Evaluation of newly isolated probiotic bacteria from various plant roots harboring growth promoting traits for the cultivation of organic tomato

A THESIS

BY

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Registration No.: 1805115

Session: 2018-2019

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY

DINAJPUR-5200

JUNE, 2019

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Submitted to the Department of Biochemistry and Molecular Biology,
Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200
In partial fulfillment of the requirements for the degree of

MASTERS OF SCIENCE

IN

BIOCHEMISTRY AND MOLECULAR BIOLOGY

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY

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ACKNOWLEDGEMENTS

All praises and appreciation are for Almighty Allah who created the universe and bestowed the mankind with knowledge and wisdom to search for its secrets.

I express my profound gratitude to my research supervisor Shefali Akter, Assistant Professor, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh, for her stimulating guidance and constant encouragement throughout my research work.

My cordial respect and pleasant appreciation to my respected co-supervisor Dr. Md. Azizul Haque, Assistant professor, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh, for his innovative guidance, cooperation, valuable suggestions, positive criticism, overwhelming encouragement and all the facilities during the entire period of this research work.

My deepest sense of gratitude to my father Abul Husain, mother Farida Begum and brother Shamim Hasan for their selfless persuasion and sacrifice. I express my heartfelt sentiments for their moral and emotional support for enabling me to carry on my academic pursuits. Their blessings and affection are not to be reciprocated in words, but are to be felt in the deepest corner of my heart.

I am extremely grateful to Dr. Md. Abu Sayed, Associate Professor and Chairman, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur, who have provided an excellent atmosphere during my work program.

My special thanks to Dr. Mst. Nur-E-Nazmun Nahar, Associate Professor, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

A special thanks to Dr. Md. Yeasin Prodhan, Assistant Professor, Department of Biochemistry and Molecular Biology, Hajee Mohammad Danesh Science and Technology University, Dinajpur.

I express my special thanks to my friends Snygdha Rani Das, Md. Bokhtiar Rahman, Simo for their love, care and valuable support. I also thank from the bottom of my heart to my juniors Md. Abdullah Al Mamun, Shamima Yeasmin, Md. Al Amin, Goutam Chandra Debnath, Shahriar Aziz, Zoherul hasan, Md. Shohorab Hossain for their constant presence and care and instant support.

I cannot finish without acknowledging lab stuff Md. Abdul Wadud, Department of Agricultural chemistry and Md. Tajul Islam, Department of Plant Pathology, Hajee Mohammad Danesh Science and Technology University, Dinajpur for their support during this work.

I acknowledge the several friends and well wishers, whom I have not mentioned above and whose best wishes have always encouraged me.

The Author

ABSTRACT

In recent year the overuse of chemical fertilizer increases day by day and their effects on the environment and human health have been reported by many studies. Due to the adverse effect of chemical fertilizer scientist should think of a new and alternative way for sustainable agriculture. The plant probiotic bacteria (Endophytic bacteria) occur inside the plants and has a diverse effect on plant growth promotion, which can solve this problem by providing sustainable agriculture. This study was carried out by 20 isolated endophytic bacteria from rice, tomato and cauliflower roots and also evaluated their role as plant growth promoters. The isolates were named as P1 – P10 form rice roots, TR1 – TR5 form tomato roots and CR1–CR5 form cauliflower roots. All the isolates except P7 were continued for next studies. On the basis of KOH string test the 15.79% isolates were gram positive and the rest of 84.21% isolates were gram negative endophytes. The biochemical tests reveal that among the isolated strains 84.21% were positive for MIU and Urease test, 73.68% were positive for CIU test, 52.63% were positive for Oxidase test, 47.37% were positive for Voges-Proskauer (VP), 15.79% were positive for Methyl Red (MR) test. All endophytic bacterial isolates were positive for Catalase and TSI test. In carbohydrate utilization test 47.37%, 78.95%, 94.77% and 100% were positive for Lactose, Maltose, Sucrose and Dextrose, respectively. For extracellular hydrolytic enzyme assay all the isolates showed positive results. Among the 20 endophytic isolate 18 isolates were molecularly characterized using 16s rRNA gene sequencing and were identified 38.89% as *Enterobacter* sp., 22.22% as *Klebsiella* sp., 16.67% as *Pantoae* sp., 11.11% as *Bacillus* sp., 5.56% as *Pseudomonas* sp., and 5.56% as *Burkholderia* sp. These identified strains have been submitted to gene bank in National Centre for Biotechnology Information (NCBI). Three Phylogenetic tree has also been described for all the 18 strains with maximum likelihood method and proper node and branch lengths has been maintained to identify all of these strains. The 19 isolated bacteria were checked for their plant growth promoting (PGP) ability and the 100% of the isolates were shown positive results for phosphate solubilization which was done on Pikovskayas Agar medium. The ability of nitrogen fixation of the isolates were checked both in N- free (*Azotobacter* agar medium) and N- containing (Yeast extract mannitol agar) medium. In both the medium all the isolates were able to fix atmospheric nitrogen and showed positive results. In N- containing (Yeast extract mannitol agar) medium the isolates P4, P5, P8, P9, CR1 and CR2 were shown the maximum

nitrogen fixation ability. For indole acetic acid (IAA) production 100% of the strains were able to produced IAA and the maximum amount were produced by P2 (3.514µg/ ml), P5 (4.038µg/ ml), TR1 (6.093µg/ ml), TR3 (7.002µg/ ml), TR4 (3.65µg/ ml) and TR5 (6.729µg/ ml). The ability of isolates to utilize ACC as a sole source of N was assessed on Dworkin and Foster (DF) minimal medium and the isolates TR3, CR4 and CR5 gave the maximum absorbance at 600nm. The growth promoting ability of all the isolates were checked on tomato plant individually and also in the consortium. The plate assay for germination effect of isolated strains were carried out on tomato seeds and the highest significant amount of germination percentage, root length, shoot length, seedling length, vigour index was found in CR4 (100%), P9 (7.19 cm), CR3 (6.57 cm), P9 (13.23 cm) and CR4 (1262.67 cm), respectively. For pot (field) application of 40 days recorded data, the highest significant root length, shoot length, fresh weight and dry weight were recorded in TR2 (7.03 cm), TR1 (18.77 cm), P9 (2.50 gm/ plant) and TR5 (0.48 gm/ plan), respectively. The application of these endophytes as consortia in pot field experiment also showed the significant development of root length, shoot length and seedling length and the results was significant in soil+bacterial treated tomato plant compared to soil+compost+bacteria treated plant. The highest significant root length was recorded with group 2 (P6+P7+P8+P9+P10) treated plant that is 13.60 cm and the highest significant shoot length was recorded with group 3 (TR1+TR2+TR3+TR4+TR5) treated plant that is 20.17 cm. The significant seedling length was recorded with group 1 (P1+P2+P3+P4+P5) treated plant that is 32.67 cm. All this result strongly suggests that the endophytic bacteria characterized in this study could be successfully used to promote plant growth.

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CERTIFICATE

This is to certify that the thesis entitled, **"Evaluation of newly isolated probiotic bacteria from various plant roots harboring growth promoting traits for the cultivation of organic tomato"**, submitted for the degree of Master Of Science (M.S.) in **Biochemistry and Molecular Biology** of the Hajee Mohammad Danesh Science and Technology University, Dinajpur-5200 , is a bonafide research work carried out by **Sharmin Sultana** (**ID-1805115**), Session: 2018-2019 under my co-supervision and that no part of this thesis has been submitted for any other degree.

_____

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CHAPTER- I

INTRODUCTION

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INTRODUCTION

The current estimated world population around seven billion people and also predicted for the next 50 years, the population will be increased around 10 billion that requires agriculture productivity to be increased to meet all these individual's needs within the few decades [1]. To fulfill this increasing demand along with reduced chemical products (chemical fertilizer, pesticides and supplements), aiming sustainable agriculture, for protecting the environmental and human health the whole world is focused on the use and research of microorganism [2]. The chemical fertilizers used in agriculture to increase yields, kill pathogens, pests, and weeds, and have a big harmful impact on the ecosystem [3]. Also, the consumption of chemical fertilizer by the world is increases day by day. The demands of nitrogen (N), phosphorus (P), and potassium (K) is gradual increases annually by 1.4, 2.2 and 2.6 percent, respectively between 2014 and 2018 and the total global nutrients (N+P2O5+K2O) consumption is 200 500 000 tonnes in 2018. The Asia region is the largest consumer of fertilizer in the world and it's consumed 58.5 percent of the world total, the bulk of which is in East Asia and South Asia by IFA (World Fertilizer Consumption Statistics) [4].

In this sense, the application of microorganisms, especially bacteria, with plant growth-promoting features, the so-called plant probiotics, maybe a possible solution to increase crop production while avoiding the above mentioned problems related to the application of chemical fertilizers and pesticides and, moreover, allowing the obtention of better quality products [5, 6].

The term Plant Probiotic Bacteria (PPB) was first mentioned by Haas and Keel [7] to name a group of microorganisms benefiting plants, which fulfills three essential criteria that combined result in better plant protection: (i) effectiveness and competitiveness in niche colonization, (ii) the ability to create induced systemic resistance (ISR) in their hosts and (iii) presence of direct antagonistic traits on pathogens. PPB can be classified according to their interactions with the host plant is divided into 2 groups: (i) free-living rhizobacteria, which live outside plant cells and enhance plant growth as a result of the metabolites that they release in the rhizosphere, and (ii) endophytes, which live inside plant tissues and/or cells and directly exchange metabolites with their host plant, positively affecting their growth [8, 9]. Most endophytic bacteria live in the intercellular spaces of

the host plant; however, there are some bacteria able to form truly mutualistic interactions with their hosts and penetrate plant cells inside. Moreover, some of them are able to integrate their physiology and even go through a process of bacterial differentiation within the plant cells, resulting in the formation of specialized structures. The best known mutualistic symbiotic bacteria are the rhizobia, which establish symbiotic associations with leguminous plants, fixing atmospheric nitrogen for the plant in a specialized root structure commonly called root nodules [10, 11]. Other examples of mutualistic bacteria associated with plants are *Frankia*, which induces the formation of nodules in actinorrhizic plants, such as *Alnus* trees, where bacterial nitrogen fixation takes place [12] or the symbiosis between cyanobacteria and cycads, amongst others [13].

