ISOLATION, SCREENING AND CHARACTERIZATION OF PLANT GROWTH PROMOTING BACTERIA FROM RHIZOSPHERE ASSOCIATED WITH *IXORA COCCINEA*

A dissertation submitted in partial fulfilment of the requirement for degree of

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CERTIFICATE

This is to certify that the dissertation titled, "**ISOLATION, SCREENING AND CHARACTERIZATION OF PLANT GROWTH PROMOTING BACTERIA FROM RHIZOSPHERE ASSOCIATED WITH *IXORA COCCINEA***" is an authentic record of work carried out by **MARY OLIVIA ROBERT** under the supervision and guidance of **Dr. ALPHONSA VIJAYA JOSEPH**, Assosiate Professor, Department of Botany & Centre for Research, St. Teresas's College (Autonomous), Ernakulam in partial fulfilment of the requirement for the Master's Degree of Science in Botany.

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CONTENTS

INTRODUCTION

Soil is a dynamic living matrix and it is not only a critical resource in agricultural and food security but it is also towards maintenance of all life process (Gupta *et al.*, 2015). Soil is rich in microscopic life forms including bacteria, fungi, actinomycetes, protozoa and algae. Of these different microorganisms, bacteria are by far the most common. Bacteria fund over 95% of the soil microbial activities and dominate also in the level of abundance. This is as a result of their fast proliferation and ability to utilize wide range of carbon and nitrogen source as energy. It has been known for some time that the soil hosts a large number of bacteria (around 10^8 to 10^9 cells per gram of soil) and that the number of culturable bacterial cells in soil is generally only about 1% of the total number of cells present (Glick, 2012). Soil conditions like moisture, temperature, chemicals, etc., influences the type of bacteria and its number in the soil. Bacteria are generally not evenly distributed in soil. The concentration of bacteria that is found around the roots of plants i.e., in the rhizosphere, is typically much greater than in the rest of the soil.

Rhizosphere is the immediate region of plant root. Bacteria associated with the rhizosphere of plant is known as rhizobacteria. Rhizosphere into three separate parts, first the exorhizosphere relates to soil adherent to the root and those attached even after vigorous shaking, the second is rhizoplane which illustrate the thin layer of soil-root and thirdly an intercellular space in the root tissues inhabited by endophyte bacteria (endorhizosphere). These sites encourages healthy competition among organisms for more competency, saprophytic abilities and potential for enhancing plant growth. In addition to it, successful organisms multiply easily through a broad spectrum of actions as a result of high nutrient and carbon source, compete favorably with other microorganisms and poses tolerance to drought.

The rhizosphere is a host to diverse group of microorganisms that are influenced by rich source of nutrients obtained through the root exudates (Odoh, 2017). Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates creating a very selective environment where diversity is low. While these bacteria utilize the nutrients that are released from the host for their growth, they also secrete metabolites into the rhizosphere. Several of these metabolites can act as signaling compounds that are perceived by neighboring cells within the

same micro-colony, by cells of other bacteria that are present in the rhizosphere, or by root cells of the host plant (Gray & Smith, 2005).

Rhizosphere is one of the first steps in the pathogenesis of soil borne microorganisms. It is also crucial for the microbial inoculants used as biofertilizers, biocontrol agents, phytostimulators and bioremediators. *Pseudomonas* sp. is often used as model root colonizing bacteria.

Since the rhizosphere is very rich in nutrients, its associate bacteria (rhizobacteria) tend to develop a unique means of communication by enabling the effective selection of its mutual partner by creating host specificity and selective sensitive environment. Plant bacterial interaction in the rhizosphere are the determinants of plant health. It also aids in soil fertility. Regardless of the number of bacteria in a particular soil sample, the bacteria may affect plants in one of three ways i.e. beneficial, harmful, or neutral. However, the effect that a particular bacterium has on a plant can change as the conditions influencing it change.

Motile rhizobacteria may colonize the rhizosphere more profusely than the non-motile ones. This results in a better nutrient transformation. They also eliminate the harmful rhizobacteria and help in plant growth by niche exclusion. Induced systemic resistance is a mechanism by which PGPR control plant diseases through manipulation of host plant's physical and biochemical properties (Sivasakthi *et al.,* 2014).

The word PGPR was proposed by Kloepper *et al.,* (1980). It was coined for fluorescent *Pseudomonas*, a plant growth enhancer that fought against pathogens. Since then, the term has metamorphose and extended to include all rhizobacteria capable of directly enhancing plants growth (Kapulnik, 1981). Recently, it was used to include wide range of rhizobacteria that improve plant growth through different mechanisms. Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). These include bacteria that are free-living and soil-borne, those that form specific symbiotic relationship with plants (e.g., *Rhizobia spp*. and *Frankia spp*.), bacterial endophytes that can colonize some or a portion of a plant's interior tissues, and cyanobacteria (formerly called blue-green algae).

Interaction of PGPR with the host plant is an intricate and interdependent relationship involving the two partners along with other abiotic characters. These microbes can promote plant growth by

regulating nutritional and hormonal balance, producing plant growth regulators, solubilizing nutrients and inducing resistance against plant pathogens. In addition to their interactions with plants, these microbes also show synergistic as well as antagonistic interactions with other microbes in the soil environment (Nadeem, 2014).

The genera of PGPR include *Azotobacter, Azospirillum, Acetobacter, Pseudomonas, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous* and *Serratia* etc. Direct use of microorganisms to promote plant growth and to control plant pest continues to be an area of rapidly expanding research.

A rhizobacterial strain is considered to be a putative PGPR if it possesses specific plant growth promoting traits and can enhance plant growth upon inoculation. An ideal PGPR strain should be highly rhizosphere-competent, eco-friendly and tolerant of physicochemical factors like heat, desiccation, radiations, and oxidants. It should colonize the plant roots in significant numbers upon inoculation, promote plant growth and exhibit a broad spectrum of action. It should be compatible with other bacteria in the rhizosphere anddemonstrate better competitive skills over the existing rhizobacterial communities.

Plant growth promoting rhizobacteria can be classified into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR). The ePGPRs may exist in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex while iPGPRs locates generally inside the specialized nodular structures of root cells. The bacterial genera such as *Agrobacterium, Arthrobacter, Azotobacter, Azospirillum, Bacillus, Burkholderia, Caulobacter, Chromobacterium, Erwinia, Flavobacterium, Micrococcous, Pseudomonas* and *Serratia* belongs to ePGPR. The iPGPR belongs to the family of Rhizobiaceae includes *Allorhizobium, Bradyrhizobium, Mesorhizobium* and *Rhizobium*, endophytes and *Frankia* species both of which can symbiotically fix atmospheric nitrogen with the higher plants.

Horticulture is one of the areas where agricultural technologies are widely and intensively used. Horticultural crops may grow both in the open and closed space such as greenhouse and tunnel, and there are several practical applications such as propagation with cuttings and grafting, pruning, and soilless culture; plant growth regulators that have little or no use with other agricultural crops were used largely in horticultural crop production. In addition, to make sure of sufficient plant

growth and development and high fruit yield and quality, these treatments should be inhered in horticultural production. Therefore, horticultural crops require more input than the other agricultural crops and sustainability maintenance is also quite significant. For these reasons, there is a need for different techniques that increase the input efficiency, and plant growth promoting rhizobacteria (PGPR) have proved to be a major tool. PGPR can affect on plant growth by production and release of secondary metabolites, lessening or preventing deleterious effects of phytopathogenic organisms in the rhizosphere and/or phyllosphere, and/or facilitating the availability and uptake of certain nutrients like N, P, and Fe from the root environment. In accordance with these action mechanisms, PGPR can be used for various purposes such as rooting of cutting, grafting union, fruit setting and thinning, lateral root formation, increasing tolerance against abiotic stress as well as growth, development, and biological control with root inoculation and or spraying (Esitken, 2011).

Most of the studies with PGPR are related to field crops. Nevertheless, in recent years, the use of PGPR in horticultural crops to promote plant growth, development, and yield has increased in various parts of the world. Several authors have reported that root inoculation and/or spraying with PGPR can result in increased germination, seedling emergence, and modified growth and yield of various horticultural crops.

PGPR are known to influence plant growth by various direct or indirect mechanisms. Direct mechanisms facilitate nutrient uptake or increase nutrient availability by nitrogen fixation, solubilization of mineral nutrients, mineralize organic compounds and production of phytohormones. Direct plant growth promotion include biofertilizer activity. Generally, plant growth promoting rhizobacteria promote plant growth directly by either often due to their ability for nutrient supply (nitrogen, phosphorus, potassium and essential minerals) or modulating plant hormone levels. These include:-

1. Nitrogen fixation
2. Phosphate solubilization
3. Phytohormone production

1. Nitrogen fixation:

Nitrogen is an essential element for all forms of life and it is the most vital nutrient for plant growth and productivity. Although the nitrogen presents 78 % of the atmosphere, it remains unavailable to the plants. No plant species is capable for fixing atmospheric dinitrogen into ammonia and expend it directly for its growth. Thus the atmospheric nitrogen is converted into plant utilizable forms by biological nitrogen fixation (BNF) which changes nitrogen to ammonia by nitrogen fixing microorganisms using a complex enzyme system known as nitrogenase. Plant growth promoting rhizobacteria have the ability to fix atmospheric nitrogen and provide it to plants by two mechanisms: symbiotic and non-symbiotic. Symbiotic nitrogen fixation is a mutualistic relationship between a microbe and the plant. The microbe first enters the root and later on form nodules in which nitrogen fixation occurs. Rhizobia are a vast group of rhizobacteria that have the ability to lay symbiotic interactions by the colonization and formation of root nodules with leguminous plants (Gupta *et al.,* 2015)

2. Phosphate solubilization:

Phosphorus is the most important key element in the nutrition of plants, next to nitrogen (N). It plays an important role in virtually all major metabolic processes in plant including photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration [20]. It is abundantly available in soils in both organic and inorganic forms. Plants are unable to utilized phosphate because 95-99% phosphate present in the insoluble, immobilized, and precipitated form. Plants absorb phosphate only in two soluble forms, the monobasic (H_2PO_4) and the dibasic (HPO_4^2) ions.

Plant growth promoting rhizobacteria present in the soil employ different strategies to make use of unavailable forms of phosphorus and in turn also help in making phosphorus available for plants to absorb. The main phosphate solubilization mechanisms employed by plant growth promoting rhizobacteria include:

(1) Release of complexing or mineral dissolving compounds e.g. organic acid anions, protons, hydroxyl ions, CO_2,

(2) Liberation of extracellular enzymes (biochemical phosphate mineralization) and

(3) The release of phosphate during substrate degradation (biological phosphate mineralization).

3. Phytohormone production:

A wide range of microorganisms found in the rhizosphere are able to produce substances that regulate plant growth and development. Plant growth promoting rhizobacteria produce phytohormones such as auxins, cytokinins, gibberellins and ethylene can affect cell proliferation in the root architecture by overproduction of lateral roots and root hairs with a subsequent increase of nutrient and water uptake

Indole Acetic Acid (IAA): Among plant growth regulators, indole acetic acid (IAA) is the most common natural auxin found in plants and its positive effect on root growth.

Cytokinins and gibberellins: Several plant growth promoting rhizobacteria Azotobacter sp., Rhizobium sp. etc. can produce cytokinins or gibberellins or both can produce either cytokinins or gibberellins or both for plant growth promotion plant growth promoting rhizobacteria produce lower cytokinin levels compared to phytopathogens.

Phytopathogenic microorganisms are a major and chronic threat to sustainable agriculture and ecosystem stability worldwide disturb the soil ecology, disrupt environment, degrade soil fertility and consequently show harmful effects on human health, along with contaminating ground water. PGPR is a promising sustainable and environmentally friendly approach to obtain sustainable fertility of the soil and plant growth indirectly. This approach takes inspire a wide range of exploitation of PGPR led to reducing the need for agrochemicals (fertilizers and pesticides) for improve soil fertility by a variety of mechanisms that via production of antibiotics, siderophores, HCN, hydrolytic enzymes etc. These accounts for the indirect plant growth promotion mechanisms activity or biopesticide activity.

The most intensively researched use of PGPR has been in agriculture and horticulture. Several PGPR formulations are currently available as commercial products for agricultural production. Recently developing areas of PGPR usage include forest regeneration and phytoremediation of contaminated soils. PGPR is also being used in green and sustainable agriculture in order to reduce the effect of chemical fertilizers, pesticides and insecticides (Jimtha *et al.*, 2020).

The success and commercialization of plant growth promoting rhizobacterial strains depend on the linkages between the scientific organizations and industries. Numerous work done showed

different stages in the process of commercialization include isolation of antagonist strains, screening, fermentation methods, mass production, formulation viability, toxicology, industrial linkages, quality control and field efficacy. Moreover, commercial success of PGPR strains requires economical and viable market demand, consistent and broad spectrum action, safety and stability, longer shelf life, low capital costs and easy availability of career materials (Gupta *et al.*, 2015)

OBJECTIVES OF THE STUDY

- Isolation of bacteria from rhizosphere associated with *Ixora coccinea*
- Screening of the isolates for specific enzyme production.
- Screening of the isolates for plant growth promoting properties.
- Morphological characterization of screened isolates.
- Biochemical characterization of screened isolates.
- Molecular identification of screened isolates using PCR.

REVIEW OF LITERATURE

Various groups of bacteria that are able to stimulate plant growth by a mechanisms of action are referred to as plant growth-promoting rhizobacteria or PGPR. They affect plant growth and development directly or indirectly either by releasing plant growth regulators (PGRs) or other biologically active substances. This causes an increase in the available nutrients, which increase nutrient uptake and reduces the negative effects of pathogenic microorganisms on plants. PGPRs have various types of effects, such as an increase in root growth and nutrient uptake, the stimulation of plant hormones, inhibition of the activity of plant pathogens, improvement of the soil structure, and mineralization of organic pollutants.

Bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology to a greater extent, especially considering their competitiveness in root colonization (Sivasakthi *et al.,* 2014).

The word PGPR was proposed by Kloepper and associates (1980). It was coined for fluorescent *Pseudomonas*, a plant growth enhancer that fought against pathogens.

Nadeem and co-workers (2014) suggested that plant growth under stress conditions may be enhanced by the application of microbial inoculation including plant growth promoting rhizobacteria (PGPR) and mycorrhizal fungi. These microbes can promote plant growth by regulating nutritional and hormonal balance, producing plant growth regulators, solubilizing nutrients and inducing resistance against plant pathogens.

