproduction or by affecting navigation. (Gill *et al*., 2012) when studied combined pesticide (Imidacloprid, Cyhalothrin and mixure of both insecticide) exposure on colony level traits in bees (Overall worker loss). They resulted that overall worker loss was seen in case of mixture of pesticide followed by lambda cyhalothrin and Imidacloprid and least was observed in case of control as shown in figure 2. As workers and their survival is vital to colony success because workers provide labor which perform various necessary activities like brood care, foraging, cleaning *etc*. Usage of different pesticides are resulting in death of workers which ultimately affecting the colony success rate.

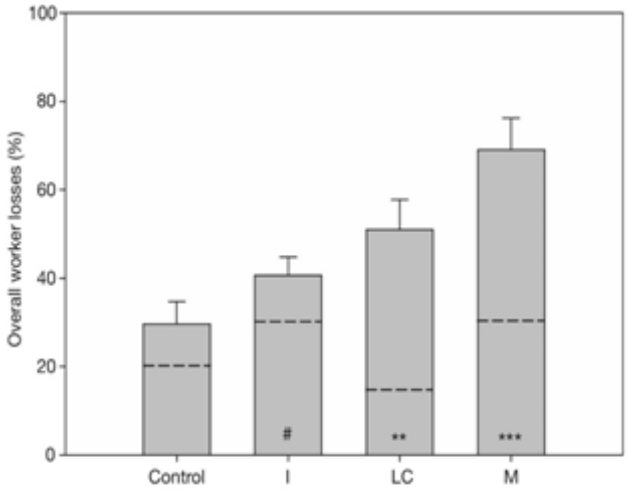

Figure 2: Worker loss is more in mixture of pesticide followed by lambda- cyhalothrin and imidacloprid (Gill *et al*. 2012)

Pesticides are used in combination to avoid resistance in target pest but there growing concern that their widespread use contributes to the decline of pollinator population by causing sub lethal effect like impaired foraging ability, reduction in reproductive output and decrease navigation performance. In pesticides group neonicotinoid group were reported to be more harmful to bees because it gets bind and activate nicotinic acetylcholine receptors (nAChr) which

cause neuronal inactivation in the honeybee brain which is strongly associated with cognition and learning ability of bees. Stanley *et al*. (2015) assessed the impairment of bumble bee learning and memory when exposed to neonicotinoid pesticide with different concentration *i.e.* 2.4ppb, 10ppb, and 250 ppb**.** They found that bees displayed higher learning level in control and 2.4ppb than those from 10ppb and 250ppb (Figure 3.). Similarly, when (Williamson *et al*., 2013) studied the effect of prolonged exposure to imidacloprid and coumaphos and their combination resulted that olfactory learning and memory formation in honeybees and reversal rate highly decreased in 24 hrs (Figure 4).

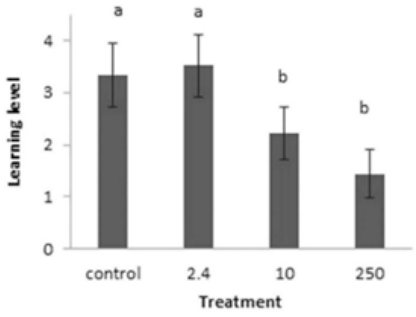

Figure 3: Effect of neonicotinoid on learning level of bee at different concentration (Stanley *et al*., 2015)

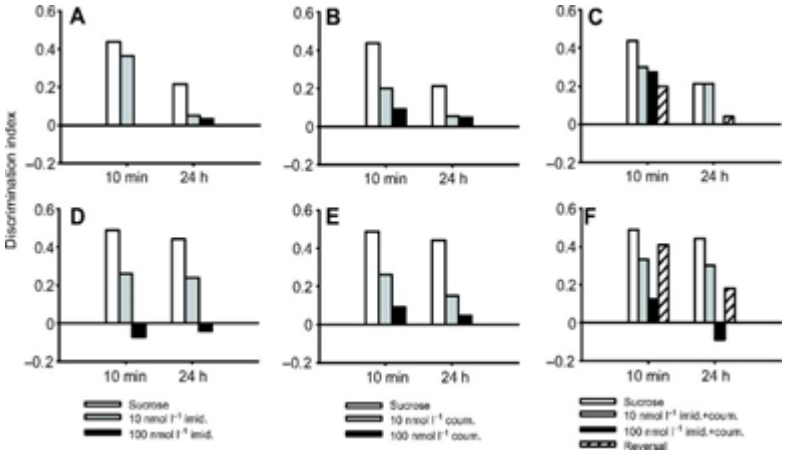

Figure 4: Effect of imidacloprid, coumaphos and their combination on learning level of bee (Williamson *et al.* 2013)

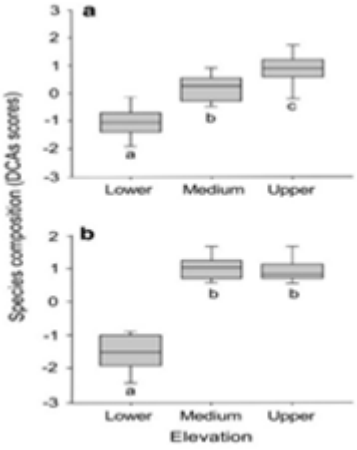

Figure 5: Species composition a-1988-1989, b- 2007-2009 (Ploquin *et al*., 2013)

Climate Change

Changing climate scenario has resulted in the "pollinator crisis" and this crisis is ultimately affecting the production of crops, due to a decrease in pollination processes. For

example, a tree may flower sooner than usual, while the pollinator may reproduce later in the year and therefore the two species no longer coincide in time (Settele *et al*., 2016). Climate change is affecting pollinators both phenologically and spatially. Phenologically means species that normally occur in similar seasons or time cycles, now have different responses to environmental changes and therefore no longer interact whereas, Spatially, means when two species that would normally share the same distribution now respond differently to climate change and are shifting to different regions. Global warming combined with changing weather condition affected the symbiotic interactions of bees and the flowering plants they pollinate resulted into mismatches between the timing of adult bee emergence and the onset of flowering is one concern. Increased temperatures changing distributions of flowering plants and curtailing the natural ranges of bees by shifting them further north towards increased elevations is another concern reported by (Gerard *et al*., 2020). This widespread alteration which includes extinction of species from native area and colonization of new area towards high latitude, reflects the ecological impact of global environment change on biodiversity. (Ploquin *et al*., 2013) studied the bumble bee community homogenization in montane areas of northern Spain in two different time period first is from 1988-1989 and second study was conducted in 2007-2009 and found difference between assemblages of bee species. Bumble bee species which earlier located at lower elevation in 1988-1989 was shifted to upper elevation in 2007-2009 study (Figure 5). They also reported higher colonization in upper elevation than the lower elevation and extinction of species from lower elevation than the middle and upper hill elevation.

Alien species

Alien species is species that is introduced intentionally or accidently outside their natural distributional range. Introduction of alien species affect the native species as it competes with native species for limited resources. (Hudewenz and Klein, 2015), conducted an experiment where he studied competition between red mason bee and honeybees (*Apis mellifera*) in cage experiment and reported that red mason bees enabled to compete with honey bees for floral resources They resulted that when red mason bees were caged with different densities of honey bee (*Apis mellifera*) @ 100 and 300 honeybees the niche breadth of honey bees significantly increased with increasing number of honeybees and niche breadth of mason bees started decreasing (Figure 6) as well as this competition affected the brood cell production of red mason bees (Figure 7). They concluded that wild bees suffer from competition with honey bees and this competition is shifting them to list of endangered species.