The plant probiotics microorganisms especially fungi and bacteria also known as plant growth promoters (PGP) for their beneficial effect in growth of plants by increasing the germination rates, shoot and length, leaf area, chlorophyll content, nitrogen content, protein content, leaf area, biomass, yield and they have the ability to get faster adaptation to environmental changes (stress condition) during drought, heat or salinity, etc. They promote growth directly through biological nitrogen fixation, phosphate solubilization, phytohormone production, inhibition of ethylene biosynthesis in response to biotic and abiotic stress, etc., or indirectly through inducing resistance to pathogen and other plant growth promotion attributes [14, 15]. Nitrogen is one of the major limiting nutrients for the growth of the plant. Plant uptake nitrogen from the atmosphere and make available by the help of symbionts in the root nodules of legumes. This process is called biological nitrogen fixation Rhizobia and nitrogen-fixing bacteria share *fix* and *nif* genes that encode for nodulation and nitrogen fixation, respectively. The nitrogen fixation is done by the nitrogenase enzyme produced by the bacteria and regulate by oxygen concentration and availability of nitrogen. The nitrogen-limited condition also interferes in plant hormone production, and hence some diazotrophs are able to produce phytohormones in addition to nitrogen fixation [16, 17]. Phosphorus is the next limited compound available for plants it plays an important role in cell metabolism and signaling. Phosphorus in $H2PO4^{-}$ and $HPO42^{-}$ can be absorbed by plants, but unfortunately, they are present in bound form with organic or inorganic molecules that are unavailable to plants. Though phosphorus is used as chemical fertilizer, excessive and unmanaged application has a negative impact on the environment. Endophytes are phosphate-solubilizing bacteria that solubilize the bound form thereby making available to plants. In addition, enzymes including phosphonates,

phytases, and C-P lyases also play a role in converting insoluble phosphorus to available phosphorus [18]. Plants produce hormones such as auxins, cytokinins, gibberellins, ethylene, and abscisic acid. Endophytic microbes have the potent to produce these hormones which influence plant growth and development. Auxin is the crucial plant hormone and fundamental component that modulates plant growth and development. Indole-3-acetic acid (IAA) is a member of the auxin family produced by bacteria, fungi, and plants. IAA induces lateral root formation in dicots and adventitious root formation in monocots. IAA combines cambial growth and vascular development. They are transported via phloem by forming concentration gradients and accumulate in different tissues [19, 20]. The enzyme ACC deaminase is produced by many endophytes which converts ACC into α-ketobutyrate and ammonia. Ethylene is produced from ACC synthase which inhibits primary root elongation and lateral root formation but promotes root hair formation, thus having a positive and negative role. Ethylene increases at a higher rate when the plant is in stressed conditions. Hence, it is also known as the stress hormone. Reduction in the ACC level reduces ethylene levels and thus decreases the plant stress [21, 22].

Tomato (*Solanum lycopersicum L.*) is one of the most important widely grown vegetables all over the world for their edible fruits and one of the major importance of vegetable for the agricultural industry. It is an excellent source of vitamins, minerals, and antioxidants [23, 24]. At present, the consumption of tomatoes have been associated with its role in the prevention of several human diseases [25, 26]. It is regarded as an excellent source of antioxidant compounds lycopene which can help prevent the development of many forms of cancer, it also contains a variety of nutrients including fiber, potassium and vitamins A and C, providing about 20 percent of the daily recommended requirements of vitamin A based on a 2,000 calorie diet (United State Department of Agriculture Nutrient Database). A medium tomato also provides about 26 percent of the daily recommended levels of vitamin C [27-29]. The average requirements of nutrients: 30 t/ha organic matter; 50 kg/ha nitrogen (N); 80-100 kg/ha phosphorus (P); 200-250 kg/ha potassium (K). Under greenhouse conditions the nutrient doses can be higher to increase the yield [30]. Combination of endophytic bacteria and reduced amount of chemical fertilizer can achieve overall or more yield equivalent to the yield that was obtained from recommended full dose of chemical fertilizer [31]. In order to make tomato cultivation sustainable, more environment-friendly and less dependent on chemical fertilizer, there is a need to use microbial preparation in the form of biofertilizer. Taking into concern the impressive role

of biopesticides and biofertilizers in the promotion of sustainable agriculture, which basically include, target-specificity, environmental safety, and biodegradability [32], several government agencies are actively funding research and enhancing development and marketing of these green inputs. In spite of the above notable mentions, it is extremely crucial to note that Asian countries consume only 5% of the total biopesticides sold globally while the North American Free Trade Agreement (NAFTA) countries claim to be the major global consumers of biopesticides. Significantly, the USA, Mexico and Canada consume about 47% of the biopesticides sold globally [33, 34].

The current shift in agricultural practices has thereby gradually incorporated minimal use agrochemicals in-field practices while simultaneously employing bacterial microbiota as bio-inoculant, either singly or in consortia. Extensive research is being pursued in the context of enhancing plant growth, yield and simultaneously conferring tolerance to unfavorable environmental or soil conditions by inoculating plants with PGPB. The microbes are amalgamated with plant roots, shoots, leaves, as well as fruits or seeds thereby inducing a symbiotic relationship between plants and microorganisms. Yet, a few research articles have been published concerning the growth promotion of tomato plants by the plant endophytes. In fact, several numbers of articles and patents were reported concerning growth promotion and organic biofertilizer. Unfortunately, none of these mentionable researches have been conducted in Bangladesh yet. Therefore, the current objective of this research may lead to developing technology towards biofertilizer in Bangladesh.

Therefore, the present study was carried out with the following objectives:

- To isolate endophytic bacteria from rice, tomato and cauliflower roots having potential for plant growth promotion.
- Biochemical characterization and Screening of endophytic bacteria for PGP traits
- Molecular characterization of isolated endophytic bacteria according to their 16S rRNA gene sequence.
- To reveal phylogenetic relationship of the identified strains.
- Germination effect (plate assay) of the isolated bacteria on seedling length of tomato.
- Pot field experiment of the isolates (single and consortium) on growth and development of tomato plant.

CHAPTER- II

REVIEW OF LITERATURE

CHAPTER- II

REVIEW OF LITERATURE

World's chemical fertilizer consumption has grown continually over the past decades. Chemical fertilizers are commonly used to fill soil nutrients to enhance agricultural yields. Although using chemical fertilizers provides significant benefits to society, it can be harmful if using heavily and improperly. In general, chemical fertilizers compose of principal nutrients requiring for plant growths such as nitrogen, phosphorous, and potassium (NPK) as well as micronutrients such as copper (Cu), iron (Fe), manganese (Mn), zinc (Zn) and heavy metal contaminants such as arsenic (As), cadmium (Cd), chromium (Cr), and lead (Pb) [35].

These chemicals elements may potentially cause adverse health effects even exposed at the low levels. Existent studies have documented that the exposure to chemical fertilizers linking to contact dermatitis [36] and respiratory problems [37]. For example, the case reports exhibited that contact dermatitis was caused by nickel in fertilizers found in chemical laboratory factory workers, by phosphates showed in fertilizer factory workers and by calcium ammonium nitrate reported in farmers used fertilizers [36]. Chemical fertilizer exposure decreased lung functions possibly causing a restrictive types of lung disorders in the long term. It is found that exposure to chemical fertilizers significantly increased the risk of respiratory symptoms. The exposure to ammonia at a higher level was significantly related to an increased prevalence of respiratory symptoms and an acute reduction of lung functions among urea fertilizer factory workers [37]. Tree planters working with chemical fertilizers did not have relation to increased inhalation or dermal exposure to metal contaminants such as As, Cd, Cr, Pb, Ni and they also found a weak relation between dermal and respiratory irritation and working with chemical fertilizers [35]. Farmers are occupationally exposed to chemical fertilizers while applying fertilizers to soil and plants. Thus, they may potentially have health effects associated with chemical fertilizer exposure such as respiratory and dermal symptoms e.g. respiratory irritations, cough, chest tightness, skin irritation, rashes, and contact dermatitis [36]. It was also evidenced that shallow groundwater in agricultural areas in this province was contaminated with heavy metals due to overusing chemical fertilizers for a long time [38] in addition, it had evidence regarding the excessive use of chemical fertilizers related to nitrate contamination in groundwater in other areas [39]. Consequently, people living in these agricultural areas might face health risks related to chemical fertilizers. Due to the

adverse effect of chemical fertilizer, the increasing connection among plants and microbial populations need to accept worldwide.

Plants and animals are normally associated with diverse microorganisms. They play a remarkable role to stimulate immunity and development in the gut [40]. In the same way, endophytic microorganisms play a role inside the plant and promote plant growth and development. The endophytic bacteria can be those that colonized within the internal tissue of the plant without causing any external sing of infection or any negative effect on their host [41]. There are about 300,000 plants exist in the earth and each of them is host to one or more endophytes [42]. Among them, only a few of the plant has been completely studied about their endophytic biology. The endophytic communities were mainly comprised of five phyla viz. *Proteobacteria* (90%), *Actinobacteria* (1.5%), *Planctomycetes* (1.4%), *Verrucomicrobia* (1.1%) and *Acidobacteria* (0.5%). The commonly found bacterial endophytic genera are *Pseudomonas*, *Bacillus*, *Burkholderia*, *Stenotrophomonas*, *Micrococcus*, *Pantoea* and *Microbacterium* etc [43]. These endophytic microorganisms play an important role in the cycling of nutrients and prevent the negative effect of phytopathogens. They promote growth by the production and secretion of plant growth regulators, antagonistic activity against nitrogen fixation and the supply of biologically nitrogen fixation [44]. Therefore, endophytic bacteria also provide an important role in a sustainable system of crop production [45].