Plant growth promoting rhizobacteria (PGPR) are an important cluster of beneficial, root-colonizing bacteria thriving in the plant rhizosphere and bulk soil. They exhibit synergistic and antagonistic interactions with the soil micro biota and engage in an array of activities of ecological significance. They promote plant growth by facilitating biotic and abiotic stress tolerance and support the nutrition of host plants. Basu and colleagues (2021) also considered PGPR as an ecofriendly alternative to hazardous chemical fertilizers due to their active growth endorsing activities.

Gupta and colleagues (2015) plant growth promoting rhizobacteria promote plant growth directly by either often due to their ability for nutrient supply (nitrogen, phosphorus, potassium and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory

effects of various pathogens on plant growth and development in the forms of biocontrol agents, root colonizers, and environmental protectors.

Saharan and Nehra (2011) detailed on how the inoculation of crop plants with certain strains of PGPR at an early stage of development improves biomass production through direct effects on root and shoots growth. They also suggested that inoculation of ornamentals, forest trees, vegetables, and agricultural crops with PGPR may result in multiple effects on early-season plant growth, as seen in the enhancement of seedling germination, stand health, plant vigor, plant height, and shoot weight, nutrient content of shoot tissues, early bloom, chlorophyll content, and increased nodulation in legumes.

Odoh (2017) pointed out that plant growth promoting rhizobacteria (PGPR) involves the utilization of large array of soil bacteria to improve yield and plant growth. As free living and symbiotic rhizobacteria, PGPR colonizes extracellular and/or intracellular rhizoenvironment in search for carbon source while indirectly aiding plant growth. He also explained that the seeds or soil application of PGPRs inoculant enhances directly phosphates solubilization, atmospheric nitrogen fixation and secretion of plant hormones (indole acetic acid, gibberellins, cytokinins and ethylene) needed for growth and adaptation in stressed environment.

Wang and co-workers (2021) provided evidence of benefits when PGPR mixtures, rather than individuals, are used for protecting crops from various diseases, and underscore the critical determinant factors for successful use of PGPR mixtures. Thy also evaluated the challenges of and limitations to achieving the desired outcomes from strain/species-rich bacterial assemblages, particularly in relation to their role for plant disease management. In addition, towards locating additive or synergistic outcomes, they highlight why and how the benefits conferred need to be categorized and quantified when different strains/species of PGPR are used in combinations.

The effect of, activators on selected soil properties, the experimental treatments including the application of the growth activators P energetic (K + P) and Azoter, which contains the bacteria *Azotobacter chroococcum, Azospirillum brasilense,* and *Bacillus megaterium,* before sowing or during vegetation were studied by Artyszak and Gosdwski (2021) .

In addition, PGPR have been used for different purposes in horticultural crops, for instance, improving grafting union in grape (Kose *et al.,* 2005), fruit setting (Esitken *et al.,* 2006) and fruit thinning (Esitken *et al.,* 2009).

Altaf and colleagues (2019) explained the utilization of microorganism with the goal at enhancing nutrient availability for plants is a significant practice and is essential for agriculture. Besides, a sole PGPR not only performs as biological control agent but it performs multiple mode of actions. The rhizosphere, rhizoplane or the space pockets between the root cortex cells are the places where ePGPRs resides whereas iPGPRs resides generally in the peculiar nodule like structure of root cells. The genera of bacteria such as Serratia, Pseudomonas, Micrococcous, Flavobacterium, Erwinia, Chromobacterium, Caulobacter, Burkholderia, Bacillus, Azospirillum, Azotobacter, Arthrobacter and Agrobacterium belong to ePGPR.

PGPR affect plant growth by indirect mechanisms that involve suppression of bacterial, fungal, viral, and nematode pathogens. A lot of study showed that inoculation with PGPR resulted in significant yield increases in different crops, rooting of hardwood and semi-hardwood cuttings, increased germination and enhanced emergence of seeds under different conditions, promoted nutrient uptake of roots, total biomass of the plants, increased seed weight, induced early flowering, etc. (Kaymak, 2010).

Evidence the involvement of several chemical signals in controlling the associative symbiosis between plant growth-promoting rhizobacteria (PGPR) and roots was enunciated by Drogue and associates (2013). In this review, recent findings about the identification of signals involved in the interaction of plants with PGPR, and of PGPR with each other's, were presented.

In agricultural management practices, they are often included to minimize chemical inputs, increase productivity and restore the natural equilibrium in agro-ecological systems. Exchange of signals between plant roots and PGPR and how these relationships modulate plant abiotic stress responses by induced systemic resistance. (Jimtha *et al.,* 2020)

The steadily increasing potentiality of PGPR in agriculture was noted by Bhattacharyya and Jha (2011) and it is as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements. Growth promoting substances are likely to be produced in large quantities

by these rhizosphere microorganisms that influence indirectly on the overall morphology of the plants.

In the review article, Zhuang and coworkers (2007) explained the role of PGPR in bioremediation. Recently, the application of PGPR has been extended to remediate contaminated soils in association with plants. Of all the present contaminants, the profound impacts of organic and heavy metal pollutants have attracted worldwide attention.

The greatest benefit of seed treatment may be inhibition of slightly parasitic or nonparasitic but toxigenic microorganisms. The PGPR were antagonistic in vitro to most of these deleterious bacteria. The role of deleterious bacteria in depressing root growth is under study (Suslow *et al.*, 2008).

van Loon (2007) investigated on PGPR and came to the conclusion that the root-colonizing *Pseudomonas* bacteria have been shown to alter plant gene expression in roots and leaves to different extents, indicative of recognition of one or more bacterial determinants by specific plant receptors. Conversely, plants can alter root exudation and secrete compounds that interfere with quorum sensing (QS) regulation in the bacteria. Such two-way signaling resembles the interaction of root-nodulating *Rhizobia* with legumes and between mycorrhizal fungi and roots of the majority of plant species.

Beneduzi and colleagues (2012) the ability of bacterial siderophores and antibiotics to suppress phytopathogens could be of significant agronomic importance. Both mechanisms have essential functions in microbial antagonism but also are able to elicit induced resistance. Resistance-inducing and antagonistic rhizobacteria might be useful in formulating new inoculants, offering an attractive alternative of environmentally friendly biological control of plant disease and improving the cropping systems into which it can be most profitably applied.

In a study conducted by Dawwam and co-workers (2013), the results of in vitro assays showed that all isolates can produce IAA, while four isolates solubilized rock phosphate. These isolates having abilities for IAA production and phosphate solubilization were tested as bio inoculant to potato tubers. The results of inoculated plants showed significant differences in vegetative growth parameters as well as photosynthetic pigments.

Glick (2012) concluded the review on PGPR stating that it is already being used successfully in a number of countries in the developing world and this practice is expected to grow. In the more developed world, where agricultural chemicals remain relatively inexpensive, the use of PGPB occupies a small but growing niche in the development of organic strategies.

While initial PGPB are likely to be non-transformed bacterial strains that have been selected for certain positive traits, it is likely in the future, as a greater understanding of the mechanisms at play in the bacterial stimulation of plant growth is gained, that scientists will genetically engineer more efficacious strains. Scientists will need to prove to both the public and to regulatory agencies worldwide that genetically engineered PGPB do not present any new hazards or risks (Gray and Smith, 2005).

Esitken (2011) explained that PGPR can be used for various purposes such as rooting of cutting, grafting union, fruit setting and thinning, lateral root formation, increasing tolerance against abiotic stress as well as growth, development, and biological control with root inoculation and/or spraying.

Bioremediation, based on bioaugmentation and biostimulation approaches, is a promising strategy for remediating contaminated soil. The use of plant growth-promoting rhizobacteria (PGPR) as a bioaugmentation tool is an effective technique for treating hydrocarbon contaminated soil (Saeed *et al.,* 2011).

MATERIALS AND METHODS

The soil from the rhizosphere of *Ixora coccinea*, which is a horticulture plant was taken and bacterial strains were isolated.

1. Isolation of Bacteria

The bacterial strains were isolated from the soil by the serial dilution and spread plate method.

i. Source of Sample

The soil sample was collected from the rhizosphere of *Ixora coccinea* plant from Vaduthala, Corporation of Kochi, Ernakulam district, Kerala.

ii. Collection of Sample

The soil sample was collected from the rhizosphere of *Ixora coccinea* with a clean trowel, transferred into a clean polythene bag and stored in the refrigerator in the department laboratory.

iii. Isolation of Bacterial Strains

Isolation was done based on the protocol by Benedetto *et al.*, (2019). 10gm of soil sample collected was weighed out. It was then added to 90 ml distilled water taken in a conical flask that was autoclaved. It was mixed well and kept undisturbed for the sediments to settle down.

4 test tubes were taken with cotton plugs. 9ml of distilled water was added to each test tube and autoclaved. 1ml of the sample was pipetted out from the conical flask and added to the first test tube with 9ml of autoclaved distilled water to get 10^{-3} dilution. It was thoroughly mixed with the pipette itself.

1 ml from the 10^{-3} sample was taken and added to the second test tube to get 10^{-4} dilution. It was mixed thoroughly. Again 1ml was taken from the previously diluted sample, transferred to the third test tube and mixed thoroughly to get 10^{-5} dilution. Finally, 1ml was taken from this and transferred to the last test tube to get 10^{-6} dilution.

Now the sample has been diluted to 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions respectively.

Agar agar bacteriological (for general bacteriological purpose) that contains-

- Moisture 12%
- Ash Content 3%
- Gel Strength 600 gm/cm^2

Was taken along with Nutrient Broth that has the constitution

- Peptone 5.00 gm/L
- NaCl 5.00 gm/L
- HM peptone B (equivalent to beef extract) 1.50 gm/L
- Yeast extract 1.50 gm/L
- Final pH 7.4±0.2

Nutrient agar medium was made by adding nutrient broth and agar in the composition

- Nutrient broth 3.9 gm
- Agar 6 gm
- Distilled water 300 ml

It was made in a 500 ml conical flask plugged with a cotton plug, covered and autoclaved. Cotton swabs were also made and autoclaved. Serially diluted samples, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} were spread on 4 agar plates each using the cotton swabs. These agar plates were kept for incubation for 24hr at 30-37° C in the incubator.

After 24hrs the plates were checked for individual colonies and the colonies that had distinct character like colour, texture and size were selected from the 10^{-3}, $10^{-4,}$ $10^{-5,}$ 10^{-6} plates . Those were streaked on to new agar plates by quadrant streaking method again kept for incubation in the same temperature requirement for 24 hrs. Pure colonies were obtained.

Nutrient agar slants were prepared for temporary storage of the colonies. For that nutrient broth and agar was added in the following constitution-

- Nutrient broth 2 gm

- Agar agar 3 gm
- Distilled water 150 ml

It was heated until dissolved and poured into test tubes, 10 ml each. The test tubes were covered with cotton plug and autoclaved. After sterilisation test tubes were placed in the LAF in a slanting position and waited until solidified. The colonies from the plates were transferred to each slant and those slants were kept for incubation at 30-37° C for 24hrs. After incubation period the slants were wrapped with cling film and stored in the refrigerator for further analysis.

2. Enzymatic Screening of Bacterial Strains

Enzymatic screening was done as preliminary screening. The isolates were screened based on the protocol by Geetha *et al.*, (2014) for the production of amylase, gelatinase and protease. A positive result was indicated by a zone of clearance around the colony. Diameter of the zone of clearance was measured using a scale in centimetres.

i. **Test for Amylase**

Starch agar medium plates were prepared by using ready-made starch agar medium along with agar.

The starch agar medium contains the following contents per litre,

- Beef extract 3.0 gm
- Peptone 5.0 gm
- Soluble starch 2.0 gm
- Agar 15.0 gm

200 ml medium was prepared. The following composition was followed for medium preparation.

- Starch agar medium 5 gm
- Agar 1 gm
- Distilled water 200 ml

The medium was prepared in a conical flask, covered with a cotton plug and autoclaved. petri plates were placed in the LAF and the medium was poured into each plates, waited for an hour to cool down. After solidification of the medium each bacterial strain was spot inoculated on to the starch agar plates. The plates were then kept in the incubator at 30°C for 48 hours. After the incubation period the plates were flooded with iodine solution at the end of the incubation period, held for a minute, and then drained off. Iodine forms a blue color complex when it combines with starch. The color blue fades quickly. The colorless zone around colonies suggests amylase production.

ii. **Test for Gelatinase activity**

The presence of gelatinase production on plates were determined using gelatin agar plates containing 0.1 percentage of gelatin. For that the following constitution was made-

- Nutrient media 2.6 gm
- Agar 4 gm
- Gelatin 0.2 gm
- Distilled water 200 ml

The media was prepared and autoclaved lower than the usual time. Extended exposure to heat may damage the gelatin. Petri plates were autoclaved separately. The medium was poured into each plates and kept to solidify. After solidification, the bacterial strains were spot inoculated onto the medium plates. They were kept for incubation at 30°C for 48 hours. Gelatinase production was indicated by a zone of clearance surrounding the colonies.

iii. **Test for Protease Activity**

The qualitative assay for protease production was performed on sterile skim milk (1%) agar plates. The media was prepared by adding nutrient medium, agar and skimmed milk powder. The contents were taken in the following composition.

- Nutrient medium 2.6 gm
- Agar 4 gm
- Skim milk. 2 gm
- Distilled water 200 ml

The media was autoclaved only for 15 mins. Extended exposure to heat damages the skimmed milk powder. Petri plates were autoclaved separately. The media was poured into the sterile plates. After solidification, isolates were spot inoculated and followed by incubation at 30 ° C for 48 – 72 hrs. After the incubation period the plates were examined for zone of clearance around the colony. This zone of inhibition indicates the enzymatic degradation of protein by the protease enzyme.

3. Screening for Plant Growth Promoting Characters

The screening for the plant growth promoting characters were conducted by following the protocol by Benedetto *et al.,* (2019). The isolates were tested for characters like production of ammonia, phosphate solubilization and production of IAA.

i. **Production of Ammonia**

Bacterial isolates were tested for the production of ammonia in peptone water. Freshly grown cultures were inoculated in 10 ml peptone water in each tube separately. Peptone water was prepared according to the Himedia composition that contains-

- Peptone 1 gm
- Sodium chloride 0.5gm
- Distilled water 100 ml

The media was prepared and transferred to test tubes 10 ml each. This was autoclaved with cotton plugs plugged in. After sterilization process, the test tubes containing the media was kept for cooling down the temperature to room temperature. Then the media was inoculated with the bacterial strains using an inoculation loop. It was then incubated for 48-72 h at 28 ± 2°C. After the

incubation period, the isolates were tested for presence of ammonia. Nessler's reagent (0.5 ml) was added in each tube.