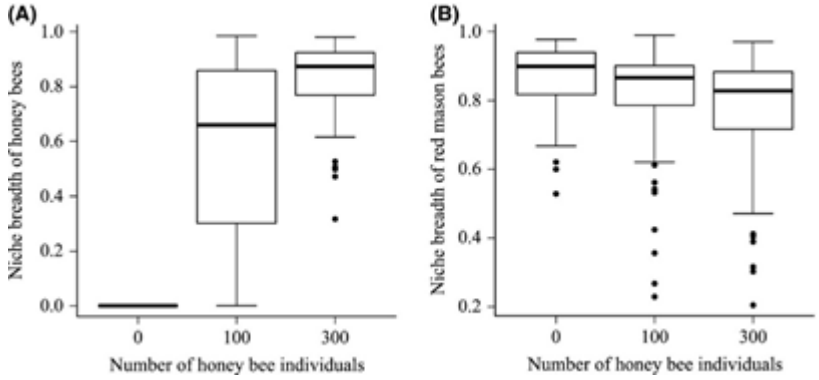

Figure 6: Niche breadth of honey bee and red mason bee (Hudewenz and Klein., 2015)

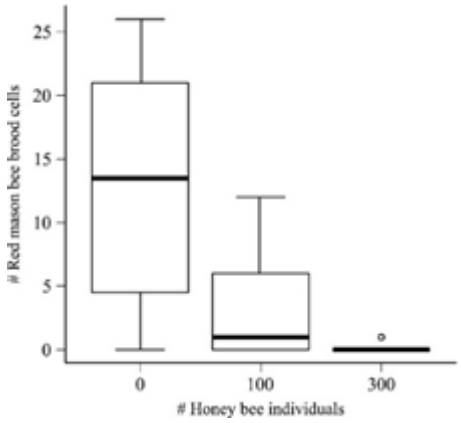

Figure 7: Effect on red mason bee brood cell (Hudewenz and Klein, 2015)

Domestication

The European honeybee *Apis mellifera* is an important domesticated bee which is used worldwide for commercial pollination of high value crops like fruits and for honey production. *A. mellifera* belongs to European country and in East Asia, it has been intensively managed by beekeepers. Although wild pollinators play an important role in the pollination of wild flowering and crop plants but our current horticultural systems also rely on managed honeybees. Although the number of honeybee hives has increased by 45% on a global scale, there have been major regional declines (e.g., a reduction of 59% in the United States from 1947 to 2005), and globally beekeepers have been reporting high overwintering colony mortalities, which threaten the sustainability of bee husbandry. Although many factors, ranging from agricultural intensification to the use of pesticides, have been implicated in pollinator declines but in domesticated honeybees, RNA viral infections transmitted by the ectoparasitic mite varroa destructor have become the potential contributors to global honeybee colony mortality. (Wilfert *et al*., 2016) reported the, deformed wing virus (DWV) as the key pathogen associated with overwinter mortality of varroa-infested colonies. The varroa mite expanded from its native host, the Asian honeybee *A. cerana*, to the domesticated European honeybee, and now has a global distribution.

Electromagnetic waves from mobile phones

The sudden growth in the telecommunication sector leads to the manifold increase of mobile phone and exponential installation of cell towers. In 2021, the number of mobile devices operating worldwide stood at almost 15 billion, up from just over 14 billion in the previous year. The number of mobile devices is expected to reach 18.22 billion by 2025, an increase of 4.2 billion devices compared to 2020 levels (Statista, 2021) and radiations generated by mobile phones are disturbing the life cycle of honeybees and affecting their reproduction system and honey producing (Halabi *et al*., 2013). Cell phone towers near beehives interfere with honeybee navigation, it was found that when a mobile phone was kept near a beehive it resulted in collapse of the colony in 5 to 10 days, with the worker bees failing to return home, leaving the hives with just queens, eggs and hive-bound immature bees (Sainudeen., 2011).

Different approaches to support pollinators

- Implementation of pollinator gardens, and beehives in urban setting
- Educate/ aware public on the vitality of bees
- Diversified cropping
- Promote integrated pest management
- Phase out existing harmful pesticides and avoid registration of those that are harmful to pollinators
- Develop mechanism to limit the spread of parasites and pathogens to managed and wild pollinators
- Prevent and minimize the risk of introducing invasive alien species harmful to pollinators and to plant resources
- Minimize deforestation and other threats that impact negatively on wild pollinators and on traditional bee keeping
- Preserve or restore pollinator and habitat distributed in natural areas including forests, grasslands and agricultural lands, urban areas and natural corridors
- Bee hotels, also called nests or houses, are a great way to attract pollinators to your family’s flower or vegetable garden.
- Bee hotels are places for solitary bees to make their nests. These bees live alone, not in hives.

Conclusion:

Nearly 90% of the world wild flowering species entirely or at least in part depends on biotic pollination and in biotic pollination bees, are one of most important group which plays pivotal role in regulating ecosystem service by participating in vital activities of nature,

agriculture, and human well-being but these pollinators are at high risk due to increasing biotic, abiotic and interaction between stressors. Different stressors like land fragmentation, climate change, competition for limited resources, different pathogens and pest attack, excessive pesticide usage, domestication and electromagnetic waves emitted from increasing cell phone usage has dragged bees to the verge of declining. so it has become extremely necessary to initiate considerable efforts to aid pollinator conservation for better and healthy future.

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SURVEY REPORT OF MACROINVERTEBRATES AS A BIOINDICATORS OF STATUS OF THE RIVER WARDHA FLOWING FROM PULGAON

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Abstract:

The rivers of India play an important role in the lives of the Indian people. The river system provides irrigation, potable water, cheap transportation, electricity, as well as provide livelihood for a large number of people all over the country. This easily explains why nearly all the major cities of India are located by the banks of river. The rivers also have an important role in Hindu mythology and are considered holy by all Hindus in the country. Many people work on water quality by applying physico-chemical parameters, but for the first time quality of water was monitored by studying macroinvertebrate fauna. Macroinvertebrates means the animals without back bone, can be seen with necked eyes and whose home is streams, rivers, wetlands and lakes. The study was carried out from January 2008 to December 2008. The present paper embodies the biodiversity of macroinvertebrates from rivers Tapi, Sipna, Khandu, Khapra, Gadga, Dolar in Satpuda. They form the bio-indicators of lentic water ecosystems.

Keywords: Macroinvertebrate, Water quality, Satpuda.

Introduction:

Fresh water is one of the sources of scarce natural resource and its conservation is assuming greater and greater significance because day by day it is going on polluting. So, up till now people are interested in water quality because they want to know if their water is safe drinking and can support healthy population of fish and other animals, that's why they perform physico-chemical analysis of water and test the water quality. But here is one another way of finding out quality of water that is the study of macroinvertebrates, (the water quality indicators) from the particular water body.

Each aquatic species from tiny bacterium to whale is unique. It is not size but the genetic composition of plants and animals that makes all the life forms special. Each species has its own inheritant genetic library that codes its ability to survive in the changing environment; the best example of this is macroinvertebrates. That's why we are dealing with Macroinvertebrates.

Macroinvertebrates are involved in the mineralization and recycling of organic matter. Many larval forms of the insects, crustaceans and mollusks serve as food source for birds and fishes.

Macroinvertebrates are very important organisms in the aquatic environment and occupy almost all conceivable habitats having wide range; they are important components of food web in an ecosystem. There interactions with other biotic communities and with abiotic factors within their microhabitat, makes them interesting and valuable indicators of water quality.