2.1 Plant-bacteria interactions

Several different microorganisms are commonly found in soil (bacteria, fungi, protozoa, and algae) and estimated that there are often around 108 to 109 bacterial cells of soil/ gram [46]. Plants are continuously in contact with bacteria throughout their lifetime. Different bacteria can affect plants differently. Bacteria can be generally divided into three classes based on their mode of action and the plant's response: 1) plant pathogens; those bacteria that inhibit plant growth and cause different disease symptoms in various parts of the plant, 2) plant asymptomatic bacteria; those that do not produce any observable effects on plant growth, and 3) plant growth-promoting bacteria (PGPB); those that support plant growth to significant levels. The effect that a particular bacterium has on a plant may vary according to the environmental conditions. Endophytic bacteria stimulates plant growth by providing fixed nitrogen, a compound that is typically present in limited amounts in many soils. Also, a particular bacterial strain may provide just the right amount of the plant

hormone indole-3-acetic acid (IAA) to stimulate the growth of one plant while the same level of IAA may inhibit the growth of a different plant. There is a growing body of literature describing the potential uses of plant-associated bacteria as agents that can be used to stimulate plant growth and manage soil and plant health [47, 48]. In addition to promoting the growth of plants used in agricultural practice, PGPB also play important roles in horticulture, silviculture and environmental remediation [48]. PGPB can be broadly classified into three categories: those that colonize the root surface and the close neighborhood (rhizospheric bacteria), those that establish a symbiotic relationship with plants (symbiotic bacteria), and those that can enter into the root interior and colonize inside the plant (endophytic bacteria) [49].

2.2 Interesting molecules and substances assimilation and/or biosynthesis

2.2.1 PPB and nitrogen fixation

Although is quite abundant in the Earth, nitrogen (N) is the most limiting nutrient for plants, which requires it for the formation of aminoacids and subsequently, proteins. Some prokaryotes have the exclusive of managing the process of combination or conversion of atmospheric nitrogen into organic forms, which can be finally assimilated by plants [50]. Amongst free-living rhizobacteria, members of the genera *Azospirillum*, *Azotobacter*, *Beijerinckia*, *Bacillus, Paenibacillus*, *Burkholderia*, *Gluconoacetobacter* and *Herbaspirillum* were reported as nitrogen-fixing microorganisms. The genus *Azospirillum* is commonly associated with cereals in temperate zones, increasing crop yields in most of them, as well as in some legumes and sugarcane [51, 52]. Members of the genus *Azotobacter* are able to fix nitrogen in rice crops. Moreover, some species of this genus are tested as biofertilizers for several cereals, such as wheat, barley, oat, rice or maize; oil plants, such as linseeds and sunflowers; and other variety of plants, such as beetroot, tobacco, tea, coffee and coconuts [53].

Some species belonging to the genera *Gluconacetobacter*, *Azospirillum* and *Herbaspirillum* are frequent sugarcane endophytes and act as nitrogen fixers contributing to this plant nutrition [54]. The genus *Herbaspirillum* has also been identified as a nitrogen fixing endophyte of several crops [54, 55]. Last but not least, the genera *Bacillus* and *Paenibacillus* are free-living nitrogen fixers and have other PGP traits, which make them suitable candidates for application [56].

Moreover, some of those free-living bacteria may enter roots of some crops, such as species of *Azoarcus*, *Azospirillum* and *Burkholderia* in rice roots, which increase the nitrogen concentration of this specific crop or nitrogen-fixing *Azorhizobium* strains in wheat plants [57]. Interestingly, some strains of the genera *Rhizobium* and *Bradyrhizobium*, which were found in association with rice and wheat roots, increase this nutrient concentration in those plant yields [58].

On the other hand, certain diazotrophic bacteria are able to establish truly mutualistic symbiosis within plant tissues, mainly through the formation of root nodules. These symbioses are found between rhizobia and legumes, rhizobia and *Parasponia*, *Frankia* and *actinorhizal* plants and *cyanobacteria* and *cycads* [59, 60].

2.3. PPB and nutrient mobilization

2.3.1 Phosphate solubilization

Phosphorous is particularly essential for plant growth and development and the plant contain phosphorus 0.2 - 0.8% of the plant dry weight. It plays an important role in plants in many physiological activities such as cell division, photosynthesis and development of root system and utilization of different carbohydrates. Phosphorous deficiency results in the leaves turning brown accompanied by small leaves, weak stem and slow development. Plants might take up several P forms but the greatest part of the applied P fertilizer is absorbed in the forms of HPO_4^{2-} or $H_2PO_4^-$ [61]. Soil microorganisms are effective in releasing P from inorganic and organic pools of total soil P through solubilization and mineralization.

Solubilization process can be accomplished by a range of different mechanisms which include excretion of metabolites such as organic acids, proton extrusion and production of chelating agents. The production of gluconic acid seems to be the most frequent agent of mineral phosphate solubilization [62]. Phosphate solubilization by phosphate solubilization bacterial (PSB) strains is associated with the release of low molecular weight organic acids mainly gluconic and ketogluconic [63], which through their hydroxyl and carboxyl groups chelate the cations bound to phosphate, therefore converting it into soluble forms. Inorganic acids e.g. hydrochloric acid can also solubilize phosphate but they are less effective compared to organic acids at the same pH. The pH of rhizosphere is lowered through biotical production of protons or bicarbonates (anion/cation balance) and gaseous (O_2/CO_2) exchanges. Phosphate solubilization ability

of PSB has direct correlation with p^H of the medium. The use of PSB in agricultural practice would not only off set the high cost of manufacturing phosphate fertilizers but would also mobilize insoluble phosphorus in the fertilizers and soils to which they are applied. Among the whole microbial population in soil, phosphate solubilizing bacteria (PSB) constitute 1 to 50%, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5% in P solubilization potential. Strains from bacterial genera *Pseudomonas, Bacillus, Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers. *Bacillus megaterium, B. circulans, B. subtilis, B. polymyxa, B. sircalmous, Pseudomonas striata* and *Enterobacter* are referred as the most important strains [64]. High proportion of PSM is concentrated in the rhizosphere and they are metabolically more active than from other sources. Release of root exudates such as organic ligands can also alter the concentration of P in the soil solution. In addition, the microorganisms involved in P solubilization as well as can enhance plant growth by enhancing the availability of other trace element such as iron (Fe), zinc (Zn) etc [65].

The 101 isolates were able to solubilize phosphate belonged to genera *Burkholderia, Cedecea, Cronobacter, Enterobacter, Pantoea* and *Pseudomona*s. The higher PSB population is found in rhizosphere than non rhizospheric soil [66]. The highest population of *Pseudomonas species* (PS) was detected in Pikovaskaya's medium. PSB inoculants with their beneficial traits considered as potential biofertilizer for the sustainable cultivation system.

2.3.2 Urease and denitrification activity

The plant growth promoting bacteria break down urea into simple forms of nitrogen which can be readily absorbed by the plants to promote growth. Urease production by bacteria is an important aspect in growth and development of plant in the case where fertilizers are applied. Urease catalyzes the hydrolysis of urea into unstable carbamic acid and rapid decomposition of carbamic acid occurs without enzyme catalysis to form ammonia and carbon dioxide. The ammonia reacts with water to form ammonium (NH_4^+) which is a plant available source of nitrogen. Plants regulate their nitrogen metabolism by the control of urease activity in the presence of urea as inducer and suppress its activity in the presence of excess ammonia [67]. The positive implication of urease is an important aspect in growth and development, as the bacteria has potential to convert urea to simpler forms of N which are readily absorbed by plants [68].

Under O_2 free conditions, many microorganisms can utilize NO_3^- as a terminal electron acceptor process called NO_3^- respiration. The pathway involving the reduction of NO_3^- to gaseous end products is known as denitrification. Denitrification is an important factor to maintain the nitrogen cycle in the atmosphere, water and soil. The ability of the bacteria to reduce nitrates to nitrogen (N_2) refers to denitrification activity. This is a critical feature of rice root endophytes, as it will give time for the plants to absorb readily available nitrogen before it can be converted to free N_2 by other denitrifying bacteria that could be present in the host plant or in the rhizosphere. Denitrification is a microbial facilitated process of nitrate reduction that may ultimately produce molecular nitrogen (N_2) through a series of intermediate gaseous nitrogen oxide products. The process is performed by heterotrophic bacteria such as *Paracoccus* denitrificans and various *Pseudomonads* [68].

2.4 PPB and phytohormone biosynthesis

2.4.1 IAA production

Plant hormones are regulators that influence plant growth and development and IAA is one of the most physiologically active auxin. IAA is a plant hormone with no apparent function in bacterial cell and it is observed that IAA production improve the fitness of the plant bacterium interaction. IAA production allows the bacteria with competitive advantages to colonize plant tissues. Bacterial IAA loosens plant cell wall and as a result facilitates an increasing amount of root exudation that provides additional nutrients to support the growth of rhizospheric bacteria [69]. Auxins are involved in several plant processes including lateral and adventitious root formation and root elongation, which elevates stress resulting in plant growth promotion. It has been estimated that as much as 80% of the rhizosphere bacteria can synthesize auxins [70]. Many rhizobacteria are capable of producing indolic compounds in the presence of precursor amino acid tryptophan (Trp). However, under natural conditions availability of Trp varies with the host plant. Studies have shown that many rhizospheric bacteria are capable of synthesizing IAA even without Trp [71]. Bacterial auxin can alter the auxin balance inside the plant and is capable of influencing the auxin homeostasis by interfering with plant auxin transport.

Bacterial IAA increase root surface area and length and therefore provide the plant greater access to soil nutrients and water uptake. Bacterial IAA can obviate to a certain extent the function of ACC deaminase, siderophore and phosphate solubilizing bacteria.

Screening of the rhizosphere and endophytic bacteria for in-vitro potential of IAA production provide a reliable base for selection of effective PGP bacteria. Many studies have shown that the interaction of IAA-producing bacteria with plants might posit that since IAA activates the transcription of ACC synthase, these bacteria should ultimately result in the production of relatively high concentrations of ACC and subsequently inhibitory levels of ethylene. However, this is infact not the case because as plant ethylene levels increase, the ethylene that is produced feedback inhibits IAA signal transduction thereby limiting the extent that IAA can activate ACC synthase transcription [1, 72].

The IAA producing endophytic bacteria *P. putida* and *Rahnella aquaqtilis* are able to stimulate the growth of some cereals and of radish [70]. Plant growth promoting rhizobacteria are believed to increase root growth and length resulting in greater root surface area that enables the plant to access more nutrients from soil. Similarly, the ability to produce IAA was extremely variable among the endophytic bacteria, even with similar ARDRA pattern (D, Pa1, and Pg), suggesting that the production of IAA is strain dependent [73]. Endophytes synthesize IAA to direct their plant hosts through signaling pathways. For example *Pseudomonas syringae* can induce IAA and abscisic acid biosynthesis in *Arabidopsis thaliana* [74].