- Mercuric chloride 10 gm
- Potassium iodide 7 gm
- Sodium hydroxide 16 gm
- Distilled water 100 ml
- pH 13.2±0.05

Development of brown to yellow color was a positive test for ammonia production.

ii. **Phosphate Solubilizing Activity**

The plates were prepared with Pikovskaya's medium. The medium has the following composition in 500 mL-

- Glucose 5 gm
- $MgCl_2$. 6 H_2O 2.5 gm
- $MgSO_4$. 7 H_2O 0.125 gm
- KCl 0.1 gm
- $(NH_4)_2 SO_4$. 0.05 gm
- Tricalcium phosphate 5 gm
- Agar 7.5 gm

The media was prepared and autoclaved along with the petri plates. Then the medium was poured into each petri plate and kept for solidifying. After solidification, the plates were inoculated with the bacterial strains by spot inoculation. It was incubated in an incubator at 28 - 30°C for 3-7 days. The plates were then examined and data were recorded. A clear zone around the colony indicated a positive result.

iii. **Production of the IAA**

Luria Bertani (LB) broth (Himedia) medium modified with tryptophan is used as the test medium. LB broth contains the following constituents.

- Tryptone 10 gm/L
- Yeast extract 5 gm/L
- Sodium chloride 10 gm/L
- Final pH 7.4±0.2

The following constitution of the contents were made to prepare the medium.

- LB broth 1.3 gm
- Tryptophan 1 gm

The media was transferred to test tubes about 5 mL each, plugged with cotton plug, covered and autoclaved for 20 mins. After sterilization the test tubes containing medium was kept to cool down to room temperature. After cooling down, the bacterial isolates were transferred to these test tubes using an inoculation loop. The inoculated tubes were incubated for 24 hours at 28°C.

After the incubation period 2 to 3 drops of orthophosphoric acid were added to 2 ml of supernatant. 4 mL of Salkowski reagent was also added and incubated at room temperature for 25 minutes. Salkowski reagent was prepared by mixing

- 0.5M $FeCl_3$ 1 ml
- 70% per chloric acid 50 ml

Positive results showed formation of pink color. This indicates IAA Production by the bacterial strains. Yellow coloration or no color change showed a negative result.

4. Morphological Characterization

i. **Gram Staining**

Gram's staining was conducted by following the protocol by Smith and Hussey (2005) for Gram's staining, the bacterial smear was dried in the vicinity of the flame and allowed to cool. It was then stained with crystal violet solution for 1 minute followed by rinsing with water for few seconds. Then the slide was flooded with mordant - Gram's iodine solution and wait for 1 minute. It was followed by adding ethyl alcohol and again wait for 1 minute. Then the slide was again washed with water, drained. The smear was counterstained with safranin for 30 seconds. Finally the slide

was washed with water, drained and air dried and observed under the light microscope at 40 x magnification. At the completion of the Gram Stain, gram-negative bacteria will stain pink/red and gram-positive bacteria will stain blue/purple.

5. Biochemical Characterization

The biochemical characterization of the bacterial isolates were done. Tests were conducted according to the protocol by Shoaib e*t al.,(*2020).

i. Indole Production Test

Tryptone broth medium is was the test medium**.** Tryptone broth medium contained the following constituents.

- Tryptone 1gm/L
- Sodium chloride 0.5gm/L
- Distilled water 100ml

The medium was dispensed 5mL into each test tubes, autoclaved at 15 lbs for 15 minutes. Inoculated the tube of Tryptone water with a loopful of overnight culture of bacterial strains. Incubated for 48 hours –99 hours at 37^{0} C. After incubation, 0.5mL of Kovac's reagent was added to each test tube and kept for 1-5 minutes. The appearance of dark red color on the surface layer which indicated the test was positive.

ii. **Methyl red Test**

Glucose-phosphate peptone water medium was used. Glucose-phosphate peptone water medium contains the following composition-

- Glucose 2.5 gm

- Potassium phosphate 2.5 gm
- Peptone 3.5gm
- Distilled water 500 ml

5ml glucose-phosphate peptone water medium was dispensed in small culture tubes, plugged and autoclaved at 15 lbs (112^{0}C) for 10 minutes. Inoculated the tubes with the bacterial strains. Incubated for 24-96 hours at 28-37 ^{0}C or until good growth was obtained. After incubation, approximately 5-10 drops of methyl red indicator was added slowly along the sides the tube to form a layer on the surface of the tube. Observe the ring of color formed. Red color indicated positive and yellow indicates negative.

iii. **Voges-Proskauer Test**

Glucose-phosphate peptone water medium was used as the test medium. Glucose-phosphate peptone water medium contains the following composition-

- Tryptone 1gm/L
- Sodium chloride 0.5gm/L
- Distilled water 100ml

5ml glucose-phosphate peptone water medium was dispensed in small culture tubes, plugged and autoclave dat 15 lbs (112^{0}C) for 10 minutes. The tubes inoculated with the bacterial strains.

After inoculation, equal amount of Barritt's reagent A and Barritt's reagent B was added to each test tube. Agitated thoroughly and maintained for 1-2 hours. Development of pink or red coloration indicates positive test and no change or faint brown color indicates negative.

iv. Citrate Test

Isolates were stabbed into Simmons citrate agar slants. It has the following composition-

- Magnesium sulphate 0.1gm/l
- Ammonium dihydrogen phosphate 0.5gm/l
- Potassium hydrogen phosphate 0.5gm/l
- Sodium citrate 1gm/l
- Sodium chloride 2.5gm/l
- Bromothymol blue 0.04gm/l
- Agar 7.5 gm/l
- Distilled water 500ml

Medium was made in a conical flask and 5ml of the medium was taken in each test tube which was covered with a cotton plug and autoclaved at 15 lbs (112^{0} C) for 10 minutes. The bacterial strains were stabbed into Simmons citrate agar slants using an inoculating loop and then zig-zagged back and forth on the surface of the slant and incubated at 28±2oC for 24hrs. Change in color from green to Prussian blue indicated positive reaction for citrate utilization.

v. Catalase Test

Luria Bertani (LB) broth (Himedia) medium modified with tryptophan ws used as the test medium. LB broth contained the following constituents.

- Tryptone 10 gm/L
- Yeast extract 5 gm/L

- Sodium chloride 10 gm/L
- Final pH 7.4±0.2

The media was prepared and 5ml of the medium was taken in each test tubes, plugged with cotton plug and autoclaved for 20 min. After sterilization the test tubes containing medium were kept to cool down to room temperature. After cooling down, the bacterial isolates were transferred to these test tubes using an inoculation loop. The inoculated tubes were incubated for 24 hours at 28°C.

Under aseptic condition a drop of H_2O_2 was placed on a clean sterile glass slide with the help of a pipette. With a sterile inoculating loop, a small amount of culture was placed on this and looked for immediate bubbling.

The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result.

vi. **MacConkey Agar Test**

The MacConkey agar media was used as the test medium**.** MacConkey agar medium contained the following constituents.

- Peptic digest of animal tissue 8.5gm/l
- Proteose peptone 1.5gm/l
- Lactose 5 gm/l
- Bile salts 0.75gm/l
- Sodium chloride 2.5gm/l
- Distilled water 500ml

The medium was prepared in a conical flask and covered with a cotton plug and autoclaved. It was then transferred to the autoclaved petri plates. It was allowed to cool. Then the bacterial strains are streaked on the petri plates. Incubated the plates for 18 to 24 hours at 35^{o} - 37^{o}. If the strains are

lactose fermenting strains then they grow as red or pink color and may be surrounded by a zone of acid precipitated bile.

6. Molecular Analysis of Screened Isolates using PCR

Molecular Characterization and 16S rRNA gene sequence analysis for identification of species of the isolates were conducted . The sequences were analysed using bioinformatic tools for species level identification.

i. Genomic DNA isolation

Genomic DNA isolation was done following the protocol (Esteban *et al.*, 1993).

- A single colony of the isolate was inoculated into Luria Broth (Hi media, India) and incubated at 37^{o}C overnight with constant shaking
- 1.5 mL culture was taken in a microfuge tube and centrifuged at 8000 rpm (Sigma, Germany) for 5 min.
- The pellet was resuspended in 567µL Tris-EDTA (TE) buffer (pH 8)
- To the suspension, 30µL of 10% sodium dodecyl sulphate (SDS) and 3µL Proteinase K (20mg/mL) were added and mixed well
- The culture was then incubated for 1h at 37^{o}C in a water bath.
- 100µL of 5M NaCl and 80µL Hexadecyl trimethyl ammonium bromide (CTAB) (10mg/mL) were added and incubated for 10min at 65^{o}C in a water bath.
- The tubes were allowed to cool to room temperature and an equal volume of chloroform-isoamyl alcohol (24:1) was added.
- The contents were mixed gently and centrifuged at 10000 rpm for 10min.
- The aqueous layer at the top, containing the DNA, was carefully transferred to a fresh microfuge tube using a blunt end sterile tip.
- An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added to the aqueous layer and centrifuged again at 10000 rpm for 10 min.
- The aqueous layer was collected in a fresh tube and 0.6 volume of isopropanol was added to it.
- The contents were mixed gently and centrifuged at 12000 rpm for 10min.

- The supernatant was discarded and the pellet was washed with 70% ethanol and centrifuged.
- The supernatant was discarded and the pellet was dried at room temperature.
- The purified DNA was then dissolved in 100µL TE buffer (pH 8).

The concentration of genomic DNA thus obtained was estimated by the spectrophotometric method (Shimadzu UV-VIS Spectrophotometer, Japan). The purity of the DNA was assayed spectrophotometrically using the automatic calculation of the ratio of optical densities at 260 and 280 nm. The quantification of DNA was done using DNA/Protein pack® software of spectrophotometer. DNA was stored at -20^0C until further use. An appropriate dilution (~80-100ng) of genomic DNA was used as template for PCR reactions.

ii. 16S rRNA sequence analysis

A PCR based method using a primer pair for 16S rRNA was used for species identification (Shivaji et al., 2000; Reddy et al., 2000, 2002a; b). A portion of the 16S rRNA gene (1.5kb) was amplified from the genomic DNA. Products after PCR amplification was purified by gene clean kit (Genei, India) and subsequently sequenced, followed by homology analysis.

Sequence	Primer	Reference
16SF	5' AGTTTGATCCTGGCTCA 3'	Shivaji et. al.,2000
16SR	5' ACGGCTACCTTGTTACGACT' 3'	Reddy et. al., 2000,2002 a; b

iii. Polymerase Chain Reaction (PCR)

PCR was performed using the genomic DNA (~100ng) as template and16S rRNA specific primers as detailed above.

a. PCR Mix composition

PCR buffer (10X)	2.0µL
dNTPs (2.5mM)	2.0µL
Forward primer (10µM)	1.0µL

Reverse primer (10μM)	1.0μL
Taq DNA polymerase (1U)	0.2 μL
Template DNA	0.5 μL
$MgCl_2$ (1.5mM)	1.2μL
Sterile distilled water to make the final volume to	20μL

b. PCR conditions

Annealing	- 56^{o}C	-30 sec.
Extension	- 72^{o}C	-2 min.

PCR assays were performed in MJ Mini Thermal cycler (Bio-Rad, USA).

iv. Agarose gel electrophoresis

The agarose gel electrophoresis was carried out for the visualization of PCR products. Agarose gels of appropriate strength (1 - 2%) depending on the size of the PCR product were prepared in Tris-Acetate-EDTA (TAE) buffer. Ethidium bromide (EtBr) solution was added at a concentration of 10mg/mL. Definite volume of PCR product was mixed with gel loading dye and loaded into the wells. DNA markers were run along with the products for confirmation of amplicon size. Electrophoresis was performed at a constant volt (5V/cm) (Genei, India) and the gel pictures were captured with Bio-Rad Gel documentation system.

vi. *In silico* nalysis of the 16S rDNA sequences

a.DNA sequencing and analysis

Products after PCR amplification were purified by gene clean kit (Genei, India). The products were sequenced by Sanger's Dideoxy method using ABI 3730 Excel at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. The sequenced PCR products were analysed online using BLAST software (http://www.ncbi.nlm.nih.gov/blast) and the identity of the sequences were established.

OBSERVATION AND RESULTS

Bacterial strains were isolated from the soil by serial dilution and spread plate method. 24 colonies were isolated from the 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} diluted samples. They exhibited difference in their colony characters such as color difference, texture and colony size.

Most of the colonies were milky white or white in color and had different textures like slimy, sticky.

The results of the extracellular enzyme screening, biochemical analysis, morphological screening and molecular identification are given below:

1. Enzymatic Screening of Bacterial Strains

The isolates were screened for the production of amylase, gelatinase and protease. The diameter of the zone of clearance formed around the colonies were measured using a scale in centimeters.

i. Test for Amylase

The appearance of clear zone surrounding the colony indicated positive result for starch hydrolysis test. Out of 24 strains tested, 20 strains of bacteria exhibited positive results for amylase activity.