Macroinvertebrates are useful indicators of stream quality for a number of reasons:

- They are affected by the physical, chemical and biological conditions of stream.
- They are relatively sedentary and cannot easily escape pollution, so their populations reflect the overall stream.
- Some are very intolerant of pollution.
- They may show impacts from habitat loss.
- They are the critical part of aquatic food web.
- Many macros spend over a year in the water.
- They are easy to sample and can be monitored any time of the year.
- Macroinvertebrates communities vary across the State and different water bodies.
- Therefore, water body health estimate can be measured by the richness of macroinvertebrates community.

Macroinvertebrates are very important organisms in the aquatic environment and occupy almost all conceivable habitat having wide range of habitats. They are important components of food web in an ecosystem. Their interaction with other biotic communities and with abiotic factors within their microhabitat makes them interesting and valuable indicators of water quality.

They form exclusive stable diet of higher animals like amphibians, reptiles, birds and mammals.

Materials and Methods:

Macroinvertebrate collection:

The macroinvertebrates were collected in the early hours of morning between 7.00 a.m. to 9.30 a.m. as most of the animals found in mud, cling on stones, rocks, rootlets, twigs, and leaves of aquatic plants.

The macroinvertebrates were collected from all water depths from still and shallow slowly flowing waters of all the five sites. They were collected from water's surface, from within water, from the sediment or bottom or from submerged gravels, rocks, stones, logs, rootlets,

twigs, aquatic plants, particularly sedges and rushes and leaf litter because each type of habitat provides a surface or space on, within or near which macroinvertebrates can live.

Various equipments were used for sampling, sorting and identification during the present investigation were sampling nets, spoons, pipettes, forceps, droppers, 2 liters capacity glass bottles, small buckets, sorting trays, ice-block trays, magnifying glass, clipboards, pencils, markers, stickers, etc.

Result:

The present study is an extremely useful tool in the assessment of water quality using macroinvertebrates.

In the present study 4 species of Annelida, 74 species of Arthropoda, 15 species of Mollusca were recorded. As compare to midge flies, worms, leeches and pouch snails, some species of stone flies, water penny beetles, mayflies, alderflies, mussels, riffle beetles, damselflies, dragonflies, cryfish, amphipods, black flies, caddies flies, isopods, crane flies were shown more diversity.

Sensitive species:

Stone flies, water penny, Mayflies, Dobsonflies, Alderflies, Mussels,Riffle beetles.

Moderately Tolerant species:

Damselflies, Dragonflies, Crayfish, Amphipods, Black flies, Caddies flies, Isopods, Crane flies.

Pollution Tolerant species:

Midge flies, Worms, Leeches, Pouch snails.

Conclusion:

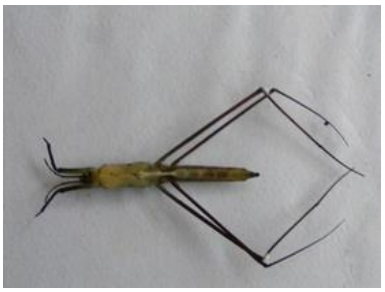
The pollution tolerant species were present only during monsoon and were scare while the sensitive species were abundant and found most of the times. This indicates thatWardha the rivers flows from Pulgaon has a good biological richness and not polluted yet.










IMPORTANCE OF SOIL AND ITS MICROBES IN NUTRIENT CYCLING

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Abstract:

Soil provides the nutrients which are important for growth of plant, animals, and billions of microorganisms. But, if soil becomes unstable, polluted and unhealthy, the life cycle stops. According to FAO the influence of fertility of soil are seen in most of the Sustainable Development Goals, as they have environmental, economic and social aspects. A fertile soil provides essential nutrients for healthy growth of plants. It also helps to produce healthy food with necessary nutrients needed for human health. Good management of soil fertility can help to lessen air, water and soil pollution. It supports various biotic communities and helps to increase vegetation. Micro-organisms transform organic matter into plant nutrients that are assimilated by plants. Microorganisms are responsible for cycling of C, N and other nutrients. They Enhance soil structure, relocate and decompose organic materials, maintain soil quality and health, increase soil aeration and penetrability and also is involved in disease transmission and control. The fertility of the soil is important for production in agricultural activity and therefore for security of food. Farmers can boost the fertility of soil and its health by improving soil nutrient management in terms of maximizing net returns, cutting down the soil nutrients depletion, and reducing the loss of nutrient or negative effects on the environment. The use of land for cultivation changes the existence of microbial populations, resulting in damages to the nutrient cycles in the soil (French *et al*., 2009). A healthy, fertile soil laid the groundwork for a strong and resilient food production system. Nowadays, one of the major tasks is to manage and maintain soil in a sustainable fashion. Every administration should promote agricultural practices which are sustainable. They must strive for improving technologies and management for fertility of soil and management of nutrient.

Introduction:

Let us begin with a statement from the revised world charter (FAO, 2015)

"Soil is fundamental to life on earth".

The term "soil", derived from L. solum, & has many definitions. Soil is defined as a mixture of mineral and organic particles of varying size and composition in regard to plant

growth. Soil is also unconsolidated material on the surface of the earth that has been put through environmental factors of parent material, climate, organisms, and landscape. Soil also provides physical support for both plants and animals including humans and the structures they build. Soil has numerous functions like it is a medium for plant growth, it filters and regulates the water supply by storing water after a precipitation event, habitat for organisms, recycle wastes and support for structures. Soils are an essential and non-renewable natural resource hosting goods and services vital to ecosystems and human life.

The importance of soil is increasing at an alarming rate due to the rapidly growing worldwide population as healthy soils will be less to meet the future food supply due to growing pressure on land for urban expansion, bio fuel production, and natural resource extraction. The soil develops by degeneration of rocks as well as minerals, through biotic actions of microbes sustained by them (Bahadur *et al.*, 2017). Soil represents an active ecological community, which makes it suitable to contemplate them in terms such as soil sustainability, wellbeing and vitality. Soils provide us with nearly 90% of all the food which we need for our consumption. We must manage soils in a sustainable way so that it will fulfill the needs of the future generation.

Soil quality can be defined as the fitness of a specific kind of soil, to function within the capacity of natural ecosystem boundaries, to support animal and plant productivity, enhance water and air quality and support human health and habitation. Agricultural sustainability depends to a large extent upon maintenance or enhancement of soil quality. Soil quality is conceptualized as the major linkage between the strategies of conservation management practices and achievement of major goals of sustainable agriculture (Andrews *et al* 2004). The quality & health of soil not only determine agricultural sustainability but also environmental quality & the plant, animal & human health.Thus the landcare & soil quality management assume great significance for ensuring agricultural sustainability which is inevitable to feed the burgeoning population.

According to FAO soil fertility is the capacity of a soil to fulfill growth of plant by providing vital nutrients and chemical, physical, and biological characteristics as a natural environment for growth of plant. Nutrients in plant include the macronutrients N, P, K, S, Ca and Mg. The micronutrients present are B, Cl, Cu, Fe, Mn, Mo and Zn. Soil is best medium for the growth and development of microorganisms.

The soils health as defined by Doran and Safley (1997) is “the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sus- tain biological productivity, promote the quality of air and water environments, and maintain plant, animal and human health.” Healthy soils are able to balance a range of functions to meet the

needs of both farmers and community. It functions to assist animal and plant life in soil, accumulate and recycle wastewater and nutrients, decompose SOM, inactivate toxic compounds, sup- press pathogens, protect soil and water quality and enhance catchment health. The soil health is the net result of undergoing conservation and degradation processes, depending highly on the biological component of the soil eco- system, and influences plant health, environmental health, and food safety and quality. The functions of healthy soil are to absorb nutrients as well as contaminants and various other substances by incorporating with clay particles and soil organic matter. The soil functions as a filter for eliminating unwanted solid and gaseous substances from air and water.