Bacterial IAA contributes to circumvent the host defense by derepressing the auxin signaling in the plant. IAA also can have a direct effect on bacterial survival and its resistance to plant defense. In plant associated bacteria, both the indole-3-acetamide (IAM) and the indole-3-pyruvate (IPyA) pathway are distributed among the genome. Phytopathogenic organisms tend to use the IAM pathway to produce IAA, whereas beneficial bacteria tend to use the IPyA pathway. Large amounts of IAA produced by bacteria together with plant IAA activate ACC synthase, leading to production of ACC, precursor for ethylene [75].

2.4.2 ACC deaminase production

Ethylene is known as the stress hormone and synthesized under various stress conditions like flooding, salination, drought, heavy metals, toxic organic compounds etc. Its precursor ACC degraded by the enzyme 1-Aminocyclopropane-1-carboxylate (ACC) deaminase, which converts ACC into α-ketobutyrate and ammonia which is present in many bacteria. ACC deaminase, which is encoded by some bacteria, can reduce the amount of ethylene, a root elongation inhibitor, and stimulate the growth of plants under

various environmental stresses. The presence of ACC deaminase activity and the regulation of ACC in several bacteria have been reported [76].

Plant growth promoting bacteria (PGPB) is acting as a sink for ACC thereby lowering plant ACC levels, decreasing the amount of ACC within the plant, can be converted into ethylene. PGP bacteria stimulate plant growth by the activity of ACC deaminase by decreasing plant ethylene level. Thereby, reducing the suppressive effect of ethylene on root elongation and thus promoting plant growth. These bacteria not only directly promote plant growth but also protect plants against flooding, drought, salt, flower wilting, metals, organic contaminants and both bacterial and fungal pathogens [1]. Plants treated with ACC deaminase containing bacteria have longer roots [77] and can better resist the inhibitory effect of ethylene stress on plant growth [69].

Evidence for the involvement of ACC deaminase in endophytic plant growth promotion comes from the demonstration of mutants of the well characterized endophytic PGPB *Burkholderia phytofirmans* PsJN that had lost ACC deaminase activity were no longer able to promote canola seedling root elongation [78].

2.5 Cell wall degrading enzymes

Cellulose is the most abundant organic carbon source. Cellulose accounts for 50% of dry weight of plant biomass. These sugars are found in lignocellulosic biomass comprised of mainly cellulose hydrolyzed by a complex enzyme cellulose [79]. Many bacteria and fungi utilize cellulose for nutritive purposes. Cellulose degrading enzymes encoded by microorganism are of great interest as they play key role in global carbon cycling. The cellulolytic property of some bacterial genera such as *Cellulomonas sp, Pseudomonas sp, Bacillus sp* and *Micrococcus sp*. was also reported [80]. A key role in the decomposition and transformation of organic matter such as plant residues in the soil ecosystem is attributed to aerobic microorganisms, especially cellulolytic bacteria that are ubiquitous in soils. Cellulases are the inducible bioactive compounds produced by microorganisms during their growth on cellulosic matter. Cellulase yield appear to depend upon complex relationship involving a variety of factors like inoculum size, p^H value, temperature, presence of inducers, medium additives, aeration and growth time etc. Cellulose degrading microorganisms can convert cellulose into soluble sugars either by their acid and enzymatic hydrolysis. Thus microbial cellulose utilization is responsible for one of the largest material flows in the biosphere. A large amount of microorganisms are

capable of degrading cellulose only few of them produce significant quantities of cell free bioactive compounds capable of completely hydrolyzing crystalline cellulose in vitro. When endophytic bacteria colonize on the plant surface, they produce enzymes to hydrolyze plant cell walls. As a result, these enzymes also have the function to suppress plant pathogen activities directly. Further, studies showed that production of cell wall degrading enzymes such as cellulase, phosphatise or pectinase in PGPR was important in facilitating the entry of the bacteria into the intercellular spaces of plant root hairs [81].

Proteases are a large group of hydrolytic enzymes that catalyze protein hydrolysis by cleaving the intra amino acid peptide bonds in protein molecules. Proteases are found in plants, animals and micro-organisms. Proteases are of great importance in the biosynthesis, bioremediation and biotransformation. Protease synthesis is also greatly affected by physical factors such as temperature, pH, incubation time and dissolved oxygen [88]. For alkaline protease production number of microbial strain were screened using skimmed milk agar media [82]. Bacteria are important alkaline protease producers and *Bacillus* genus is most prominent producer, because of their ability to produce large amount of extra cellular protease with significant proteolytic activity and stability at high p^H and temperature. Several *Bacillus species* involved in protease production are *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megatarium* and *B. subtilis* [83].

Stenotrophomonas maltophilia plays a key role as a PGP bacterium which suppress development of disease by secretion of antibiotics, production of extracellular enzymes such as protease and chitinase and potential root colonizer. *Pseudomonas fluorescence* (AUPF25) producing protease used as a biocontrol. Similarly, different mode of action of biocontrol such as inhibition of pathogen by antimicrobial compounds, parasitism may involve production of extracellular enzymes (Protease and chitinase) that lyse pathogen cell wall and induction of plant resistance mechanism [84].

The *Pseudomonas fluorescence* CHA0 mutation of *GacA* controlled *aprA* gene (encoding the major extracellular protease) or the *gacA* regulatory gene resulted in reduced biocontrol activity against the root-knot nematode *Meloidogyne incognita* in tomato and soyabean. Culture supernatant of strain CHA0 inhibited egg hatching and induced mortality of *M. incognita juveniles* more strongly than did supernatants of *aprA* and *gacA* mutants, suggesting that *aprA* protease contributes directly to biocontrol [85].

2.6 Plant colonization

Prior to colonizing the inside of a plant, an endophyte first colonizes the rhizosphere or the phyllosphere, where rhizosphere refers to the area of the soil surrounding a plant's root and phyllosphere represents the aboveground surfaces of a plant that are used as microbial habitat [86]. Microorganisms can gain entry into plants by chance as well; however, those microbes that enter a plant's interior accidentally generally do not survive for long periods of time. Thus, a microorganism that is able to enter the interior of the plant is not necessarily a true endophyte. True endophytic colonization must be confirmed with proper spread among different organs of the plant (if required) and the maintenance of endophytic state for bacterial generations within the plant environment [87]. These two most important abilities of endophytes distinguish endophytes from other transient visitors.

Like plant pathogens, endophytes employ different mechanisms to gain entry into plants. They may enter the plant through different points; these entry points include tissue wounds, stomata, lenticels, root cracks and germinating radicles [88]. Entry through root cracks is recognized as the main portal of entry for bacterial colonization. Other mechanisms may include penetration of bacteria via root hair cells and through the production of cell wall degradative enzymes [89].

Upon reaching the inside of the plant, an endophyte may localize itself at the point of entry or may be spread throughout the plant. If endophytes are systemically spread then they can reside in the intracellular spaces, in the vascular system, and/or within the cells [90]. Specialized cellular environments are selected by certain bacterial species because of their higher tendency to become established in those environments [87]. For example the plant xylem was demonstrated to be a selective environment for nitrogen-fixing endophytic bacteria such as *Acetobacter diazotrophicus, Bacillus pumilus, Gluconacetobacter diazotrophicus, Klebsiella pneumoniae* and *Serratia marcescens* [91, 92]. The effects of endophytes on their host plants vary widely; they may be beneficial to some hosts while harmful for some others (in that case they are considered as plant specific endophytes). This mainly depends on the response of their host at various growth stages and different environmental conditions to the mechanisms employed by the bacteria [87].

2.7 Isolation of bacterial endophytes

The endophytic niches likely protect the bacteria from many environmental stresses. A wide range of plants can serve as hosts for endophytic bacteria, ranging from herbaceous plants to woody plants. Bacterial endophytes have been isolated from almost all kinds of plants including monocotyledonous plants and dicotyledonous plant genera. A large number of bacterial endophytes have been isolated from almost every part or tissue of the plant [93]. Bacterial endophytes are widely abundant in all types of plants; no known plant species are completely free of endophytes [94]. However, endophytic population densities are lower than those of rhizospheric bacteria or bacterial pathogens [94, 95]. Furthermore, apparent absences of endophytes in a plant species may be either due to the procedure used for the isolation of the endophytes or the microorganism is not culturable. Operationally, the most reliable method for isolating endophytes is the tissue surface sterilization method that comprises three main steps: 1) surface sterilization of plant tissue, 2) maceration of sterilized tissue, and 3) culturing of serially diluted macerated plant tissues. Bacterial endophytes can also be extracted from the internal plant tissues [95].

CHAPTER- III

MATERIALS AND METHODS

CHAPTER- III

MATERIALS AND METHODS

Following part shows all the methods and materials related to this work, including isolation, biochemical, molecular characterization and plant growth-promoting traits of these isolated endophytic bacterial strains. Finally, there applications to promote tomato growth.

3.1 General

3.1.1 Place of study

The investigation was planned and carried at Biochemistry and molecular biology research lab in Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur.

3.1.2 Glassware and apparatus

All the laboratory equipment for conducting this work such as test tube, petri dishes, conical flasks, glass pipettes, beakers, glass rods, micropipette tips, eppendorf tubes, falcon tubes, PCR tubes were first clean with soap solution, then rinsed with distilled water and finally autoclaved at 121°C in 15 psi for 15 minutes before using it.

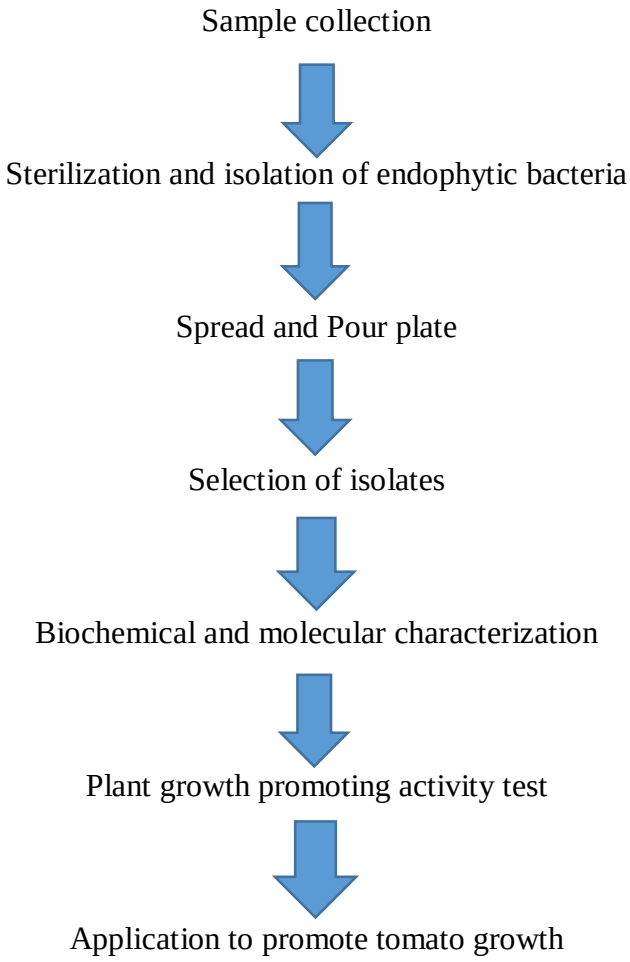
3.1.3 Media, solutions and reagents

Different types of media, reagents and solution were used for endophytic bacterial isolation, morphological biochemical and their plant growth-promoting traits analysis. All the required reagents and solutions were freshly prepared before use and those were acquired from the laboratory. None of the solutions or reagents were further purified since they were of a reagent grade.