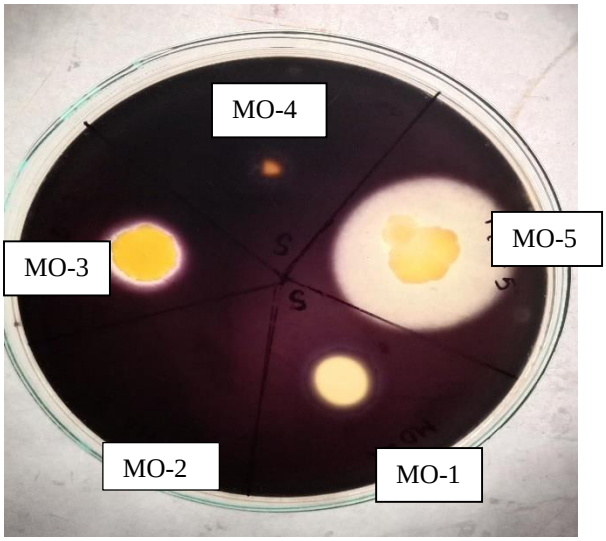


Plate. 1. MO-1 -, MO-2 -, MO-3 +, MO-4 -, MO-5 +

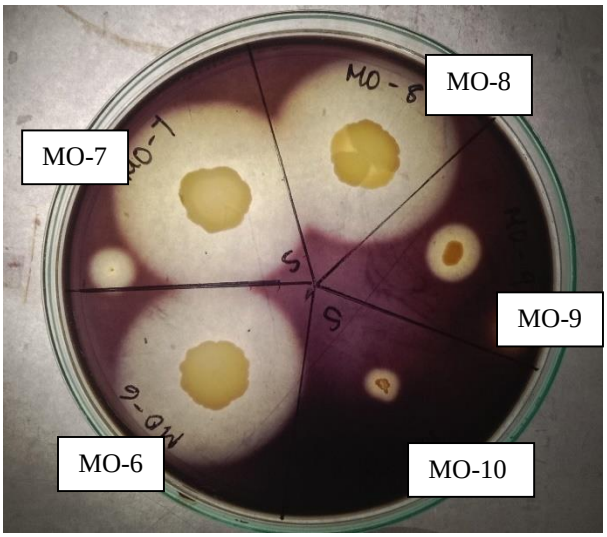


Plate. 2. MO-6 +, MO-7 +, MO-8 +, MO-9 +, MO-10 +

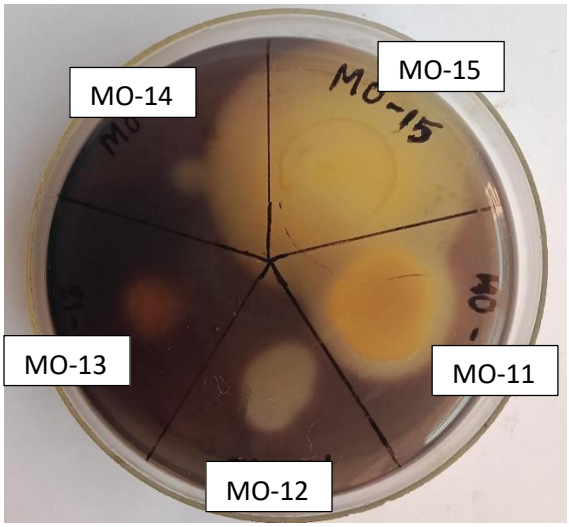


Plate. 3. MO-11 +, MO-12 +, MO-13 +, MO-14 +, MO-15+

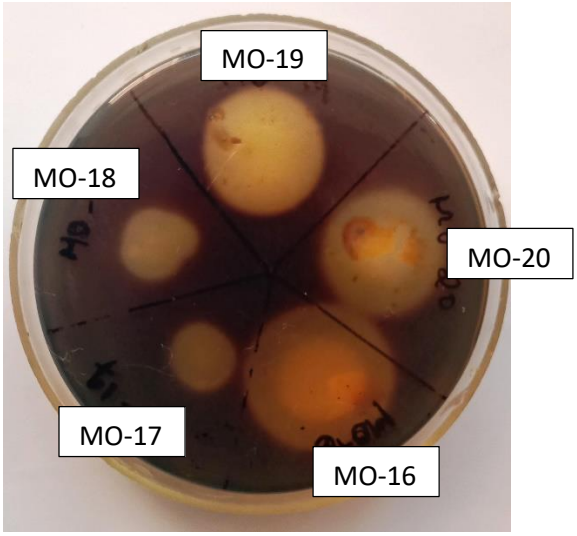


Plate. 4. MO-16 +, MO-17 +, MO-18 +, MO-19+-, MO-20+

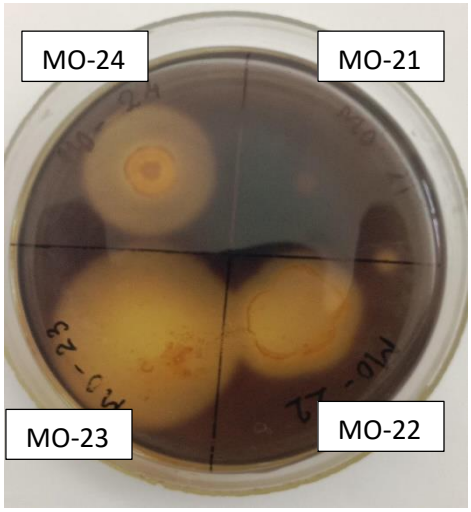


Plate. 5. MO-21 +, MO-22 +, MO-23 +, MO-24+

Bacterial strains showing positive results for amylase production.

ii. Test for Gelatinase

None of the strains exhibited the gelatinase production.

iii. Test for Protease

Proteolytic enzyme production was detected as formation of a clear zone around the colony on skim milk agar medium. Out of 24 strains tested, 20 strains showed proteolytic activity.

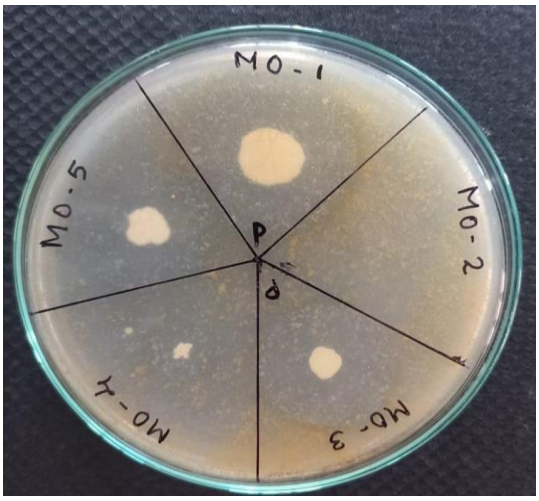


Plate 6. MO-1 +, MO-2 -,MO-3 +, MO-4 +, MO-5+

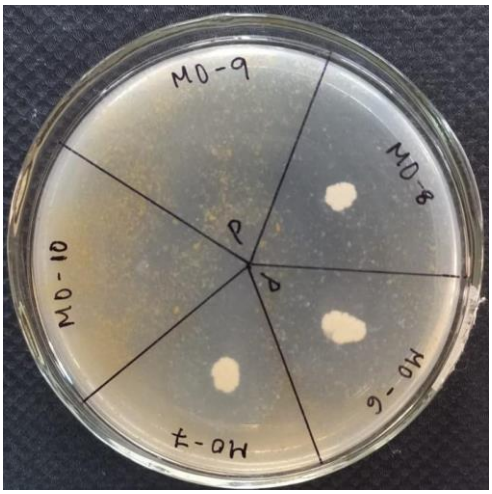


Plate. 7. MO-6 +, MO-7 +, MO-8 +, MO-9 +, MO-10 +

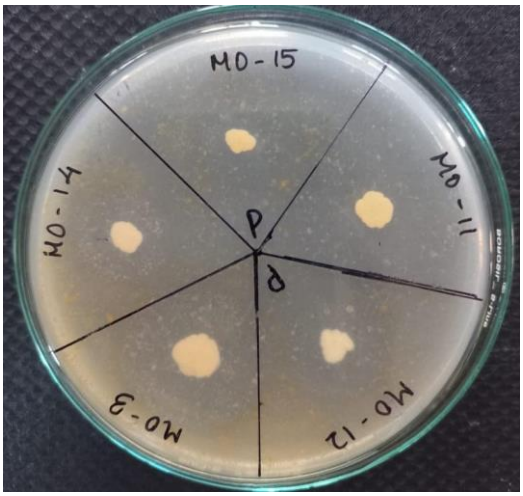


Plate. 8. MO-11 +, MO-12 +, MO-3 +, MO-14 +, MO-15+

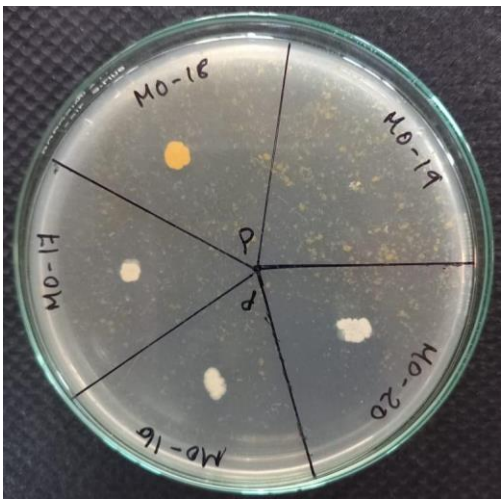


Plate. 9. MO-16 +, MO-17 +, MO-18 +, MO-19 -, MO-20+

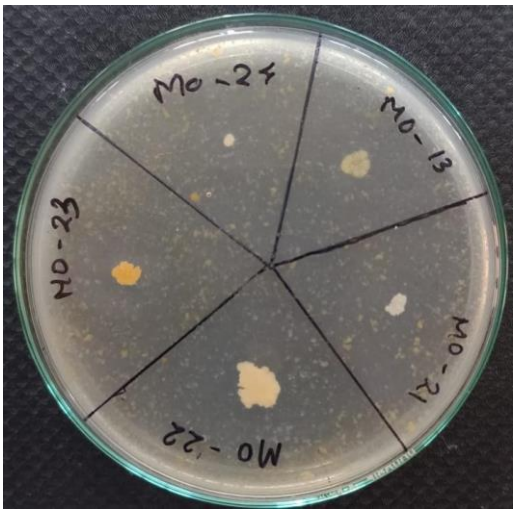


Plate. 10. MO-21 +, MO-22 +,

MO-23 +, MO-24+, MO-13+

Bacterial strains showing positive results for protease production.

The following table provides the results of the enzymatic screening of the strains. The diameter of the zone of clearance in centimeters is given:-

Table 1. Enzymatic screening of the bacterial strains.

Bacterial strains	Amylase production (in cm)	Protease production (in cm)	Gelatinase production (in cm)
MO-1	--	+ 3.4cm	--
MO-2	--	--	--
MO-3	+ 1.5cm	+ 2.6cm	-
MO-4	--	+ 1cm	-
MO-5	+ 3.1cm	+ 2.4cm	-
MO-6	+ 3.4cm	+ 2.9cm	-
MO-7	+ 3.4cm	+ 2.5cm	-
MO-8	+ 3.3cm	+ 3cm	-
MO-9	+ 3.2cm	--	-
MO-10	+ 0.6cm	--	-
MO-11	+ 2.2cm	+ 2.4cm	-

MO-12	+ 1.5cm	+ 2.5cm	-
MO-13	+ 1.2cm	+ 1.9cm	-
MO-14	+ 0.5cm	+ 2cm	-
MO-15	+ 4.2cm	+ 2.4cm	-
MO-16	+ 2.6cm	+ 1.7cm	-
MO-17	+ 1.1cm	+ 1.9cm	-
MO-18	+ 1.3cm	+ 2cm	-
MO-19	+ 2cm	-	-
MO-20	+ 2.1cm	+ 1.6cm	-
MO-21	-	+ 1cm	-
MO-22	+ 2.2cm	+ 3cm	-
MO-23	+ 3cm	+ 2cm	-
MO-24	+ 2.3cm	+ 1.2cm	-

Out of the 24 strains tested, best performing 10 strains were selected for the next stage of screening – plant growth promoting characters. They are, MO-5, MO-6, MO-7, MO-8, MO-11, MO-15, MO-16, MO-20, MO-22, and MO-23.

2. Screening for plant growth promoting characters

i. Production of Ammonia.

In the test for ammonia, after addition of Nessler's reagent all the bacterial strains were tested positive for ammonia production. 4 strains showed dark yellow color. They are: -MO-5, MO-7, MO-8, and MO - 20.

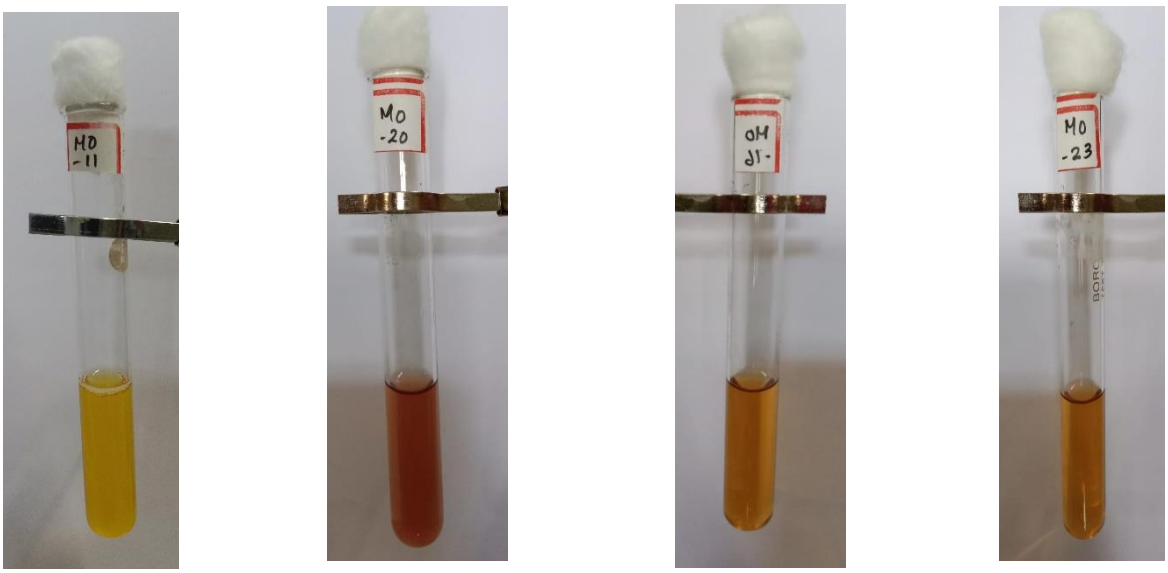


Plate 11. Bacterial strains showing positive results for ammonia production.

ii. Test for phosphate solubilizing activity.

None of the bacterial showed a clear zone around the colonies. All the bacterial strains were tested negative for phosphate solubilization. None of the strains solubilized phosphate present in the medium.

iii. Production of IAA.

On adding Salkowski reagent to the cultures, 9 out of the 10 isolates were tested positive. The negative cultures did not have any color change. Among the 10 isolates 5 exhibited dark pink color. They are - MO- 5, MO- 6, MO- 7, MO- 8, MO-20.

Plate 12. Bacterial strains showing positive results for IAA production.