Microbes in soil and its importance:

Living organisms present in the soil are grouped into two categories as Soil flora and Soil fauna. Soil is not an inert static material but a medium pulsating with life. Soil is now believed to be dynamic or living system. Microorganisms comprise of about 0.5 percent of the total mass of the soil, yet they have a considerable influence on the properties of soil and its processes. Around 70 per cent of the total soil metabolism is due to the micro flora. These are the organisms less than 0.1 milli meter in diameter are extremely rich and varying. They include fungi, bacteria, algae, cyan bacteria, yeasts etc. Most of the microorganisms are able to deteriorate any naturally existing substances. They can alter organic matter into nutrients that are absorbed by plants. Microorganisms are responsible for cycling of C, N and other nutrients. They enhance soil structure, relocate and decompose organic materials, maintain soil quality and health, increase soil aeration and penetrability and also is involved in disease transmission and control. Soil microorganisms are essential for the growth of plants by providing nutrients to the plants through the process of mineralization of organic content.

Microorganism in soil is not only an essential part of soil, but also an active source of substances for transformation of soil. Microbes in soil help in the processes of nitrification (a process by which nitrogen compounds are converted to nitrite and nitrate) , ammoniafication (process of decomposition with the production of ammonia) and nitrogen fixation (process by which nitrogen from the atmosphere is taken and converted into nitrogen compounds), to breakdown the organic matter and produce nutrients. They also act as sources of carbon and nutrients, which are released after their death through mineralization by other microbes (Anderson and Domsch, 1980). Microorganisms are important for the production of humus.

Due to urbanization, flooding erosion, drought and salination, soils are losing their fertility much faster than they can be replaced by natural weathering processes. The constant increase in the yield of crops based on plant breeding technology is slowing down and cannot

keep pace with population growth. So awareness are shifting to the soil and understanding their structure and function which will give us methods and plans to manage agricultural land which will maintain yields of crop for farming. The soil microorganism including bacteria, fungi, and other organisms plays various roles in soils, yet we understand very little about them but we do know that organic matter in soils which are derived from microorganism are essential for growth and development of plants and animals. Microorganisms absorb and bind together organic matter and minerals to create the structure on which the fertility of soil depends upon. It's important to note that soil with various microbial community tend to have more large soil particles called macro aggregates. These are water-stable particles larger than two hundred fifty micrometer in diameter, which improves water and nutrients maintenance in the soil. Microorganisms in soil also play a role in the soil ecosystem, particularly in the transfer of nutrients between trophic levels and the dispersal of organic material in the soil.

The base for microbial activity and agriculture crop production are provided by soil and is very crucial to improve soil health for healthy growth of crops because microorganisms play an important role in building a complex link between plants and soil. Soil microbes are a active component of soil and performs many important functions in the soil system. Moreover, they enhanced the availability of nutrients to the plants. Microbes support biological nitrogen fixation of different biological transformations that support the accumulation and utilization of key nutrients, support root and shoot growth processes, disease control, and improve soil quality in crop cultivation. The microorganism in soil offer nutrient-dense nourishment and improves crop production and recycles soil. They play an important role in fertilizing the soil. Besides, they improve plant growth on various physiological parameters of plants by a number of mechanisms. The mechanism involved in growth promotion includes plant growth regulators, production of different metabolites, and conversion of atmospheric nitrogen into ammonia in direct and indirect ways. In addition, soil microbes offer resistance against diseases. Nutrient cycling is a dynamic and functional property of ecosystem. The life cycle on earth would be impossible without nutrient cycle as important nutrients would quickly be taken up by organisms and locked in a form that cannot be used by others. Soil is one part of the ecosystem and supports all terrestrial life-forms; therefore, protection of soil is of high priority, and a thorough understanding of soil enzyme activities is a critical factor in assuring that soil remains healthy. A better understanding of soil enzymes in maintaining the soil health will provide an opportunity for an integrated biological assessment of soils. The response of microbes to environmental stress is rapid compared to higher organisms, due to their high surface to volume ratio (Pankhurst and Hawke, 1995). There are three mechanisms which are used to describe how microbial

activity can boost plant growth. They are manipulating the hormonal signaling of plants, repelling or outcompeting pathogenic microbial strains and increasing the bioavailability of soil-borne nutrients. There is no doubt that agricultural production has been able to feed the world's expanding population so far, but this may not last long.

It is found out that soil microorganisms have the ability to bind metals from the aqueous solution (called biosorption), where an organism is able to isolate the toxic metals. Soil microorganisms drive the sulfur cycle because of which sulfur undergoes many changes in soil, including oxidation and reduction reactions, mineralization and immobilization reactions, and volatilization reactions. The soil microbial biomass is the key driving force behind all sulfur transformations.

Lastly, soil microbes help plants in nutrient uptake by conversion of unavailable nutrients into available form. Due to lack of knowledge regarding their importance, people think about the negative impact of microorganisms because in many cases microbes act as disease-causing agents.

Conclusion:

Soil microorganisms are a crucial element of soil ecosystems and play a necessary function in terrestrial ecosystem processes, especially the regulation of carbon and nutrient cycles. They depend upon carbon sources provided by means of plant litter and root exudates and they can be influenced through modifications in plant-derived organic matter. Microorganisms play an essential function in the weather, ecosystems, and plant health. They inhibit plant illnesses, improve crop performance, activate the germination capacity of seeds, and stimulate stress tolerance and general health. Soil organisms play an important role in nutrient cycling. However, the complexity of soil structure and fluctuations in environmental conditions and nutrient availability combine to make it difficult for soil microorganisms to achieve their metabolic potential.

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ECTOMYCORRHIZA: A BOON TO THE LIFE OF PLANTS AND *IN VITRO* SYNTHESIS OF ECTOMYCORRHIZAE BETWEEN *BOLETUS EDULIS* BULL. AND *ABIES PINDROW* ROYLE EX D. DON

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Abstract:

The purpose of study is to synthesize *in vitro* ectomycorrhizae between *Abies pindrow* and *Boletus edulis*. The synthesis was achieved between *A. pindrow* seedlings inoculated with pure culture of *B. edulis* which resulted in the formation of short, branched lateral roots. Synthesized mycorrhizae were dark brown in colour. The transverse sections of the synthesized roots revealed the presence of thick fungal mantle and well developed "Hartig net". Pure culture of *B. edulis* was also reisolated from both vermiculite peat moss mixture and synthesized ectomycorrhizae to confirm the *in vitro* mycorrhizal synthesis between two. The inoculated seedlings were healthy and showed more growth of the root and shoot system as compared to seedlings which were kept as control.

Keywords: *Abies pindrow*, *Boletus edulis*, ectomycorrhiza, *in vitro*.

Introduction:

The term "Mycorrhiza" was coined by Frank (1885) for highly evolved, mutualistic associations between soil fungi (Basidiomycetes, Ascomycetes and Zygomycetes) and plant roots (most vascular plants). About 90% of the world's present species of vascular plants belong to different families that are characteristically mycorrhizal (Molina *et al*., 1992). Basically mycorrhizae are of two types ectotrophic and endotrophic (Frank, 1885), later they were named as ectomycorrhiza and endomycorrhiza (Peyronel *et al*., 1969). The five more types have been added to them i.e. ectendomycorrhiza, orchidaceous, ericarious, arbutoid and monotropoid mycorrhiza (Harley and Smith, 1983).