3.1.4 Instruments

Electrical balance, Centrifuge machine, Autoclave, Magnetic strirrer hot plate, Spectrophotometer, Shaking Incubator, Laminar Air Flow Clean Bench, Digital pH meter, Microscope, Refrigerator etc.

3.2 Follow chart of study



3.3 Sample collection

3.3.1 Sample collection sites

The healthy rice, tomato and cauliflower roots were collected from the different fields beside the Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur, Bangladesh. The collected samples were then taken to the Biochemistry and Molecular Biology lab HSTU, Dinajpur.

3.3.2 Sample preparation

The sterilization step is an important part of the isolation of endophytic bacteria. First, the root samples were washed out by running tap water. Then it was surface sterilized by 70% ethanol for 1 minute then with 0.01% mercuric chloride for 5 minutes and followed by 0.5% Sodium chloride for 5 minutes. Finally, it was treated with sterile distilled water

5 times and then the root samples were cut down into small pieces. Then the samples were macerated separately in phosphate buffer of pH 7.2 with a sterile pestle and mortar. After that, the samples were spread on Pikovskaya's (PKV) agar medium for screening the phosphate solubilizing endophytic bacteria [96]. Then the plates were incubated at 37°C for 24 hours. After 24 hours the verities of the colony according to color, shape and size were selected for further purification. Finally, the bacterial isolates were picked from plates and purified by streaking techniques and incubated at 37°C.

3.3.3 Media preparation

The Pikovskaya's (PKV) agar medium was used for the isolation of endophytic bacteria [96]. The PKV agar medium was prepared with distilled water. The composition of the medium is g/l: Glucose- 10 g, tricalcium phosphate (TCP) – 5 g, ammonium sulphate– 0.5 g, sodium chloride- 0.2 g, potassium chloride- 0.2 g, magnesium sulphate– 0.1 g, yeast extract- 0.5 g, manganese sulphate– trace, ferrous sulphate– trace, agar- 15 g, the pH was adjusted to 7.0 ± 0.2 and then the prepared media was autoclaved at 15 psi. pressure (121°C) for 15 minutes.

3.4 Morphological & Biochemical characterization

The selected endophytic bacterial strains were differentiated based on their metabolic pattern by using different biochemical tests [97] including KOH staining, MR-VP, oxidase, catalase, MIU, TSI, citrate utilization, urease test, carbohydrate utilization test (dextrose, lactose, maltose and sucrose) [98], and activity on different growth media (CMC, Casein, Xylan, Amylase).

3.4.1 KOH staining test

The alternative technique, Potassium hydroxide (KOH) string test was used to identify the gram-negative bacteria. The KOH made the gram-negative bacterial cell wall viscous by forming mucoid with the loop [99]. For KOH staining test a bacterial colony from the culture plate was emulsified over glass slide in the suspension of 3% KOH. Then the suspension was stirred continuously for one minute and the loop was gently pulled up from it. The test was considered positive and the strain was identified as gram-negative if string was seen within the first 30 seconds after mixing in the KOH solution.

3.4.2 Biochemical test

3.4.2.1 Methyl Red Voges-Proskauer Test (MR-VP) Test

Methyl Red (MR) and Voges-Proskauer (VP) broth are used to identify the metabolic pathway of an organism, this identification is performed by detecting the ability to produced stable acids end products. In medium, the MR positive organism creates an acidic environment that will overcome the buffers (Potassium phosphate that resists pH). So, after adding methyl red, the broth stayed red. The positive VP result organisms further metabolize pyruvic acid to form acetyl-methyl carbinol (acetoin). This end product, in the presence of atmospheric oxygen and 40% potassium hydroxide is converted to diacetyl and it under the catalytic action of alpha-naphthol and creatine, is converted into a red complex [100, 101].

MR-VP broth was prepared with autoclaved distilled water and 5 ml broth was transferred to each test tubes. After that the selected isolates were inoculated into each tube and incubated at 37°C for 48 hours. After incubation, five drops of methyl red reagent was added for detecting MR positive isolates by searching for red colors in the test tubes. For VP test after inoculation and incubation at 37°C for 48 hours 0.6 ml of 5% alpha-naphthol was added to individual test tubes. Following after 0.2 ml of 40% KOH was added and the test tubes were shaken gently in atmospheric oxygen. After that the tubes were allowed to remain for 10-15 minutes, they were observed for a red color development which indicates a positive result. Both MR-VP tests negative will produce a yellow color.

3.4.2.2 Oxidase test

Oxidase test was used to check the endophytic isolates can utilize cytochrome c oxidase which is basically an enzyme of the electron transport chain. On a clean filter paper a drop of oxidase reagent (1% tetramethyl-p-phenylenediaminedi hydrochloride) was added. After that a small amount of bacterial pure culture of the selected isolates were streaked onto the oxidase droplet by an inoculating loop. The positives results indicates that the isolates are aerobic and can use oxygen [97].

3.4.2.3 Catalase test

The catalase test was used to check the presence of the catalase enzyme. A small amount of 3% H_2O_2 was added into the bacterial colony on a clean glass slide. The rapid production of bubbles indicates that the organism can utilize catalase. A negative result

indicates the absence of catalase enzyme. This test identifies members of *Enterobacteriaceae* family which are catalase-positive [97].

3.4.2.4 Motility Indole Urease (MIU) Test

The MIU test used to detect if the selected isolate is mobile or not, it can utilize urea and also check if it can produce indole [97]. The media was prepared using MIU agar medium and autoclaved at 15 psi. pressure (121°C) for 15 minutes. After that, 5 ml sterile 40% urea solution was added per 95 ml basal medium. Then 5 ml solution was transferred to each sterilized test tube and the media was left to cool down completely until it had a semi-solid consistency by gently placing the tubes in a horizontal manner. The selected isolates were inoculated and incubated at 37°C for 24 hours. The results were interpreted as per table 1.

Table 1: MIU result evaluation parameters [97]

Conditions	Positive	negative
Urea	A color change from yellow to orange to pink-red	No color change
Motility	A cloudy mass of bacteria is seen	Hazy medium without any accumulation
Indole	pink-mauve color on the bottom of the tube	No pink-mauve color is formed

3.4.2.5 Triple Sugar Iron (TSI) test

The Triple Sugar Iron (TSI) media contain three sugars (Lactose, Sucrose, and Glucose) and also iron which is done to confirm the ability of the bacteria to utilize these sugars. After preparing the media 5 ml of the broth was distributed to each test tubes and sterilized by autoclaving at 15 psi, 121°C for 15 min.

Table 2: TSI result evaluation parameters [102]

Slant/Butt condition	Slant color/Butt color	Decision taken	Gas formation
Alkaline slant/Alkaline butt	Red/Red	glucose, lactose and sucrose non-fermenter	Depending on the visibility of bubbles or cracks in the test tubes
Alkaline slant/acidic butt	Red/Yellow	Glucose fermentation only	Depending on the visibility of bubbles or cracks in the test tubes
Acidic slant/acidic butt	Yellow/Yellow	glucose, lactose and/or sucrose fermenter	Depending on the visibility of bubbles or cracks in the test tubes

Note: Production of black color means the bacterial isolate can utilize iron and have produced H_2S.

After sterilization, the media was left to cool by keeping the test tubes in a slanted position in order to form a butt and a slant. The selected isolates were inoculated and the tubes were subjected to incubation at 37°C for 24 hours. After incubation, the appearance of the media was observed by following below protocol (Table 2) [102].

3.4.2.6 Citrate Utilization Test (CIU)

The citrate test is used to identify the ability of the bacterial isolate to utilize citrate as a carbon and energy source. CIU basically identify gram negative bacteria based on their metabolic product. The gram negative isolates contain citrate-permease which uses citrate present in the medium as a carbon source of energy. This test is also distinguish between members of the *Enterobacteriaceae* family. A CIU positive test depends on the production of the alkaline products of citrate metabolism which will subsequently increase the pH of the medium identified by the color change of a pH indicator. Citrate enters into the cell and is cleaved by citrate lyase releasing oxaloacetate and acetate, this oxaloacetate

is metabolized to pyruvate and CO_2. After that CO_2 reacts with water and sodium carbonate to release an alkaline by-product. The ammonium salts that are present in the media are also used by the microorganisms which ultimately produces another alkaline product ammonia and turning the media from deep green to intense Prussian blue [103].

Media was prepared and each of the test tube was contained 5ml of media. After autoclaving at 15 psi, 121°C for 15 min the media was left to cool until it had a semi solid consistency. The selected isolates were inoculated in individual test tubes and incubated at 37°C for 24 hours. The intense Prussian blue appearance of the media and the rise in pH above 7.6 (which is due to carbonates and bicarbonates produced as by-products of citrate catabolism) indicates a positive result. Also some bacterial growth will be visible on the surface of the test tube.

3.4.2.7 Urease test

In urease test microorganism produces ammonia from urea by using the urease enzyme. The urease enzyme hydrolyzes urea into ammonia and CO_2 and changes the media color changes from light orange to magenta pink (pH 8.1) since ammonia alkalines the medium. If the organism utilizes urease, the medium will change color indicating positive results due to the presence of phenol red indicator in the urea agar media [104, 105].