Table 2. Screening for plant growth promoting characters:-

Bacterial strains	**Ammonia production**	**Phosphate solublisation**	**IAA production**
MO-5	+	-	+
MO-6	+	-	+
MO-7	+	-	+
MO-8	+	-	+
MO-11	+	-	+
MO-15	+	-	+
MO-16	+	-	+
MO-20	+	-	+
MO-22	+	-	+
MO-23	+	-	-

Finally 4 strains that exhibited best results were selected for the morphological analysis and biochemical screening. They are: - MO- 5, MO- 7, MO- 8, & MO-20.

3. Morphological characterization

The previously screened 4 strains i.e., MO- 5, MO- 7, MO- 8, & MO-20 were subjected to Gram's staining and the following results were obtained. All the 4 strains were tested Gram positive. i.e., MO- 5, MO- 7, MO- 8 & MO- 20. They showed a violet color on staining.

4. Biochemical Analysis

i. Indole Production Test

On adding Kovac's reagent to the Tryptone broth no appearance of dark red color on the surface layer was detected. All of the strains were tested negative for indole production test.

ii. Methyl red Test

On adding methyl red indicator to cultures incubated in the glucose-phosphate peptone water medium no red colored ring was formed on the surface of the tube. This indicated a negative result for methyl red test.

iii. **Voges-Proskauer Test**

No red/pink color was formed while adding Baritt's reagent to the cultures incubated in the glucose-phosphate peptone water. They were tested negative.

iv. Citrate Test

There was no change in color of the Simmons citrate agar slants from green to Prussian blue. This indicated a negative result.

v. Catalase Test

No bubbles were formed during addition of the fresh inoculum to a drop of hydrogen peroxide. Therefore no oxygen was evolved and it indicated a negative result.

vi. MacConkey Agar Test

None of the bacteria were able to grow on the MacConkey agar medium. This indicated that none of the strains were gram –ve and enteric bacteria.

Table 3. Biochemical Analysis

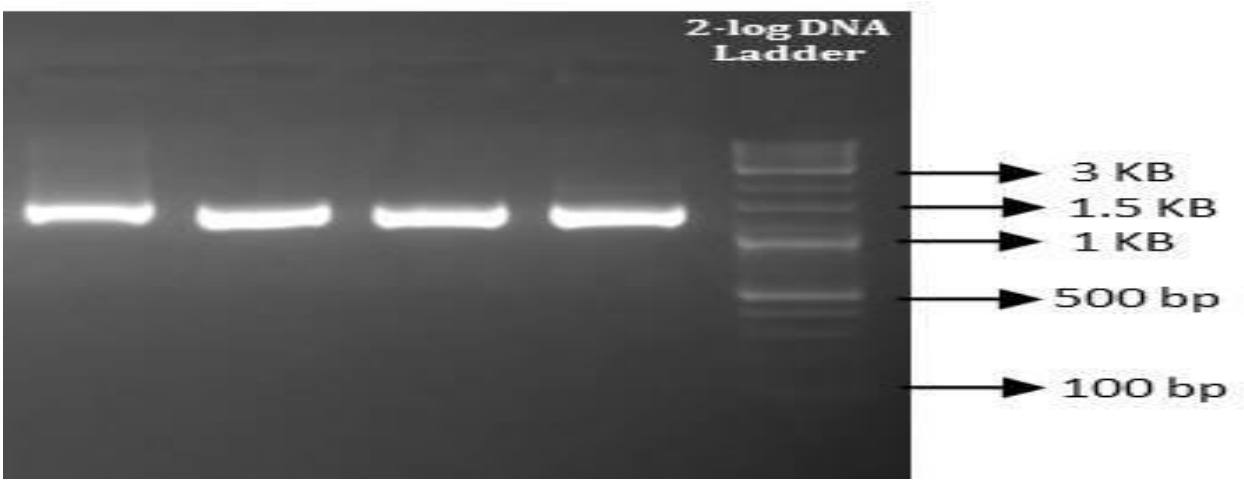
Bacterial strains	Indole test	Methyl red test	Voges-proskauer test	Citrate test	Catalase test	MacConkey test
MO-5	-	-	-	-	-	-
MO-7	-	-	-	-	-	-
MO-8	-	-	-	-	-	-
MO-20	-	-	-	-	-	-

5. Molecular identification of screened isolates using PCR

The 4 isolates were subjected to molecular level of identification based on 16S rRNA Gene Sequence Analysis for identification of species. All the 4 strains belonged to the genus *Bacillus*. The identity of the strains are listed below.

Strain	Identification	% of identity
MO- 5	*Bacillus tropicus* strain 6TM-12	99.85%
MO- 7	*Bacillus cereus* strain MUGA205	99.84%
MO- 8	*Bacillus cereus* strain ES-4a1	99.70%
MO-20	*Bacillus cereus* strain AN630_A3	100%

i. The agarose gel electrophoresis was carried out for the visualization of PCR products.

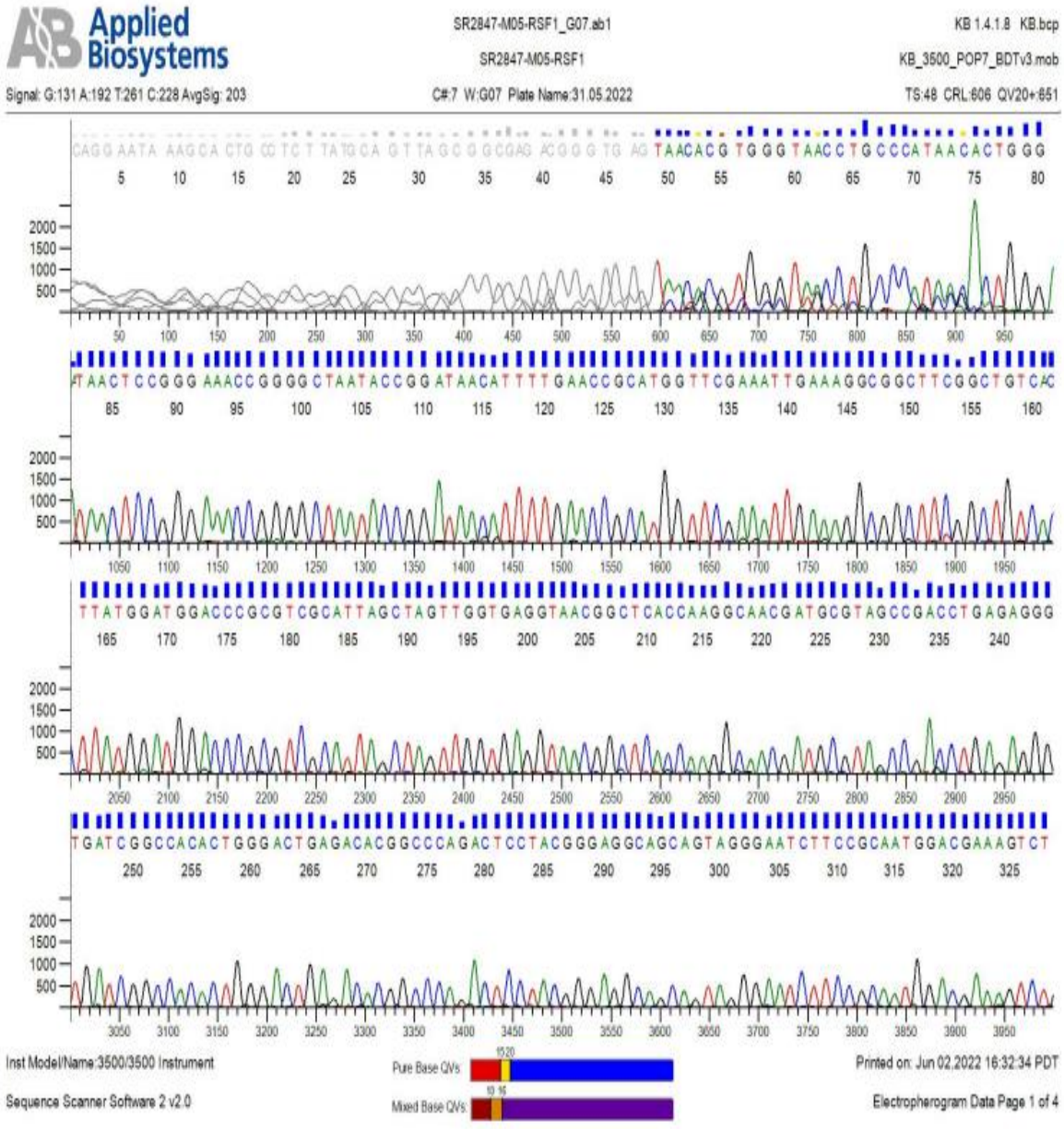


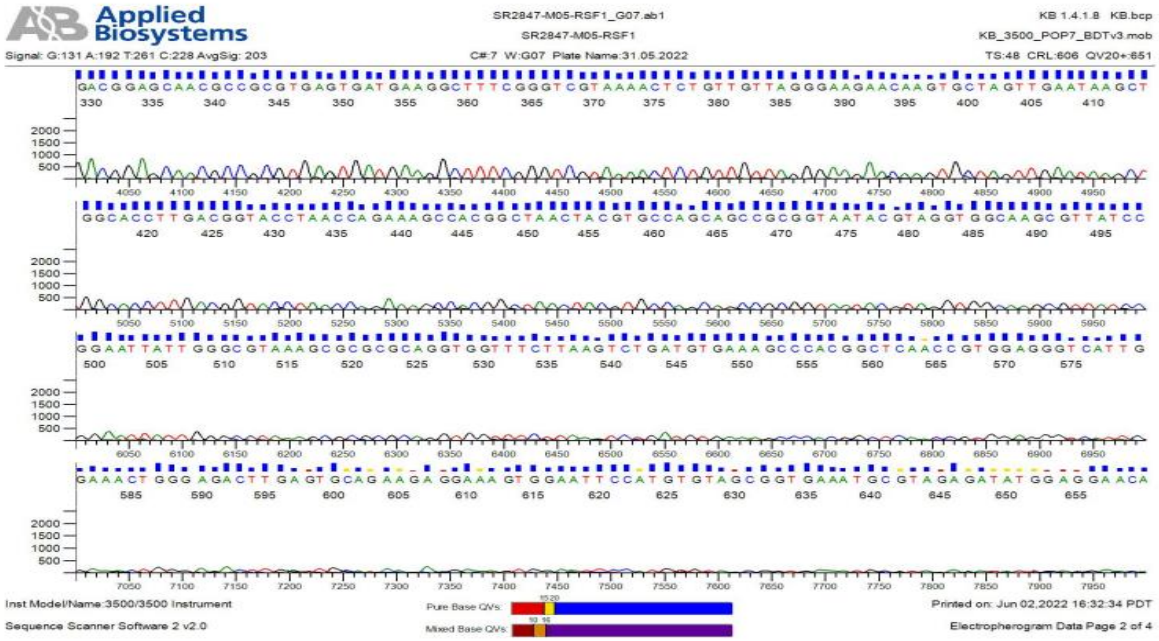
Lane 1- MO-5, Lane 2- MO-7, Lane 3 – MO-8, Lane 4 – MO-20

ii. **Electropherogram of the Identified Bacterial Strains**

Electropherogram was produced when the electrophoresis is used as the analytical technique.

Figure 2. shows the electropherogram of MO-05

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Mixed Base QVs
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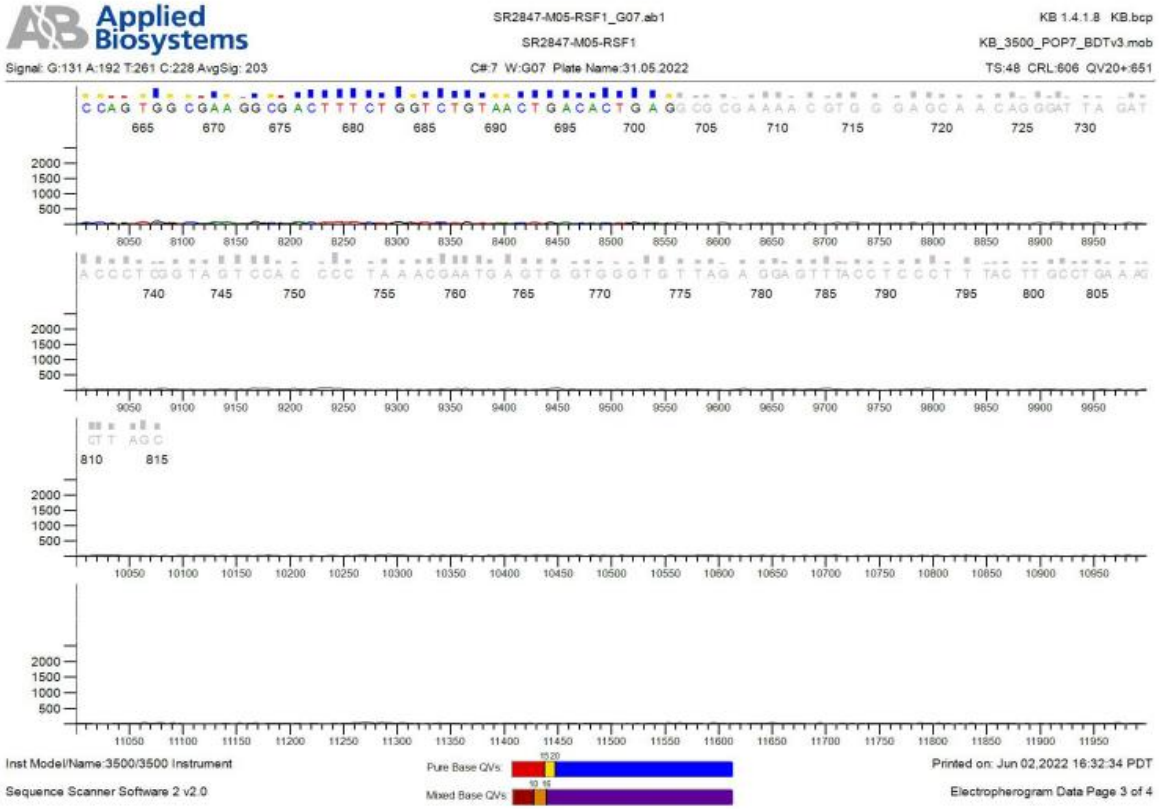
Electropherogram Data Page 2 of 4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Electropherogram Data Page 3 of 4