Numerous fungi have been identified as forming ectomycorrhizae. Molina *et al*. (1992) reported 6000 species of fungi that form ectomycorrhizae. The fungi that form ectomycorrhizae primarily belong to Basidiomycotina and Ascomycotina, which include many of the common forest mushrooms, puffballs and truffles respectively. Well-known fungal genera that form ectomycorrhizae include *Amanita*, *Boletus*, *Hebeloma*, *Laccaria*, *Lactarius*, *Pisolithus*,

Rhizopogon, *Russula*, *Suillus* and *Tricholoma* (all Basidiomycotina), *Cenococcum* and *Tuber* (Ascomycotina) (Miller, 1982).

Colonisation of roots by particular ectomycorrhizal fungi, as the consequences of particular cultivation practices in forest nursery or achieved by artificial inoculation, both in the nursery (Trappe, 1977) or in field (Dunabeitia *et al*., 2004), may significantly promote survival, establishment and growth of young trees in newly established forest plantations (Kropp and Langlois, 1990; Garbaye and Churin, 1997; Pera *et al*., 1999; Ortega *et al*., 2004).

The main mechanisms behind survival, establishment and growth of the forest plants are thought to be due to the increased uptake of water and nutrients through a greatly increased root-absorbing surface (Hatch, 1937; Smith and Read, 1997), increased longevity and growth of root system (Wilcox, 1996) and protection against environmental stress factors such as drought, pathogens and heavy metal pollution (Colpaert and Vanassche, 1992; Van Tichelen *et al*., 2001; Ortega *et al*., 2004). In natural forests, the root system of a tree is almost invariably formed ectomycorrhizae with many different ectomycorrhizal mushroom species (Dahlberg, 2001; Guidot *et al*., 2003).

Mycorrhizal symbiosis is an important facet of plant health in established ecosystems. Thus, ectomycorrhizal symbiosis is the subject of a considerable body of research which has clearly shown that the mycorrhizal associations enhance tree seedling performance (Dixon *et al*., 1984; Hatchell and Marx, 1987; Burgess *et al*., 1994). Inoculation in nurseries with selected ectomycorrhizal fungi is important in improving the establishment of plantations in the field. Out of the wide range of ectomycorrhizal genera only a few have been studied extensively in controlled experiments (Cairney and Chambers, 1999).

Many studies are related to the selection and isolation of mycorrhizal fungal species which adapt to the different environmental conditions (Kuek *et al*., 1992). These fungi were used to produce effective inoculum by using different techniques (Kuek *et al*., 1992), and the inoculation and production of forest tree species in the nursery (Hung and Trappe, 1987; Browing and Withney, 1991). Various *in vitro* systems of ectomycorrhizal symbiosis have been developed and observed for the efficiency of many fungi to synthesize ectomycorrhizae with their host plant in controlled conditions (Guerin-Laguette *et al*., 2000; Eros-Honti and Jakucs, 2009; Geng *et al*., 2009).

Generally commercial nursery seedlings which are used for forestation are either lack mycorrhizal fungi or have a very limited flora associated with their root systems. Hence for the natural regeneration of conifers, the present study will be useful to utilize the natural ectomycorrhizal fungi in artificial mycorrhizal synthesis. After successful mycorrhizal synthesis, further utilize fungal inoculum to produce ectomycorrhizal seedlings under nursery conditions in large scale for different forestation programmes.

Importance of ectomycorrhizae

Ectomycorrhizal symbiosis is very important on the globally because the dominant tree species in the most of the world's temperate and boreal forests and in large areas of tropical and subtropical forests are ectomycorrhizal (Allen, 1991; Read, 1991).

Mycorrhizal fungi have been shown to be of importance in natural revegetation of anthracite wastes in the Eastern U.S. and have been successfully used to establish conifers in these areas (Marx, 1975; Marx and Artman, 1979). Large scale inoculation experiments have been done in the U.S.A. with *Pisolithus tinctorius* on different pine species including *Pinus taeda, P. virginiana, P. ponderosa, Pinus strobes* and *Pinus resinosa* (Marx *et al.,* 1984).

Apart from nutritional benefits to their hosts, some mycorrhizal fungi can enable seedlings to withstand high soil temperatures (Marx and Bryan, 1971) and increase resistance to drought (Parke *et al*., 1983). Whereas some mycorrhizal fungi can protect roots against certain pathogens (Sinclair *et al*., 1982) and consequently can improve growth of the seedlings (Smith and Read, 1997).

Sharpe and Marx (1986) found that *Carya illinoensis* seedlings grown in a sandy loam soil showed a significant increase of 6.5% over controls colonized with naturally occurring mycorrhizae. Dixon *et al*. (1987) showed an increase in dry weight of *Pinus taeda* (loblolly pine) seedlings grown in a *Sphagnum* moss-vermiculite mixture inoculated with *P. tinctorius* over controls. In *Eucalyptus grandis* growth rates were increased by *P. tinctorius* inoculation by upto 45 times in a study comprising different *P. tinctorius* isolates (Burgess *et al*., 1994).

Hatchell and Marx (1987) studied *Pinus palustris*, *P. taeda* and *P. clausa* grown in a mixture of topsoil, sand and milled pine bark during a long-term experiment. Positive growth responses were seen on all three species when colonized with *P. tinctorius*. In another mycorrhizal study, *Quercus robur*, *Q. velutina* and *Q. alba* growing in a fumigated vermiculite based media were inoculated with a variety of ectomycorrhizal fungi and an increase in growth resulted in all three species of Oak (Dixon *et al*., 1984).

Vegetative mycelial inocula of *Scleroderma* were shown to promote growth of *Eucalyptus* (Burgess *et al*., 1993; Dell *et al*., 1994), *Acacia* (Founoune *et al*., 2002; Duponnois *et al*., 2005), *Castanopsis* (Chen *et al*., 2001) and several tree species in the Dipterocarpaceae (Omon, 1996). Promotions in plant growth resulting from spore inoculation have been reported for containerized seedlings of *Eucalyptus globulus* (Lu *et al*., 1998), *Pinus pinaster* and *Psedotsuga menziesii* (Parlade *et al*., 1996) and *Shorea pinanga* (Turjaman *et al*., 2005). Other examples of growth stimulation in the field using spore inoculum include, *Abies pinus* and *Abies* sp. (Parlade *et al*., 1997); *E*. globulus (Xu *et al*., 2001); *E. urophylla* (Chen *et al*., 2000) and *Pseudotsuga menziesii* (Parlade *et al*., 1997).

Yasushi and Ippei (2006) reported the positive role of ectomycorrhizal fungi in the growth and development of coniferous seedlings. Rincon *et al.,* (2007) inoculated seedlings of *Pinus halepensis* with mycobionts like *Suillus collinitus, Amanita ovoidea* and *Rhizopogon roseolus* and reported significant improvement in growth and development of tailored seedlings. Mycorrhizae increase the absorptive surface of roots resulting in increased uptake of water and nutrients from soil. Further mycorrhizal colonization of roots had been found to have positive effects on plant growth such as increased resistance to pathogens (Hampp *et al*., 1999), drought (Shi *et al*., 2002) or heavy metal stress (Blaudez *et al*., 2000).