The media was prepared by using a urea agar base and a pH of 6.8 was maintained. After formulation, 5 ml solution was transferred to each sterilized test tube and autoclaved at 15 psi. pressure (121°C) for 15 minutes. The media was left to cool down completely and after reaching room temperature the selected isolates were inoculated and incubated at 37°C for 24 hours for observation of urease activity.

3.5 Carbohydrate utilization test (Dextrose, Lactose, Maltose and Sucrose)

Carbohydrate utilization test was done using phenol red carbohydrate broth to observe if the selected isolates can utilize these sugars. Dextrose, lactose, maltose and sucrose-containing media were prepared according to protocol provided by the manufacturers and 5ml of the media were transferred into each test tubes at pH of 7.4 ± 0.2 [102]. After that, the selected isolates were inoculated and incubated at 37°C for 24 hours. After incubation, the tubes that turned yellow as a result of acid production by the

bacteria were taken as a positive and the ones with no color change were indicated as negative.

3.6 Extracellular hydrolytic enzyme assay

The detection of the extracellular hydrolytic enzyme activity of the isolated endophytic bacteria were done by agar diffusion method. The isolates were grown on different enzyme activity indicator media for the detection of cellulase, xylanase, amylase, and casein. The cellulose media was prepared in autoclaved distilled water with the composition of: $(NH_4)_2SO_4$ 0.1%, KH_2PO_4 0.05%; K_2HPO_4 0.05%; $CaCl_2$ 0.01%; NaCl 0.01%; $MgSO_4.7H_2O$, 0.02%; yeast extract 0.1%, and CMC with related dye 1% and then it was autoclaved at 15 psi. pressure (121°C) for 15 minutes. For xylan, amylase and casein the media was prepared as same composition like cellulose media. After autoclaved the purified colonies were subjected on the specific substrate agar plate for screening the extracellular enzyme, cellulose/xylan/casein/amylase and incubated at 37°C for 24 hour [106, 107].

3.7 Molecular Characterization by using 16s rRNA gene sequencing

3.7.1 Genotypic identification of bacterial strain

The selected endophytic isolates were characterized by 16s rRNA gene sequencing. Without this ubiquitous housekeeping genetic (16s rRNA) marker ribosomes can't translate. By this gene sequencing which itself is very much conserved with a bp of 1500, the new species of bacteria can be identified by performing phylogenic and taxonomic analysis [108, 109]. The protocols used for this method has been described below:

3.7.2 DNA sequencing protocol

To identify the sequence of any unknown sample numerous important steps need to be carried out carefully without any error. DNA sequencing have 4 steps:

- DNA isolation using 'Boiling method'
- PCR and PCR product purification
- Measurement of concentration of PCR purified DNA
- Cycle sequencing and product purification
- Data analysis

3.7.2.1 DNA isolation

Colony PCR is a method of extracting DNA from our sample via direct colony from cultured plate and doing heat block method used for the extraction of DNA.

For sufficient amount of extraction of DNA from the selected isolated strains isolates 300 µl deionized water had been taken in all the selected 1.5 ml eppendorf tubes. After that two or three single pure of bacterial colonies had been taken using a sterilized inoculating loop and mixed with the deionized water (using hands).

3.7.2.2 Heat Block Method

Then the tubes were subjected to heat shock at 100° C for 10 minutes using 'Heat Block' machine. After that, the tubes were immediately put into ice (Icebox utilized) for 10 minutes. Then these tubes were centrifuged for 15 minutes at 10,000 rpm. The pellet was removed and the supernatant was transferred into new tubes for individual samples. For extraction confirmation, the measurement was taken in a nano-drop machine immediately after the procedures were finished [110].

3.7.3 PCR and PCR product purification

For PCR amplification of the extracted DNA 16s universal primers were used as shown in table 3

Table 3: Universal Primer sequences

Primer	Sequence	Type
Forward: 27F	5'-AGA GTT TGA TCM TGG CTC AG- 3'	Universal
Reverse: 1492R	5'-GGT TAC CTT GTT ACG ACT T- 3'	Universal

For individual samples 12.5 µl master mix, 7.5 µl nuclease-free water, 1 µl each for forward and reverse primer and 3 µl DNA sample were mixed into a PCR tube making a total volume of 25 µl and vortex properly eliminating any chance of bubble formation. After the procedures, the PCR was run [111] following the accurate PCR steps described in table 4

Table 4: PCR parameters

Number of Cycle	Step Name	Temperature	Time
1	Pre Heat	95°C	3 min
35 Cycle	Denaturation	95°C	30 sec
	Annealing	48°C	30 sec
	Extension	72°C	90 Sec
1	Final Extension	72°C	5 min
1	Hold	4°C	Over Night.

After finishing the PCR samples were kept in -20° C for later use.

3.7.4 Agarose gel electrophoresis

The PCR products were subjected to agarose gel electrophoresis in which case 1% agarose gel (0.25 agar+ up to 25 ml TBE buffer) was used to stain the gel and the samples were loaded into the well with dye (3µl samples + 2µl dye). After running for 1 hour in the gel the samples were visualized using a gel documentation machine [114].

3.7.5 DNA Purification after Gel Documentation

After completing PCR, DNA was purified from the amplicon using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The individual PCR tubes holding each sample were taken and transferred into a 'spin column tube'. Binding buffer was added to each of them 4 times the amount of the sample and mixed using a vortex machine. After mixing the tubes were centrifuged in 10,000 rpm for 1 minute. After centrifugation, the unnecessary precipitate was discarded from the tubes and 650 µl wash buffer was added. The tubes were centrifuged again in 10,000 rpm for 1 minute and the precipitate was discarded. The tubes were subjected to a centrifuge for the third time in 12,000 rpm for 2 minutes to ensure the wash buffer is discarded. At the last step of purification 50 µl of elution buffer was added to each tube and allowed to stand for 1 minute. And lastly, the tubes were centrifuged again in 12,000 rpm for 2 minutes and stored at -20°C [111].

3.7.6 Measurement of PCR purified DNA using Nano-drop

Nano-drop is used to determine the DNA concentration present in each test tube. The standard DNA concentration for the next step of the molecular characterization was considered to be 9-10 nano-gram per µl (ng/ µl). To the nano drop, 2µl elution buffer was used as blank. The blank was removed and 2 µl of the sample was loaded. The DNA concentration was measured in ng/µl unit. The sample holder was sterilized each time after being used. The OD of 260/280 ratios was measured with the integrated software which indicated the purity of the sample [113].

3.7.7 Cycle Sequencing Preparation

Before cycle sequencing, the sample DNA was diluted for it to be in the exact concentration as the working solution which was 5 ng/µl. After diluting, reagents were prepared for cycle sequencing which is mentioned in table 5.

Table 5: Reagent preparation for Cycle Sequencing

Items	Volume	Reaction Number	Total Volume
Master Mix	10 µl	X1	10
T DNA (Concentration 25-65 ng/µl)	1 µl	X1	1
Primer F (Concentration 10-20 pMol)	1 µl	X1	1
Primer R (Concentration 10-20 pMol)	1 µl	X1	1
Water	7 µl	X1	7
Total	20 µl		20

Master Mix contains $MgCl_2$, dNTPs, DNA polymerase and addition of $ddNTP_S$ for chain termination. After reagent preparation for each sample, each tube was rotated to eliminate bubbles by using a rotator and finally the samples were placed in the PCR machine for cycle sequencing. The process was taken up to two hours [111].

3.7.8 Purification after Cycle sequencing

After cycle sequencing the individual DNA samples were purified in a three-step process. At first, 2 µl 3M Sodium acetate, and 50 µl 100% Ethanol were added into each PCR tube holding cycle sequencing product. Then incubated at -20℃ temperature for 15 minutes and after that, the tubes were centrifuged at 13,000 rpm for 15 minutes. Then the supernatant was discarded and 200 µl of 70% Ethanol was added and centrifuged at 13,000 rpm for 15 minutes. The supernatant was discarded and the tubes were dried overnight inside the PCR cabinet for the final step [111].

3.7.9 Sequencing DNA sample using Genetic analyzer 3130

Before running the samples in the genetic analyzer, 10 µl highly de-ionized form amide (HIDI) was added to each tube containing individual samples. The samples were spun for the elimination of bubbles and incubated at 95℃ for 5 minutes inside a thermocycler for denaturation of DNA. Immediately after incubation, the samples were put into ice (using icebox) for making sure the DNA stayed denatured. Then, the samples were loaded into ABI 3130 Genetic Analyzer which uses 4 capillary systems. For each sample, it took up to 4 hours to get a sequence [113].

3.8 Bioinformatics analysis

3.8.1 Conversion

After getting the sequence output from the genetic analyzer as '.ab1' files, the sequences were converted into 'FASTA' format using DNA sequence assembler v4 (2013), HeracleBioSoft. After getting the FASTA sequences the files were kept for identification at a species level.

3.8.2 Identification

The FASTA format files of the sequences were checked for species level identification using National Center for Biotechnology Information or 'NCBI Blast' (nucleotide blast) tool for which 'megablast' optimization program was used.

3.8.3 Analyzing DNA sequence chromatogram

After getting the sequence output from the genetic analyzer as '.ab1' files, the sequence were inserted Finch TV to get the Chromatogram picture of DNA sequence. Finch TV is a designed to view DNA sequence files like the Chromatogram. In

Chromatogram, the signal intensities are presented in a graph with the four bases, each identified by different colors. Finch TV uses green for adenine, red for thymine, black for guanine and blue for cytosine.

3.8.4 Phylogenetic tree analysis

The isolated cleaned genetic sequence was then compared to different 16S rRNA gene of different bacteria in the reference RNA sequences (16S ribosomal RNA) database of NCBI Nucleotide BLAST website using Blastn tool in order to identify the genus of the endophytic isolate. rRNA gene (rDNA) sequence analysis and subsequent maximum likelihood analysis offers a powerful tool for bacterial species identification [114]. With the help of the maximum likelihood method the optimal tree was formed and is in the units of the number of base substitutions per site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site [115]. The analysis involved 29 to 32 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 3111 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [116].

3.9 Functional properties of the endophytic bacterial isolates

3.9.1 Qualitative analysis of Phosphate solubilization by the isolates

Phosphate solubilization activity was done on the isolates to check if the isolates can solubilize the insoluble tri-calcium phosphate (TCP) by indicating the production of multiple organic acids. The qualitative analysis of phosphate solubilizing activity of the selected isolates was conducted by plate screening method which was done on Pikovskaya's (PKV) agar medium containing insoluble tri-calcium phosphate (TCP), the colonies showing clear halo zones around the bacterial growth were considered as phosphate solubilizers [96].