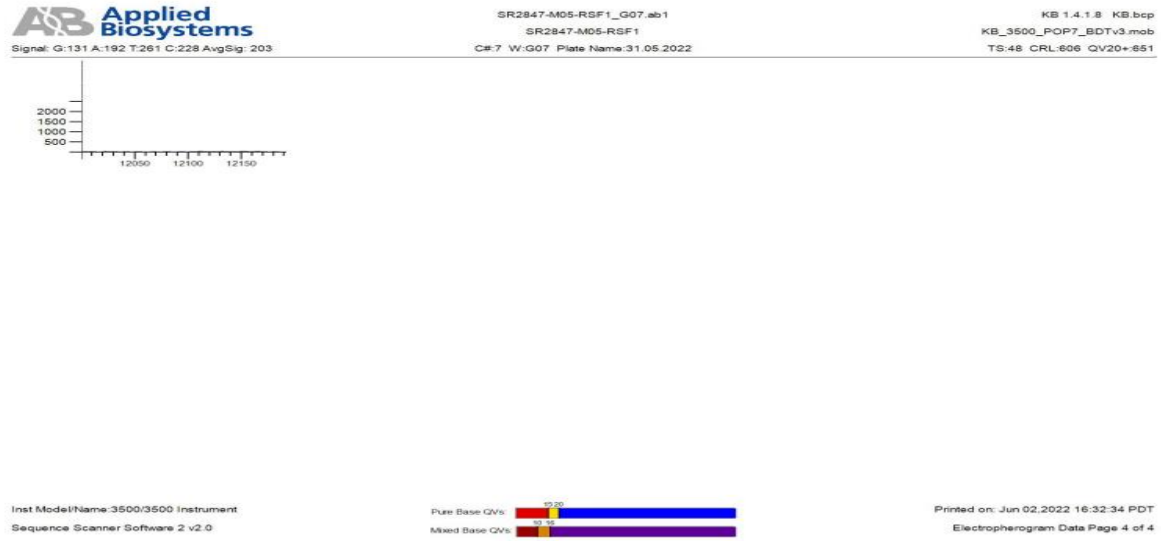
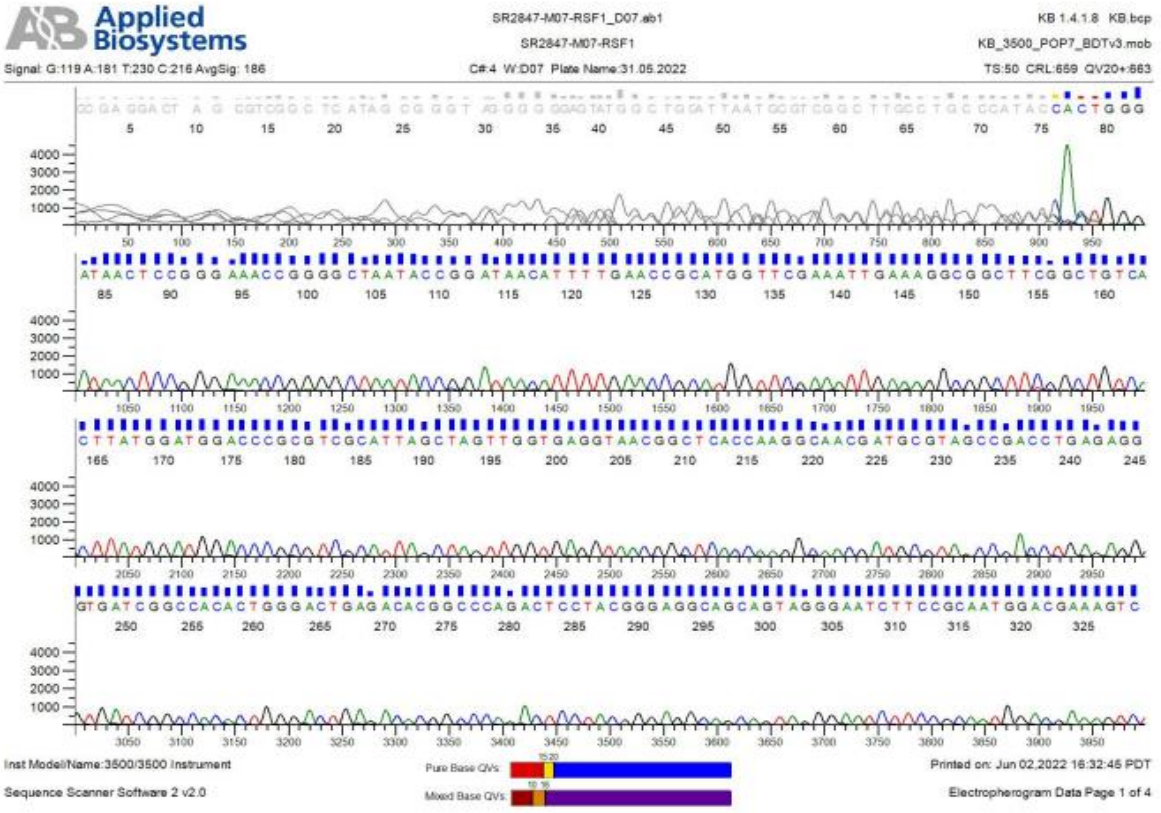
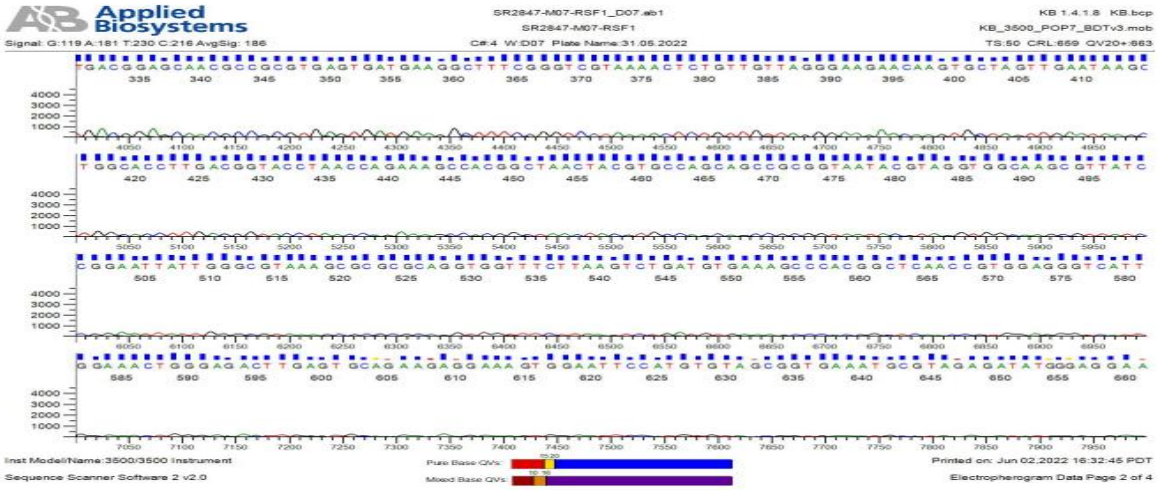


Figure 2. shows the electropherogram of MO-07

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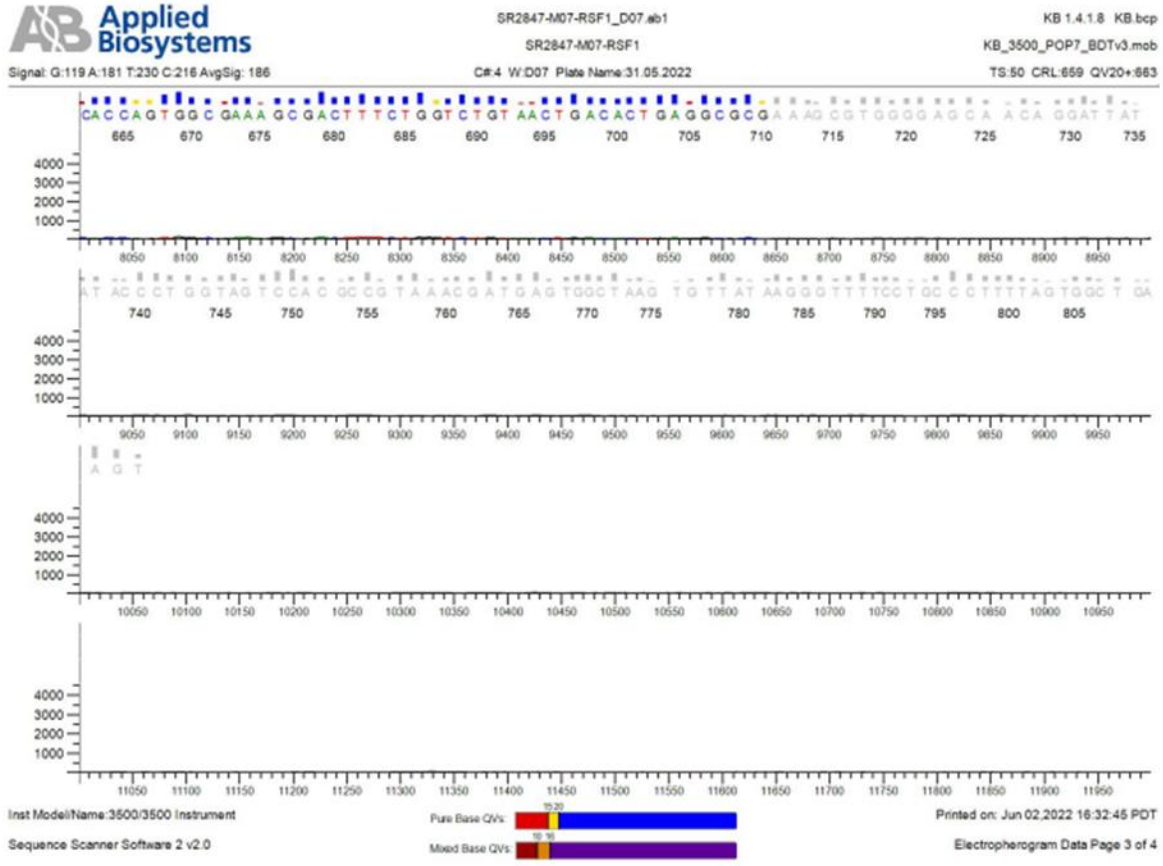
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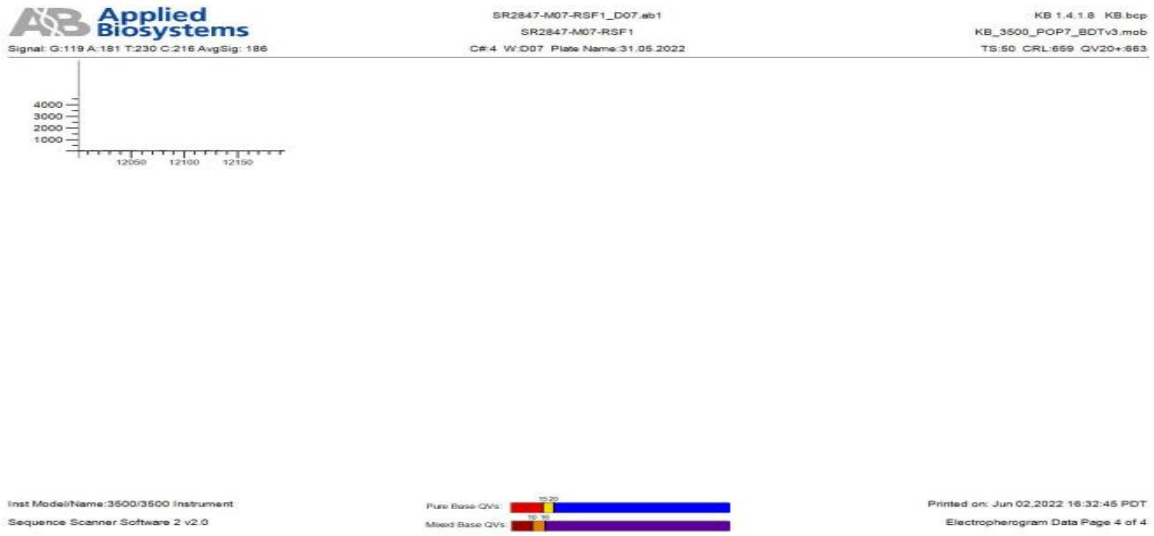

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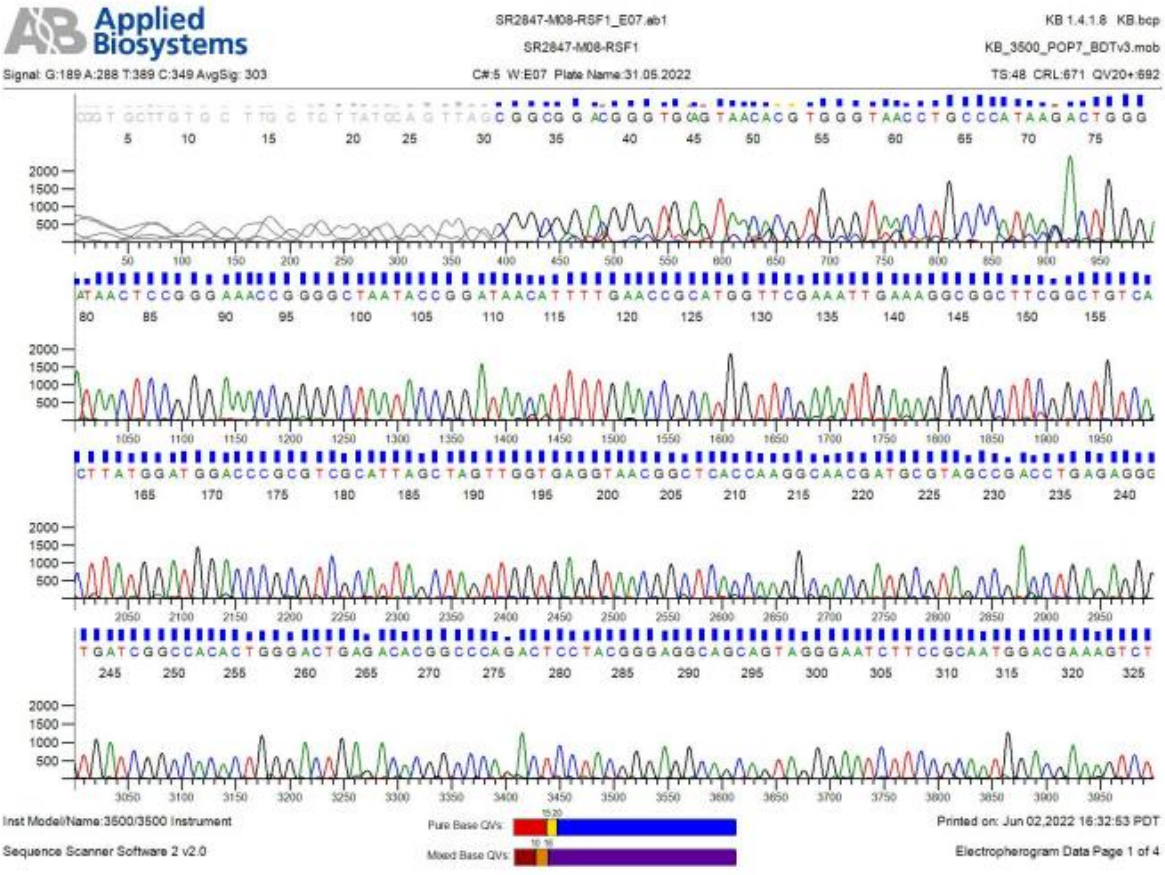