Harley (1989) suggested that production of phosphatases by ectomycorrhizal fungi is important in the solubilization of organic phytates, which constitute a large fraction of total phosphate in humic soils. These enzymes are many times more active than those on non-mycorrhizal roots (Williamson and Alexander, 1975; Mitchell and Read, 1981). The evidence for the induction of phosphatase activity in response to the lack of inorganic phosphate by ectomycorrhizal fungi have been given by many workers (Alexander and Hardy, 1981; Mousin *et al.,* 1988).

Ectomycorrhizae have been shown to produce large amounts of calcium oxalate (Malazczuk and Cromack, 1982; Lapeyrie, 1988) which may be involved in the chelation of Fe and A1 and thereby release P for plant uptake (Graustein *et al.,* 1977; Treeby *et al.,* 1989).

Jentschke and Godbold (2000) has reported that ectomycorrhizae protect plants against typical soil pollutants such as Cd, Pb, Cu, Mn, etc. by binding the metals to fungal cell wall components e.g. chitin, cellulose, cellulose derivatives and melanins, leading to metal exclusion or by activation of defense pathways including polyamines and glutathione (Zarb and Walters, 1996; Schutzendubel and Polle, 2002).

Materials and Methods:

***In vitro* synthesis of ectomycorrhizae**

In vitro synthesis of ectomycorrhiza was carried out following Molina (1979). The method involves using of 10ml peat, 90ml of vermiculite and 70ml of fungal medium for each 200ml synthesis tube. A disc of 5mm containing fungal culture was added to each autoclaved tube and ten replicates were used for each fungus and the uninoculated controls. After the mycelium colonise the peat-vermiculite medium for 2 weeks, sterile and aseptically germinated seedlings of *Abies pindrow* were introduced into synthesis tube and tubes were placed in growth chamber. The shoot remains outside of the synthesis tube through the slit and the root remains in aseptic condition. Tubes were sealed with aluminum foil to keep the roots and fungus in darkness. So this method provides seedlings with a natural shoot-root compartmentation by exposing the shoots to the atmosphere.

Tubes were periodically checked for mycorrhization and seedlings harvested after 5 months. For observation of early morphological changes during ectomycorrhiza formation, technique of Fortin *et al*. (1980) was followed. On the conclusion of synthesis experiment, small bits of inoculum were removed aseptically from the substrate (in synthesis vessel) and also from the synthesized ectomycorrhizae and were grown in Petri plates containing nutrient medium. The isolates were compared with the original culture for growth characteristics to confirm the symbiotic association.

Results and Discussion:

Boletus edulis is a wild edible and ectomycorrhizal mushroom belonging in the family boletaceae and this mushroom grow in the conifers forest and form symbiotic ectomycorrhizal association with the roots of living conifer plants. Mushroom fruiting bodies produced during rainy season near host trees are the evidences of mycorrhizae already present in the soil and host tissue. Conifer have huge diversity of ectomycorrhizae in their forest ecosystems and are primarily studied by many scientist (Izzo *et al*., 2005; Korkama *et al*., 2006; Tedersoo *et al*., 2006; Kennedy *et al*., 2007).

The *B. edulis* was observed to be growing in association with *Abies pindrow* in the natural forest. The present study was carried out to check the ectomycorrhizal synthesis of *B. edulis* with this conifer tree. Experiments showed that the *in vitro* ectomycorrhizal synthesis was achieved between *A. pindrow* and *B. edulis* successfully in six months. The seedlings inoculated with mycelial culture of *B. edulis* resulted in the formation of short branched lateral roots which ultimately form the ectomycorrhizae (Table 1 and Fig. 1).

Table 1: Morphological characteristics of *Abies pindrow* ectomycorrhiza formed during *in vitro* synthesis with *Boletus edulis*

Sr. No.	Characters	
A	**Macroscopic**	
i	Colour	Dark Brown
ii	Shape of mycorrhiza	dichotomously branched
iii	Texture	Smooth
iv	Odour and taste	Not distinct
v	Emanating Hyphae	Missing
vi	Root Hairs	Absent
B	**Microscopic**	
i	Thickness of Mantle	25-30 µm
ii	Degree of development of “Hartig net”	Well developed

The transverse section of the synthesized mycorrhizal roots of *A. pindrow* showed the presence of thick fungal mantle and well developed "Hartig net" (Fig. 1). The roots of seedlings kept as control were non-mycorrhizal. No Fungal mantle and no Hartig net were present in transverse section of these roots. Pure culture of *B. edulis* was reisolated from both vermiculite peatmoss mixture and synthesized ectomycorrhizae and these were compared with the original culture and were found to have same cultural characteristics, thus confirming the ectomycorrhizal symbiotic association between the *A. pindrow* and *B. edulis*.

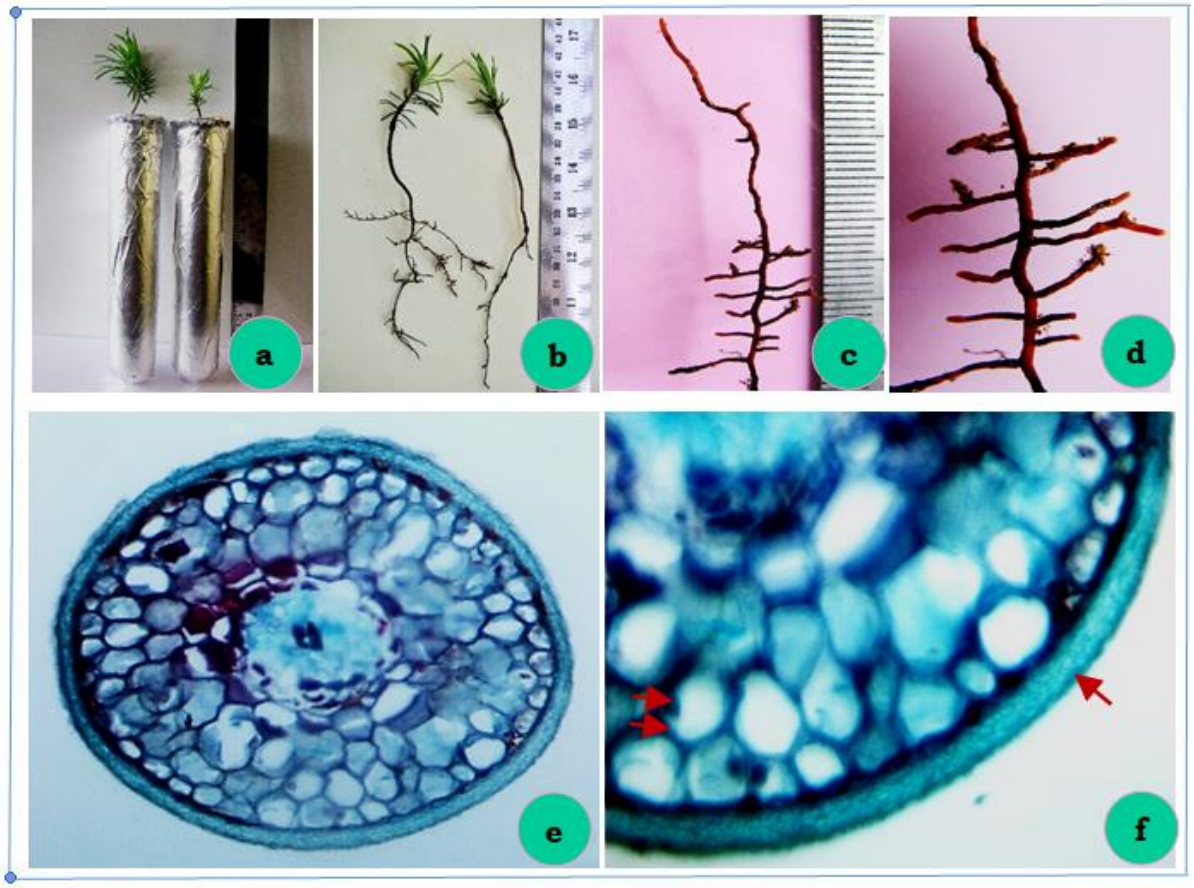

Figure 1: (A,B) Test tubes containing seedlings and Uprooted of *A. pindrow* (Seedling with large shoot inoculated with culture of *B. edulis* and with smaller shoot kept as control) for *in vitro* synthesis of ectomycorrhiza. (C) Root system of inoculated seedling showing the synthesized ectomycorrhizae. (D) A close view of synthesized ectomycorrhizae (E) T. S. of synthesized ectomycorrhizal root (20X). (F) Enlarged view (40X) of E showing well developed fungal mantle (single arrow) and Hartig net (double arrow)