The Pikovskaya's (PKV) agar medium was prepared with distilled water. The composition of medium g/l: Glucose- 10 g, tricalcium phosphate (TCP) -5 g, ammonium sulphate- 0.5 g, sodium chloride- 0.2 g, potassium chloride- 0.2 g, magnesium sulphate-

0.1 g, yeast extract- 0.5 g, manganese sulphate- trace, ferrous sulphate- trace, agar- 15 g, the pH was adjusted to 7.0 ± 0.2 and then the prepared media was autoclaved at 15 psi. pressure (121°C) for 15 minutes. After completing the autoclave the media plate was prepared with the laminar air follow and the isolates were inoculated into the plate. Then the plates were incubated at 37°C for 48 hours and the formation of clear halo zones around the bacterial growth was considered as phosphate solubilizers. The zone formation also could be due to the activity of phosphatase enzyme in bacterial isolates.

3.9.2 Qualitative analysis of Nitrogen fixation by the isolates

The fixation of atmospheric nitrogen by the isolated endophytic bacterial strains were tested qualitatively using both N-free (*Azotobacteragar* medium) and N-containing (Yeast extract mannitol agar) medium. The composition of N-free medium: 0.1 g of dipotassium hydrogen phosphate [K_2HPO_4], 0.4 g of KH_2PO_4, 0.2 g of $MgSO_4$, 0.1 g of sodium chloride [NaCl], 0.02 g of $CaCl_2$, 0.01 g of ferric chloride [$FeCl_3$], 0.002 g of sodium molybdate [$NaMoO_4$], 10 g of glucose and 15 g agar in 1000 ml of distilled water [117]. Secondly, the N-containing medium composition is 0.02 g $MgSO_4$, 1 g yeast, 1.25 g Congo red and 15 g agar in 1000 ml of distilled water [118]. Both the prepared media were autoclaved at 15 psi. pressure (121°C) for 15 minutes. After completing the autoclave the media plate was prepared in the laminar air follow and the isolates were inoculated into the plate. Then the plates were incubated at 37°C for 72 hours. The bacterial growth in the medium considers that the isolates can fix atmospheric nitrogen. In N-containing medium, the absorption of dye congo red by the isolates was observed clearly and all the zone were recorded.

3.9.3 Quantitative analysis of Indole Acetic Acid (IAA) production

The amount of indole-3-acetic acid production by isolated endophytes was done by Patten and Glick (2002) [119]. The isolates were grown in 100 ml flasks containing 50 ml Luria broth (LB) supplemented with L-tryptophan (100 µg/ ml) for 48 h on a rotary shaker. Then, cultures were centrifuge at 10,000 rpm for 15 min and the supernatants were collected. Two ml of Salkowsky reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$) with one ml of the supernatant was left to react with at 28 ± 2°C for 30 min. The pink color developed indicating the presence of IAA that was determined by measuring the absorbance in a spectrophotometer at 530 nm at the end of the incubation. A standard curve

was plotted with IAA and Salkowski's reagent dissolved in LB medium to quantify the IAA (μg/ ml) present in the culture filtrate.

3.9.4 Quantitative estimation of ACC-deaminase activity

The ability of the isolates to utilize ACC was assayed based on their ability to use 1-aminocyclopropane-1-carboxylate (ACC) deaminase as a sole nitrogen source [120]. At first, the isolates were cultured in TSB rich medium and incubated at 28°C at 120 rpm for 24 h. Then the bacterial cell pellets were suspended in 5 mL of 0.1 mol/ L Tris-HCl, pH 7.6 and transferred to a microcentrifuge tube. The contents of the tubes were centrifuged at 16000 rpm for 5 min and supernatant were removed. The pellets were suspended in 2 mL of 0.1 mol/ L Tris-HCl, pH 8.5. Then 30 μL of toluene was added to the cell suspension and vortexed for 30 s. In a fresh microcentrifuge tube 200 μL of the toluenized cells were placed and 20 μL of 0.5 mol/ L ACC was added to the suspension, vortexed, then the suspension was incubated at 30°C for 15 min. Following the addition of 1 mL of 0.56 mol/ L HCl, the mixture was vortexed and centrifuged for 5 min at 16000 rpm at room temperature. Two ml of the supernatant was vortexed together with 1 mL of 0.56 mol/ L HCl. Thereupon, 2 mL of the 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2 mol/ L HCl) was added to the glass tube and the contents were vortexed and incubated at 30°C for 30 min. Following the addition and mixing of 2 mL of 2 mol/ L NaOH, the absorbance of the mixture was measured by using a spectrophotometer at 540 nm.

3.10 Effect of all 19 isolates on tomato growth

3.10.1 Application of endophytic bacterial isolates on germination percentage, seedling growth of tomato (In dark condition)

3.10.2 Experimental details

Experiment design	CRD
Treatments	20
Control	1
Replications	3
Variety	Tomato

3.10.3 Seed bacterization

The tomato seeds were collected from BRACK nursery. The collected tomato seeds were first surface sterilized with 70% ethanol for 1 minute. After that, it was rinsed with autoclave distilled water for thrice. These sterilized seeds were dried overnight and the required amount of sterilized seeds were soaked in 1.5 ml of the bacterial suspension for 6 hours. The bacterial suspension was prepared by growing the selected isolates on TSB broth at 37°C overnight [108]. The seeds only soaked in sterile distilled water were maintained as a control.

3.10.4 Seed germination test

The germination test was done in dark condition and for each treatment, there were 39 seeds with 3 replications. All the dish was watered with sterile distilled water and incubated in the growth chamber until the data was collected. The growth-promoting activity of selected 19 bacterial strains was assessed based on the germination percentage, seedling length and vigour index. The root length and shoot length of each seedling were measured and the germination percentage was also calculated.

Germination percentage: GP= (Total germinated seeds)/(Total number of seeds)×100

Shoot length= The shoot length was measured with randomly selected seedling for each treatment and the length was measured from the collar region to tip of the shoot. The mean shoot was expressed in centimeter.

Root length= The seedlings that were used for recording the shoot length were also used to measure the root length. It was measured from the collar region to the tip of the longest root and it was expressed in centimeter.

Vigour index = Germination percentage × Seedling length (Root length + Shoot length) [121].

3.11 Application of endophytic bacterial isolates in pot (field) experiment on growth of tomato

The pot application of endophytic bacterial isolates was done in single and also in consortium.

3.11.1 Experiments details

3.11.1.1 Pot (field) experiment (single treatment)

The pot (field) experiment for single treatment was done with 23 treatments including one control and two consortia (P1 – P10, TR1 – CR5).

3.11.1.2 Experimental design for consortia pot field experiment

The consortium pot field experiment was done with 8 treatments and 3 controls. This was carried with 25 days tomato seedling. Treatment details of consortia pot field experiment:

Table 6: Experimental design for consortia treatment

No.	Treatments	Details
T11	Control 1	Only Soil
T1	Control 2	Soil + Fertilizer
T2	Control 3	Soil + Compost + Fertilizer
T3	Soil+ Bacteria+ Compost	Soil+ Compost+ Group 1 bacterial consortium
T4	Soil+ Bacteria+ Compost	Soil+ Compost+ Group 2 bacterial consortium
T5	Soil+ Bacteria+ Compost	Soil+ Compost+ Group 3 bacterial consortium
T6	Soil+ Bacteria+ Compost	Soil+ Compost+ Group 4 bacterial consortium
T7	Soil + Bacteria	Soil + Group 1 bacterial consortium
T8	Soil + Bacteria	Soil + Group 2 bacterial consortium
T9	Soil + Bacteria	Soil + Group 3 bacterial consortium
T10	Soil + Bacteria	Soil + Group 4 bacterial consortium

3.11.2 Soil preparation for pot experiment

Tomato is usually grown in light or sandy loam soils that provide good drainage and favorable soil temperatures. The soil was collected from the research plot of HSTU. The best optimal temperatures for tomato plantation are 26°C at days and 20°C at nights [122]. For this experiment a little compost and 5-10-5 fertilizer was mixed into the soil to provide additional nutrition and the 5-10-5 fertilizer contains fairly mild concentrations of nitrogen, phosphorus and potassium. Soil samples were mixed well to obtain a homogenous mixture.

3.11.3 Pot collection and preparation

All the pots were collected from the market and were cleaned up by tap water. Then the pots were filled up by soil without any fertilizer.

3.11.4 Seed germination and plantation of seedlings

The seeds were germinated for single treatment and the previously seeds sterilization procedure was followed. The seeds were soaked only with sterile distilled water for 6 hours. For consortium studies, the healthy seedling of 25 days aged was bought from the market. Healthy seedlings aged 25 to 30 days with four fully expanded leaves are suitable for transplantation. The plantation of seedling was done by digging the soil of pot according to the length of seedlings. All the pots were watering properly based on their requirements.

3.11.5 Treatment of tomato seedlings with the isolated endophytic bacteria

The first treatment of endophytic bacterial isolates for all pots including consortium was done after 10 days of plantation with 2 ml of isolates which were grown in TSB broth for 24 hours at 37°C. For each treatment, all the 2 ml of isolates were diluted with sterile distilled water and sprayed on the soil and leaf in the early morning or evening. All the controls were also treated with pure water and for fertilizer, fertilizer; compost control a little fertilizer and compost were used at one time for the treatment period. The treatments were continued at the age of 10 days, 15 days and 20 days and the treated plants were monitored for growth development over a 25 day period after treatment.

3.11.6 Treatment of control with fertilizer

During the endophytic treatment, the control of soil+fertilizer and soil+compost+fertilizer were also treated with following composition of fertilizer; urea 1.2586 gm/6.0 kg soil, TSP 0.301 gm/6.0 kg soil, MOP 0.3868 gm/6.0 kg soil and gypsum 0.8062 gm/6.0 kg soil.

3.11.7 Observation recorded

The root length, shoot length, seedling length, plant height and plant biomass production were recorded. For the single treatment, 27 days and 40 days data were collected and for consortium, only 25 days data were collected after plantation.

3.11.7.1 Plant height

The plant height was measured from the top of longest root to the tip of the shoot and it was expressed in centimeters.

3.11.7.2 Fresh weight

The fresh weight was measured after harvested in electrical balance and it was expressed in gram.