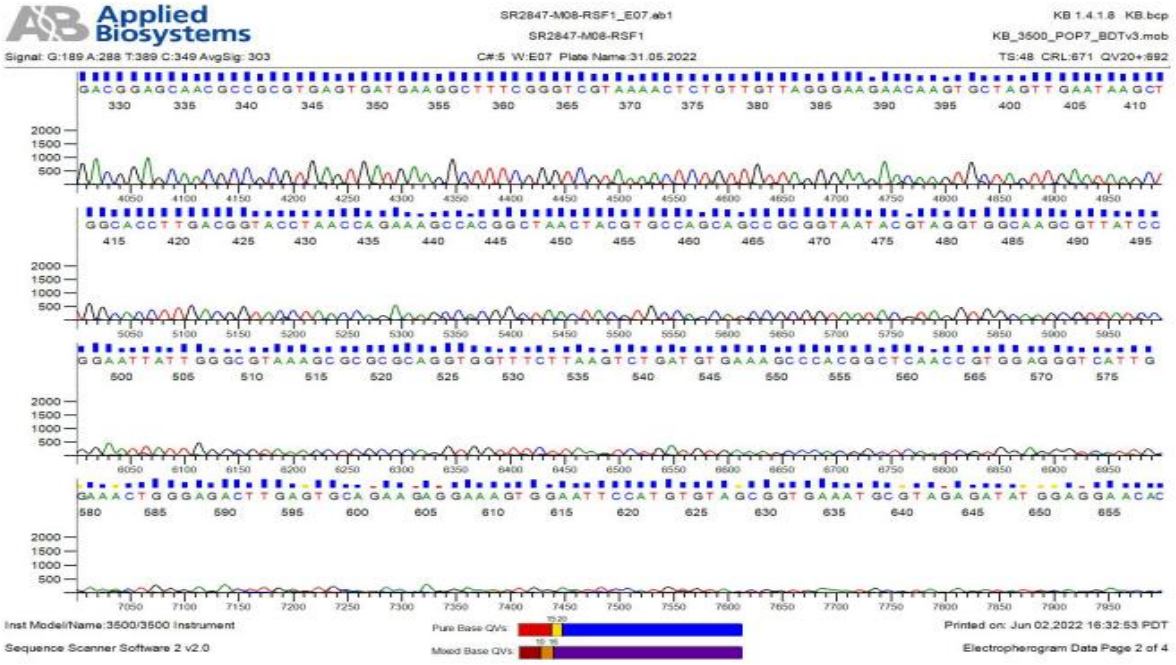
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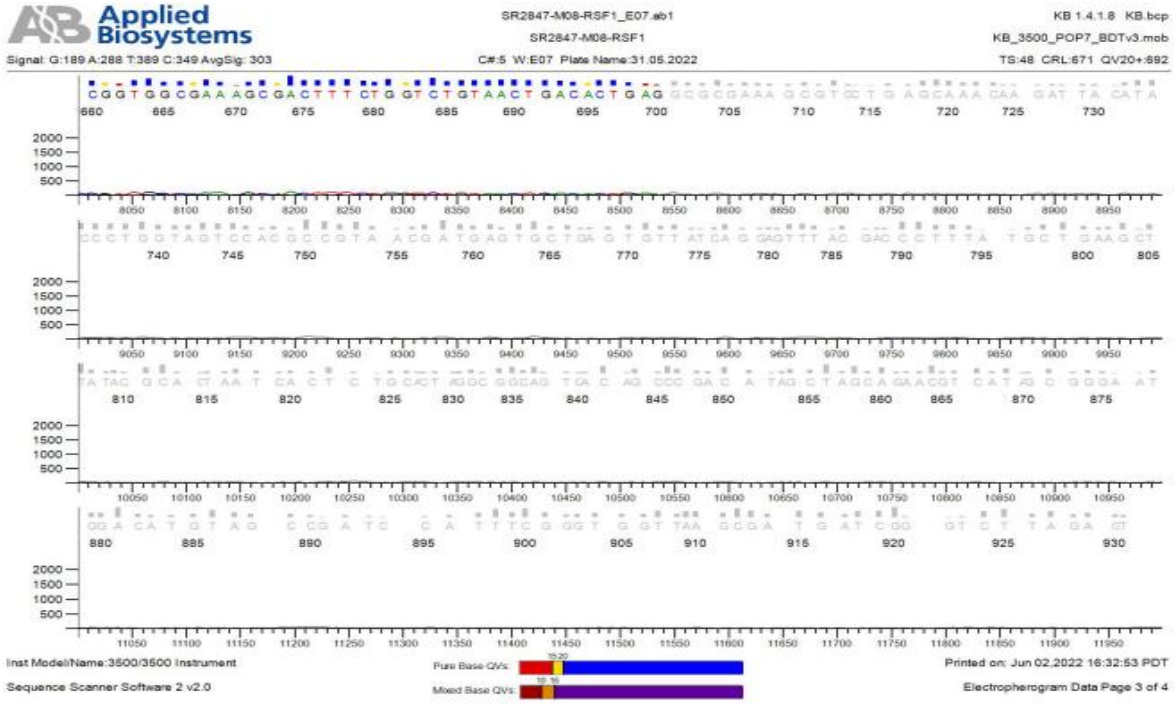
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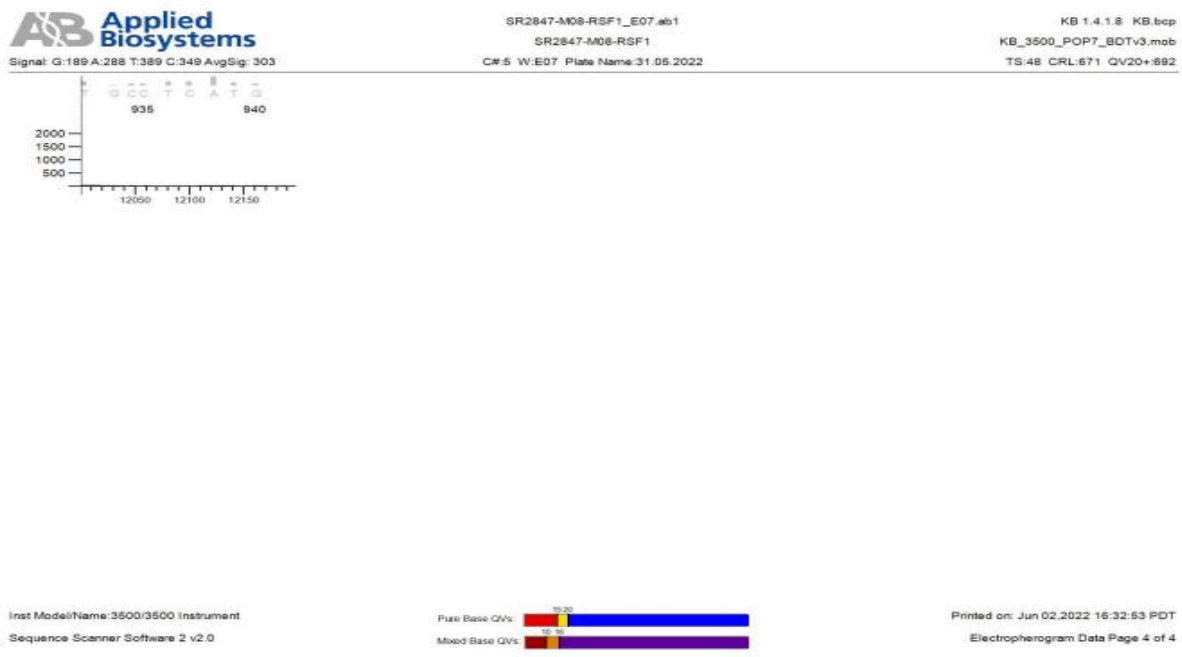
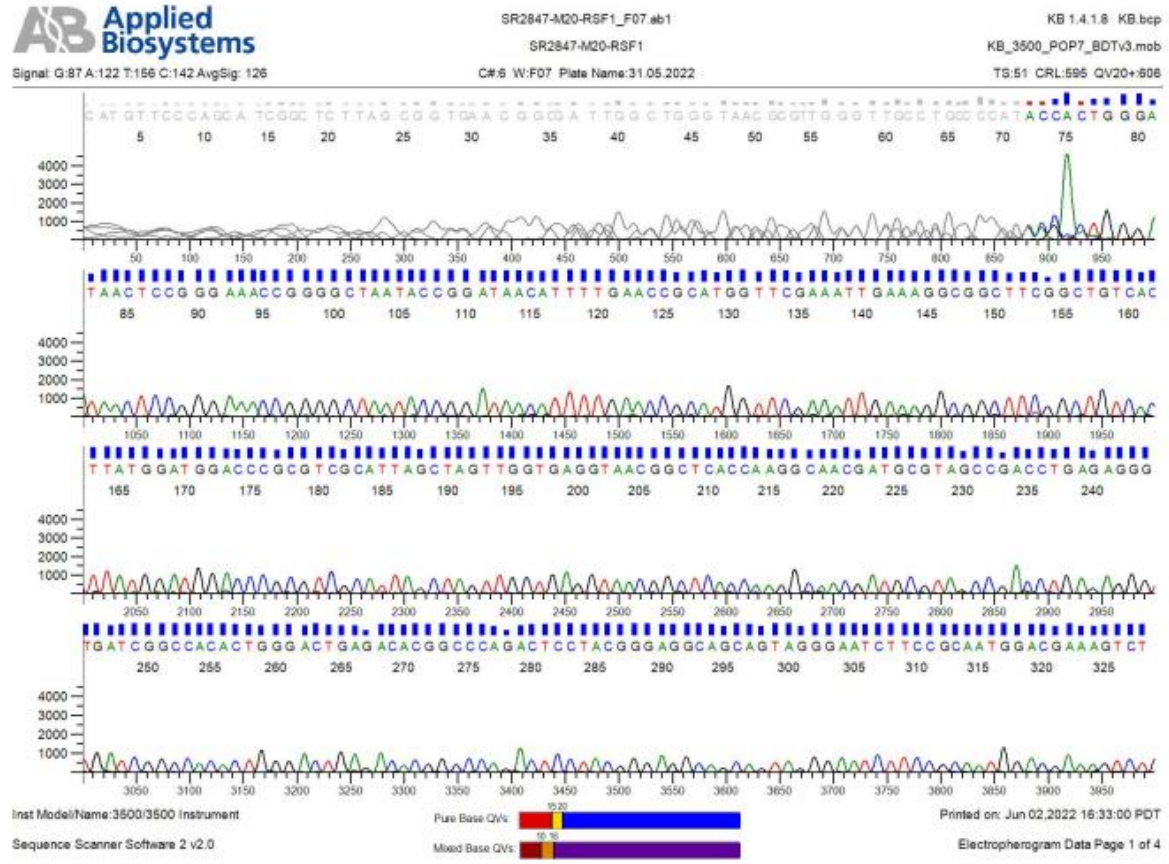
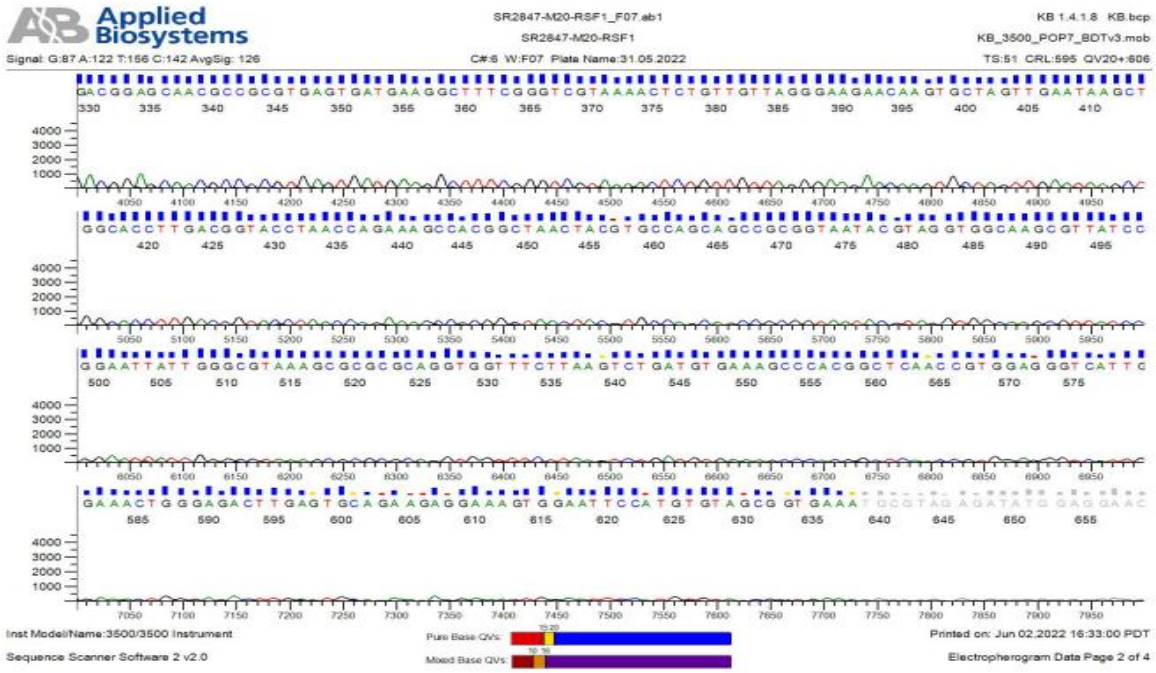