In similar studies *in vitro* aseptic synthesis of mycorrhiza between *Abies firma* and *Pisolithus tinctorius* and *Cenococcum geophilum* was achieved by Vaario *et al.*, (2000). Kumar *et al.* (2019) synthesized ectomycorrhiza between *Abies pindrow* and *Lactarius sanguifluus*. Piche and Fortin (1982) synthesized *Pinus strobus* ectomycorrhizae with seven different species

of ectomycorrhizal fungi (*Cenococcum geophilum*, *Hebeloma cylindrosporum*, *Paxillus involutus*, *Pisolithus tinctorius*, *Suillus granulatus*, *Suillus tomentosus* and *Thelephora terrestris*). Hartig net and mantles were observed in all cases. *P. involutus*, *P. tinctorius* and *T. terrestris* were much more rapid in forming ectomycorrhizae than the other species. Yamada and Katsuya (1995) also reported mycorrhizal synthesis between *P. densiflora* and 21 fungal species, including two species of *Russula* (Taylor and Alexander, 1989).

In vitro synthesis of mycorrhizae also carried by many researchers on different plants like *Castanea mollissima* and *Pinus armandii* (Geng *et al*., 2009); *Nothofagus obliqua* and *N. glauca* (Perez *et al*., 2007); *Pinus densiflora* (Yamada *et al*., 2001); *Pinus strobes* (Piche and Fortin, 1982); *Pinus densiflora* (Yamada and Katsuya, 1995); *Pinus wallichiana* (Sagar and Lakhanpal, 2005). Samson and Fortin (1988) studied the characteristics of ectomycorrhizae synthesized on *larix laricina* with six species of *Fuscoboletinus* and two species of *Suillus*. The synthesized ectomycorrhizae were olivaceous yellow, white to pale yellow, white to grayish brown, white to pinkish grey in colour.

Conclusions:

Abies pindrow seedlings synthesized ectomycorrhizae with the pure culture of *Boletus edulis*. The synthesized mycorrhizae were dark brown in colour. The transverse sections of the synthesized roots showed typical ectomycorrhizal anatomical characteristics. The synthesized ectomycorrhizae showed thick fungal mantle and well developed “Hartig net”. The inoculated seedlings were more healthy and showed more growth of the root and shoot system as compared to seedlings which were kept as control.

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THE ABILITY TO RECOVER PLANTS FROM PROTOPLAST CULTURES NEW GENETIC CONSTITUTION *INVITRO* REGENERATED FROM LEAF EXPLANTS OF *MANTHA PIPERITA*

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Abstract:

Protoplast culture therefore provides one route whereby plants can be multiplied, but it is not yet used for routine micro propagation work, although the number of species in which plant regeneration has been achieved is steadily increasing. At present isolated protoplasts are used chiefly in research into plant virus infections, and for modifying the genetic information of the cell by inserting selected DNA fragments Bruneton J (1995).A protoplast is the living part of a plant cell, consisting of the cytoplasm and nucleus with the cell wall removed. Protoplasts can be isolated from whole plant organs or tissue cultures. U A Devi and M Venkateshwarlu 2019, Wink (2003). If they are then placed in a suitable nutrient medium, they can be induced to re-form a cell wall and divide. A small cluster of cells eventually arises from each cell and, providing the protoplasts were originally plated at a relatively low density, can be recognized as one of many discrete "callus colonies. Plants can often be regenerated from such callus. Protoplasts may also be fused together to create plant cell hybrids Seigler DS (1998). Genetically modified cells will be only of general practical value if whole plants having the new genetic constitution can be regenerated from them. The ability to recover plants from protoplast cultures is therefore of vital importance to the success of such genetic engineering projects in plant science. Garcia-mas, J (2000)

Keywords: Protoplast culture, Micro propagation, Regeneration *M. piperita*

Introduction:

The peppermint *Mantha. piperita* is cultivated on a large scale in the states of Oregan, Indiana, Idaho, Ohio and Michigan. whereas spearmint cultivation is localized in Indiana and Michigan. Spearmint is also cultivated in France, the United Kingdom, Italy, Yugoslavia, Hungary, Bulgaria, Russia, South Africa, Thailand and Vietnam. Bergamot mint is commercially cultivated in China, Taiwan and India, and menthol or Japanese mint in India, China, Taiwan, Thailand, Japan and Brazil.

The first complete chemical defined nutrient medium was developed. A standard culture medium consists of a balanced mixture of macro and micro nutrients, vitamins and plant growth regulators which play an important role in cell metabolism and cell membrane synthesis hardly any progress was made in the field of plant tissue culture.

Methods of protoplast preparation

There are several different methods by which protoplasts may be isolated:

- By mechanically cutting or breaking open the cell wall;
- By digesting away the cell wall with enzymes;
- By a combination of mechanical and enzymatic separation.

For successful isolation it has been found essential to cause theprotoplast to contract away from the cell wall, to which, when the cell is turgid, it is tightly adpressed. Contraction is achieved by plasmolysing cells with solutions of salts such as potassium chloride and magnesium sulphate, or with sugars or sugar alcohols (particularly mannitol). These osmotica must be of sufficient concentration to cause shrinkage of the protoplasm, but of insufficient strength to cause cellular damage. In the past, protoplasts have been mechanically isolated from pieces of sectioned plant material, but only very small numbers were obtained intact and undamaged. This method has therefore been almost completely replaced by enzymatic isolation techniques. Commercially available preparations used for protoplast isolation are often mixtures of enzymes from a fungal or bacterial source, and have pectinase, cellulase and/or hemicellulase activity: they derive part of their effectiveness from being of mixed composition.

Protoplasts are usually isolated using a combination of several different commercial products. Plasmolysis helps to protect the protoplast when the cell wall is ruptured during mechanical separation and also appears to make the cell more resistant to the toxic effects of the enzymes used for cell wall digestion. It also severs the plasmodesmata linking adjacent cells and so prevents the amalgamation of protoplasms when the cell walls are digested away. Tissue from an entire plant to be used for protoplast separation, is first surface sterilised. Some further preparation to allow the penetration of osmotic solutions and the cell wall degrading enzymes, is often advantageous. For instance, when protoplasts are to be separated from leaf mesophyll, the epidermis of the leaf is first peeled away, or the leaf is cut in strips and the tissue segments are then plasmolysed. The next step is to incubate the tissue with pectinase and cellulase enzymes for up to 18 hours in the same osmoticum, during which time the cell walls are degraded. Agitation of the incubated medium after this interval causes protoplasts to be released. They are washed and separated in solutions of suitable osmotic potential before being transferred to a

culture medium. Less severe and prolonged enzymatic cell digestion is required if plant tissue is first treated to mild mechanical homogenisation before cellulose treatment. Another technique calls for the sequential use of enzymes; firstly pectinase to separate the cells, and then, when separation is complete, cellulase to digest the cell walls. The yield of viable protoplasts can sometimes be increased by pre-treatment of the chosen tissue with growth substances before separation is attempted.

Protoplasts are also commonly isolated by enzymatic treatment of organs or tissues that have been cultured in vitro. Cells from suspension cultures, which have been sub cultured frequently, and are dividing rapidly, are one suitable source. The successful isolation of viable protoplasts capable of cell division and growth, can depend on he manner in which the mother plant was grown. For example, found that consistently successful protoplast isolation from haploid Nicotianasylvestris plants depended on having reproducible batches of young plants in vitro. The composition of the medium on which these plants were cultured had a striking effect on protoplast yield and on their ability to divide. A low salt medium devoid of vitamins was particularly disadvantageous. The light intensity under which the plants were grown was also critical.

Methods and procedure for protoplast isolation from plant tissues have long been known (Keller *et al.,* 1992) Recent advance in the isolation, culture and regeneration of plants from protoplasts of a wide diversity of species have been reported (Gleddie *et al.*, 1989).

Essential in gredicent of the technique of genetic modification of plant cells through the protoplast system are:

1. Isolation of protoplast
2. Culture of protoplasts to raise whole plant
3. Cell fusion
4. Cell organelles the protoplasts

Source of material

The most convenient and populous source of plant protoplasts is isolation of large number of relatively the necessity of killing the plants. Moreover, the mesophyll cells are loosely arranged, the enzymes have an easy access to the cell wall. When protoplasts are prepared from leaves, the age of the plant and the conditions under which it has grown may be critical. To achieve maximum control on the growth conditions of source plants several workers have used in vitro grown shoots (Binding, 1975). In some species where it is difficult to isolate culturable protoplasts from leaf cells alternative source material of cultured cells have been used. The yield of protoplasts from cultured cells depends on the growth rate and growth phase of cells.

Frequently sub cultured suspension cultures, and cells taken from the early log phase are almost suitable (Kao *et al.,* 1971; Vasil and Vasil, 1979).

Enzyme treatment

The release of protoplasts is very much dependent on the nature and concentrations of enzymes used. The two enzymes regarded essential to isolate protoplasts from plant cells are cellulose &macerozyme. Driselase, having and number of Zymolytic activities such as Cellulose, Pectinase Laminarinase and Zylanase (Kao *et al.,* 1974) has proved especially useful for isolating protoplasts from cultured cells. Increase in yield of mulberry proplasts by treatment with chemical substances has been reported earlier (Ohnishi and Kiyama, 1987).

Osmoticum

A variety of solutes, ionic and non-ionic, have been tested for adjusting the osmotic pressure of the various solutions used in protoplast isolation and culture, but the most widely used osmotica are sorbitol and mannitol.

With advancement in plant tissue culture technology, plant regeneration systems from protoplasts have been developed in may mulberry species (Ohyama and Oka, 1975). Establishment of protoplast regeneration system using new technologies such as protoplast fusion and gene transfer would contribute to the improvement of mulberry varieties (Tohjima *et al.,* 1996).

With advancement in plant tissue culture technology, plant regeneration systems from protoplasts have been developed in may mulberry species (Ohyama and Oka, 1975). A fundamental property of isolated protoplasts is their osmotic fragility and hence the need for suitable osmotic stabilizer to the enzyme solution, the protoplast washing medium and protoplast culture medium is necessary.

Material and Methods:

Isolation of protoplasts from mesophyll cells Seeds of Mentha arvensis were collected from CIMAP (Central Institute of Medicinal Aromatic Plant) Uppal, Hyderabad. Seeds which were initially soaked overnight and then washed with running tap water for 30 min to remove adherent particles, thoroughly washed seeds were then immensed in 5% (v/v) Teepol for 10 min and then rinsed 3 times with sterile double distilled waster. This was followed by the surface sterilization with 05 % (m/v) HgCl, under the sterile conditions for 5 min. these were rinsed 5 times in sterile double distilled water to remove all traces of HgCl,. The sterilized seeds were then placed on to the basal Murashige and Skoog(1962) medium for germination.

The leaves of 2-3 cms in length and 1- 1.5 cm in width were excised from 6 weeks old seedlings. The leaves were cut into pieces Smaller than 1mm and incubated in filter sterilized

enzyme solution. The enzyme solution consistsed of 1% (w/v) Macerozyme R-10 and 1% callulose. R-10 in 0.4 m mannitol pH 5.8 and osmoticum the sliced leaf pieces of incubated in 10 ml of enzyme solution at 27" C and Shaken at 40-50 y pm for 4-5 hrs in dark.

Purification of mesophyll derived protoplasts

Gently transfer the protoplast enzyme solution and undigested tissues with a sterile medium bore Pasteur pipette on to a sterile 64 or 44 um pole size fitter inside a sterile funnel. Collect the filtrate in sterile 50 ml centrifuge tube cover with sterile tin foil or autoclaved metal closure and at foil or autoclaved metal closure and centrifuge at 500 g for 5-10 min. remove the enzyme solution carefully with a sterile pastern pipette. Add 12 ml of 0.4 M sucrose to pellet, and gently disperse and resuspend the protoplsts in the solution. Use gently with drawing and delivery with the pipette. When protoplasts are completely dispersed add carefully with a pastevr pipette by layering over the sucrose solution 3 ml of a solution of 0.4 M sorbitol 10 mm CaCl, 2 H,O, 5 mm H MES, pH, 5.8 and centrifuge at 250 g for min.

To wash the proto plasts use a Pasteur pipette to collect the protoplasts carefully at the interphase of the two solutions and transfer to 4 ml of salt solution containing 0.2% w/v CaCl, and 2.5% Kcl pH 6.0 Gently suspend and centrifuge at low speed to collect the protoplasts. Remove the washing solution carefully with a sterile Pasteur pipette. Resuspended the protoplasts in the centrifuge tubes to a density of 1 X 10 per ml in medium for Mentha to determine the density a hemacytometerand inverted microscope are used to make a count of the protoplasts.

Results:

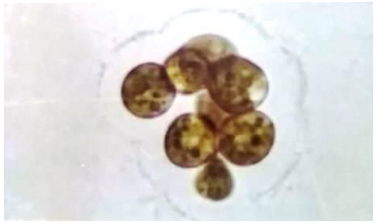

Plate –I: The ability to recover plants from protoplast cultures new genetic constitution *invitro* regenerated from leaf explants of *Mantha piperita*

A mixture of 1% cellulose and 1 % macerozyme was suitable for isolation of viable protoplasts from merophyll tissues of leaf explant culture of *Mantha. piperita* were investigated each enzyme was in effective by itself but when used in combination it resulted satisfactorily (Evans and Bravo 1983). For in vitro leaf explants above combination of macerozyme and

cellulose gave a optimum yield. Plate –I fig – a In the present study, attempts have seen made to study protoplast isolation and purification using leaf explants of *Mantha piperita*.

Conclusion:

The results showed a variable shoot forming capacity depending on the combination of growth regulators used in the culture medium. The number of shoots produced increased with the concentration of BAP and Kn until 1.5 mg/l or 0.5 mg/ l of the cytokinin and showed high frequency of explants exhibiting compact green callus with shoots (4-6), growth and also for shot induction.

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