Figure 2. shows the electropherogram of MO-07

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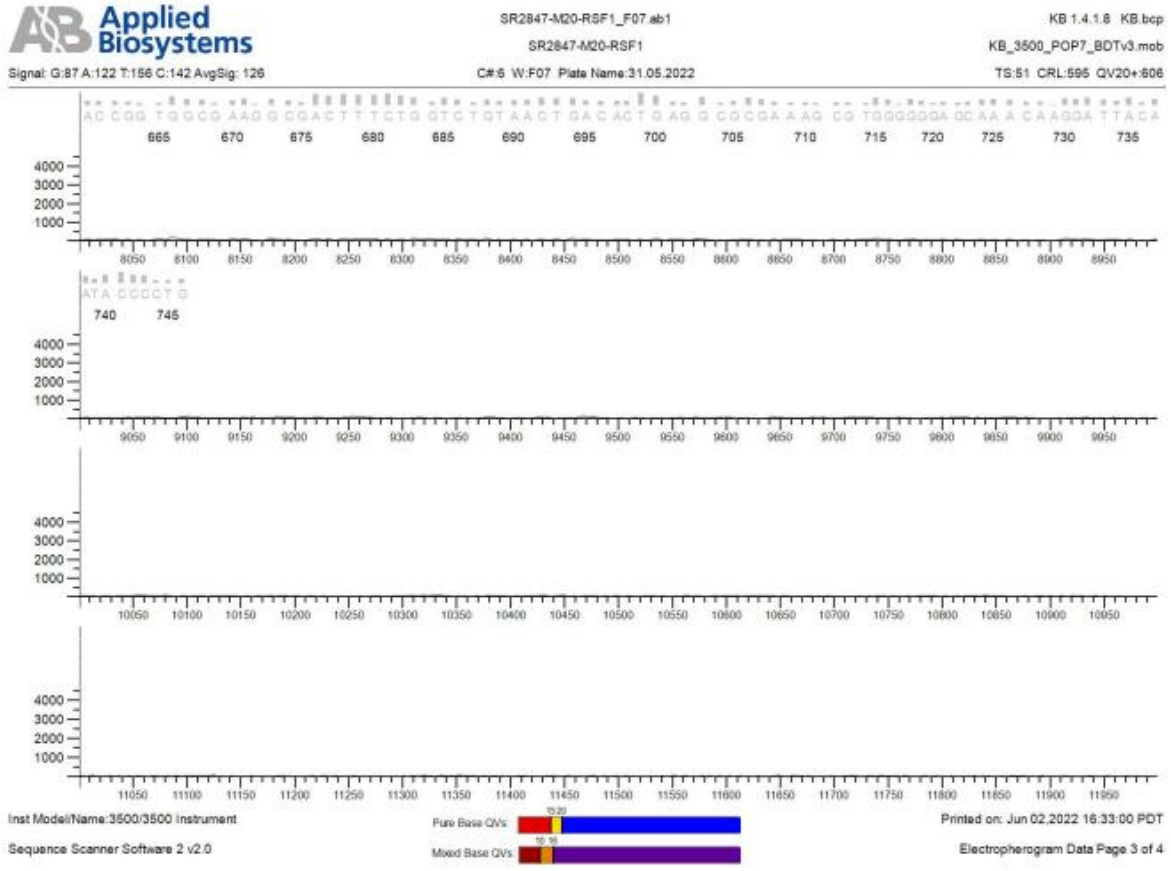
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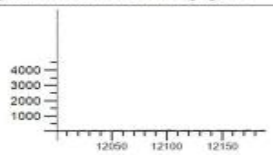

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DISCUSSION

The soil from the rhizosphere of Out of the 24 isolates that were isolated finally 4 isolates were screened on the basis of enzyme test, plant growth promoting characters and biochemical analysis. They are MO- 5, MO- 7, MO- 8, MO- 20.

21 bacterial strains each from the amylase and protease test showed positive results.

Strain MO – 02 exhibited – ve results for all the three enzyme tests. Thus MO-02 does not help directly in plant growth.

MO - 15 isolate had the maximum zone of clearance of 4.2 cm in amylase test. This indicates that MO-15 produces maximum amylase enzyme.

Amylases' main function is to hydrolyze the glycosidic bonds in starch molecules, converting complex carbohydrates to simple sugars. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. There are three main classes of amylase enzymes; Alpha, beta- and gamma-amylase, and each act on different parts of the carbohydrate molecule. In plants, the amylase can be found in seeds to break down starch into sugar to be used by the embryo to induce growth (Esitken, 1970). Amylase enzymes help plants develop as the seeds germinate, sprout, and root. Amylase is an enzyme that acts as a catalyst to hydrolyze carbohydrates. The role of amylase in plants is for breaking down starches. Starches are usually processed in this way during seed germination, and turned into sugars.

The production of α-amylase is essential for conversion of starches into oligosaccharides. Starch is an important constituent of the human diet and is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. Starch-converting enzymes are used in the production of maltodextrin, modified starches, or glucose and fructose syrups (Esitken, 2006).

None of the strains showed a positive result in gelatinase test.

Gelatinase allows the organisms that produce it to break down gelatin into smaller polypeptides, peptides, and amino acids that can cross the cell membrane and be utilized by the organism.

Gelatinase hydrolyzes gelatin, releasing peptides and amino acids into the environment that may be utilized by bacteria as nutrients. Gelatinase is expressed in several bacteria including *Pseudomonas aeruginosa* and *Serratia marcescens.* Mostly gelatinase is produced by fungi. Strains of Bacillus does not produce gelatinase (Goswami *et al.,* 2016).

In the protease test 3.4 cm round clear zone was exhibited by MO-01 strain indicating a high protease production.

Proteases are involved in the physiology of the plants during their entire cycle life (chloroplast photoinhibition, defense mechanisms, photomorphogenesis, and seed germination. The function of proteases is to catalyze the hydrolysis of proteins, which has been exploited for the production of high-value protein hydrolysates from different sources of proteins such as casein, whey and soy protein (Saharan & Nehra, 2011).

From the preliminary screening for enzymatic production, 10 best performing bacterial strains were selected for PGP characterization.

The strains MO-5, MO-6, MO-7, MO-8 and MO-20 exhibited the maximum production of IAA.

IAA is a derivative of indole, containing a carboxymethyl substituent. It is a colorless solid that is soluble in polar organic solvents. IAA is predominantly produced in the apical bud of and young leaves of plants and is known to be an inducer of cell division and elongation (Saharan & Nehra, 2011). IAA is the main auxin in plants, regulating growth and developmental processes such as cell division and elongation, tissue differentiation, apical dominance, and responses to light, gravity, and pathogens. Roots are most sensitive to fluctuations in IAA level (Goswami *et al.,* 2016).

Phosphorus is involved in the photosynthetic core of any high-yield crop production system, the crucial point at which energy is transformed from light into sugar and then into compounds which plants provide. Phosphate solubilization is usually exhibited by group of fungi (Goswami *et al.,* 2016). Phosphorus (P) is vital to plant growth and is found in every living plant cell. It is involved in several key plant functions, including energy transfer, photosynthesis, transformation of sugars and starches, nutrient movement within the plant and transfer of genetic characteristics from one generation to the next.

None of the strains exhibited a positive result for phosphate solubilizing test.

MO-5, MO-7, MO-8, MO-20 had the maximum ammonia production.

Ammonium is an important source of nitrogen for plants. It is taken up by plant cells via ammonium transporters in the plasma membrane and distributed to intracellular compartments such as chloroplasts, mitochondria and vacuoles probably via different transporters in each case. Ammonia binds air borne nitrogen and makes the most important crop nutrient, nitrogen, available for nitrogen fertilizer production. It is an important base material for fertilizers. Today, roughly 80% of the annually produced ammonia is used for fertilizer production (Goswami *et al.*, 2016).

From these results it is understood that bacterial strains MO-5, MO-7, MO-8, and MO-20 exhibited the best results in the enzymatic screening and PGP characterization. These strains were further tested for biochemical, morphological and molecular characterization.

Tests for indole, glucose utilization, organic and acetoin production and citrate utilization were conducted.

In the study conducted by Kloepper and associates, (1980) it is noted that indole derives auxin molecules that can control the division and elongation rate, as well as the identity or differentiation state of cells. Accumulation of and response to auxin have been shown to regulate many growth and developmental processes. Indole test was done to identify production of indole.

Methyl red (MR) test detects the production of sufficient acid during the fermentation of glucose and the maintenance of conditions such that the pH of an old culture is sustained below a value of about 4.5, as shown by a change in the color of the methyl red indicator which is added at the end of the period of incubation (Saharan & Nehra, 2011).

Some bacteria can utilize glucose and convert it to a stable acid like lactic acid, acetic acid or formic acid as the end product (Adesemoye et al., 2008). Organic acids play a role in plants in providing redox equilibrium, supporting ionic gradients on membranes, and acidification of the extracellular medium.

The majority of plant growth promoting rhizobacteria (PGPR) confer plant immunity against a wide range of foliar diseases by activating plant defenses that reduce a plant's susceptibility to

pathogen attack. Acetoin trigger induced systemic resistance (ISR) and protect plants (Saharan & Nehra, 2011). It is detected by Voges-proskeur test.

Citrate test useful in selecting bacteria that use citrate as its main carbon and energy source. Citrate is one of the most abundant stored form of carbon in plants that utilizes citrate. Simmons citrate agar is the medium used (Saharan & Nehra, 2011). Plants maintain their metabolic balance by transforming a part of stored malate to citrate via the TCA cycle. None of the strains were tested positive for biochemical analyses.

In molecular characterization, all the 4 strains were reported as different strains and species of *Bacillus.* In a study conducted by Sivasakthi and co-workers, (2014), obtained various PGPR members including Bacillus. It is the most abundant genus in the rhizosphere and the PGPR activity of these strains has been known for many years, resulting in broad knowledge of the mechanisms involved. There are a number of metabolites that are released by these strains which strongly affect the environment by increasing nutrient availability of the plants. Bacillus species are naturally present in immediate vicinity of the roots.

MO- 20 had difference in their production of amylase when compared to other strains but belonged to the same species. This difference may be caused due to any genetic mutation that had occurred.

MO-5, MO-7, MO-8, MO-20 exhibited the best results in the enzymatic screening and PGP characterization.

These strains can be used as a consortium or as individual strains for plant growth tests that is expected to have a higher growth rate than the control and development of biofertilizers.

These biofertilizers can be encapsulated in a biofilm thus making a more ecofriendly way to sustainable agriculture.

Since these bacterial strains exhibited hydrolysis of organic compounds like starch and protein, these have a potential use in waste management.

Biochar is a carbon-rich residue, which acts as a source of nutrients, and is also a bio-stimulating candidate to enhance the activities of oil-degrading bacteria. The application of biochar as a nutrient source to bioremediate oil-contaminated soil is a promising approach for reducing PHA contamination. These PGPR strains can be used for making biochar (Saeed *et al.*, 2011).

In horticulture plants, the production of IAA is of prime importance were proper flowering, growth of the plant and timely production of flowers are needed (Goswami *et al.*, 2016).

Inoculation in PGPR by dipping and seed priming before sowing in field under greenhouse condition, displayed increase in seedling growth up to 65% and rootstock up to 79% with PGPR strains treatment (Odoh, 2017). The inoculation of phosphate- solubilizing bacteria (PSB) along with phosphate fertilizer enhanced the performance of fertilizer and also abated it requirement by 75%. This helps in better leaf growth and can be utilized for foliage leaves that are important part of horticulture.

The blend of microbial inoculation revealed more efficiency than the inoculation of sole strain (Adesemoye et al., 2008).

There is a direct need of increasing the growers' interest to adopt the technology of PGPR along with the use of chemical fertilizers in order to enhance their efficiency, and mitigate the negative impact of chemical fertilizers by abating their excessive use, which not only rise the input cost of crop production but also harm the environment and human health (Odoh, 2017).

A lot of study showed that inoculation with PGPR resulted in significant yield increases in different crops, rooting of hardwood and semi-hardwood cuttings, increased germination and enhanced emergence of seeds under different conditions, promoted nutrient uptake of roots, total biomass of the plants, increased seed weight, induced early flowering. Thus PGPR is a better option for sustainable and ecofriendly agriculture practice (Kaymak, 2010).

CONCLUSION

Out of the 24 strains isolated finally 4 bacterial isolates were screened out after the preliminary screening by enzymatic activity, plant growth promoting characters and biochemical analysis. These can be further tested for effect of these strains in seed germination and the growth to the plantlet stage can be done in order to find their direct effect on the plants.

These isolates were tested positive for the enzyme production and exhibited best results among the 10 finally screened plant growth promoting characters.

Since these strains were the best performing in the tests conducted it can be used as a potential biofertilizers.

It has been suggested that development of plant growth promoting consortium (PGPC), could be a feasible strategy for increased activity and better viability of plant growth promoting rhizobacteria. The triad of interactions existing between the bioinoculant microorganism(s), resident soil microbiota, and host plant(s) is vital not only for the overall growth and higher productivity of the crop plants but also for maintaining the integrity of our planet's health and proper biogeochemical cycling.

Chemical fertilizers prove detrimental to soil and environmental health, while biofertilizers are natural products and do not pose threats to the ecosystem. Thus, to manage long-term soil fertility and sustain crop productivity, natural-products-based fertilizers prove to be an integral and vital component of sustainable agriculture. Thus PGPR needs to be produced commercially in order to meet these needs.

The more widespread utilization of PGPB will necessitate that a number of issues be addressed. They offer the potential to address multiple modes of action, multiple pathogens, and temporal or spatial variability. PGPR offer an environmentally sustainable approach to increase crop production and health. The application of molecular tools is enhancing our ability to understand and manage the rhizosphere and will lead to new products with improved effectiveness.

Further studies:

The seed germination studies can be conducted by immersing the seeds in the cultures of the bacterial strains individually and as a consortium. A consortium is considered to be more effective. The bacterial inoculum can be further studied in developing biofertilizers and commercialized.

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