3.11.7.3 Dry weight

The dry weight was measured after the harvested seedling was dried for 4 days. The dry weight was expressed in gram.

3.12 Statistical analysis

The results of the experiments were subjected to IBM SPSS statistic 22 for multiple analysis. One way analysis of variance (ANOVA) was performed on the data and significance difference between means were compared using Fisher's protected LSD test at $\rho = 0.01$ and 0.05. The graphical analysis with standard error bar was done by Microsoft office Excel 2013.

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CHAPTER- IV

RESULTS

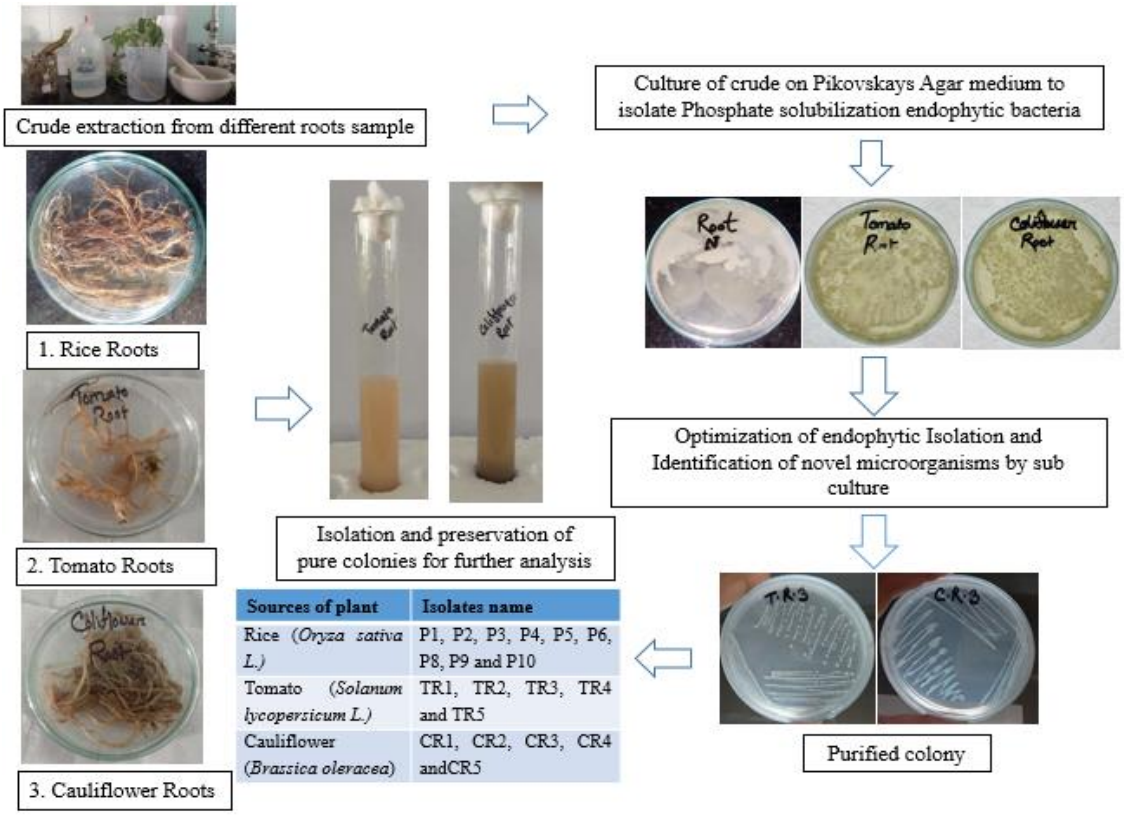
CHAPTER- IV

RESULTS

Endophytic bacteria perform several benefits to the host plant, particularly growth promotion, protection from pathogens and under diverse environmental conditions. The exploration was the isolation and characterization of endophytic bacteria from the root plants and to ensure the growth promotion activity they possess the isolates were applied to the tomato plant. The result and discussion are presented in this chapter under the following headings.

4.1 Isolation of endophytic bacteria from root samples of rice, tomato and cauliflower

The endophytic bacteria were isolated from the three varieties of plants roots; rice *(Oryza sativa L.),* tomato *(Solanum lycopersicum L.)* and cauliflower *(Brassica oleracea).* The plant samples were collected from beside the different fields of HSTU, Dinajpur. The collected samples were then taken to the Biochemistry and Molecular Biology lab at HSTU for further exploration where the phosphate solubilizing endophytic bacteria were isolated from growing healthy roots of rice, tomato and cauliflower in Pikovskayas agar media [96] (Fig 1).



Sources of plant	Isolates name
Rice (*Oryza sativa L.*)	P1, P2, P3, P4, P5, P6, P8, P9 and P10
Tomato (*Solanum lycopersicum L.*)	TR1, TR2, TR3, TR4 and TR5
Cauliflower (*Brassica oleracea*)	CR1, CR2, CR3, CR4 andCR5

Fig 1: Isolation and purification of phosphate solubilizing endophytes from rice, tomato and cauliflower roots

4.1.1 Naming of the isolates

A total of 20 phosphate solubilizing endophytic bacteria were isolated and purified by streak technique over the surface of Pikovskayas agar media. Further, isolates were maintained on their respective media and stored at 4°C throughout the period of study. Out of 20 isolates, 10 isolates were from rice root, 5 isolates from tomato root and 5 isolates from cauliflower root (Fig 2a and 2b). They were named according to the medium and source and those are presented in Table 7.

Table 7: Isolated endophytic bacteria from different plant roots sample

Sources of plants	Part	Name of Isolates
Rice (*Oryza sativa L.)*	Root (10)	P1, P2, P3, P4, P5, P6, P7, P8, P9 and P10
Tomato (*Solanum lycopersicum L.)*	Root (5)	TR1, TR2, TR3, TR4 and TR5
Cauliflower (*Brassica oleracea*)	Root (5)	CR1, CR2, CR3, CR4 and CR5

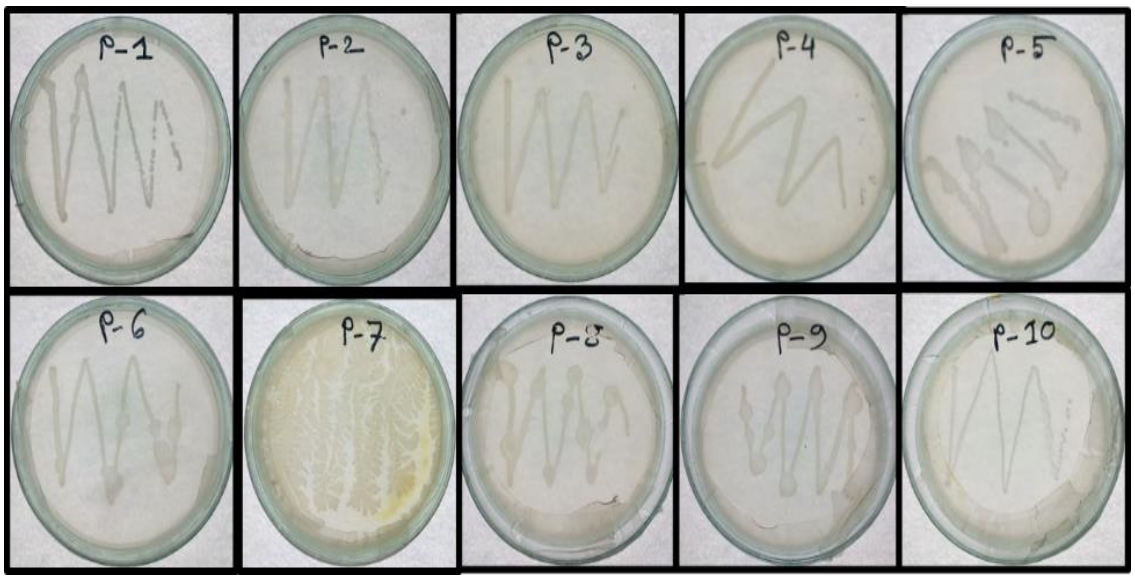


Fig 2 (a): Isolated endophytic bacteria on Pikovskayas agar medium

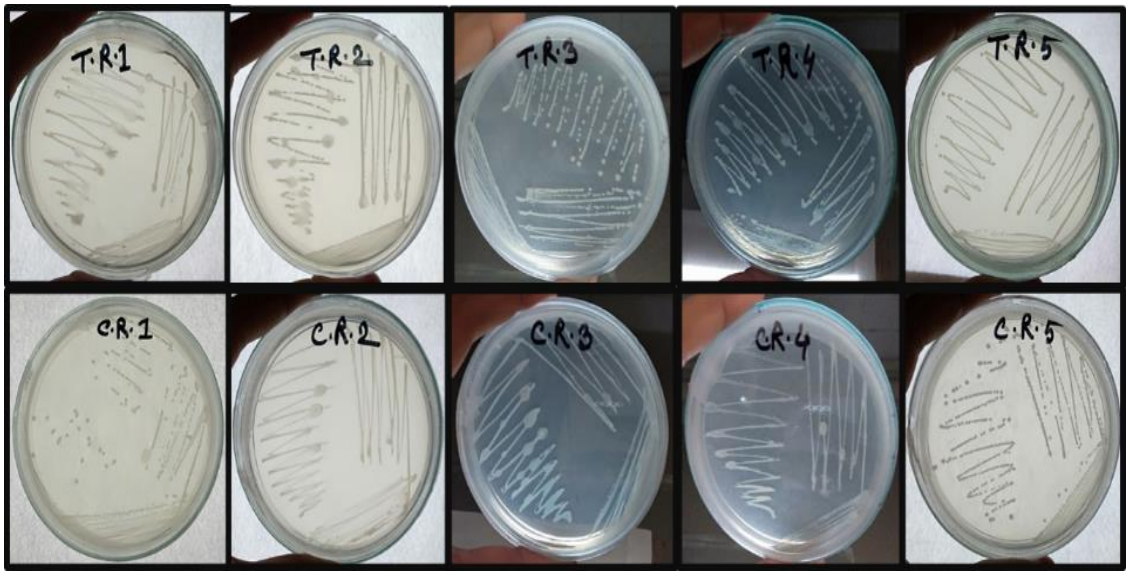


Fig 2 (b): Isolated endophytic bacteria on Pikovskayas agar medium

4.2.1 KOH String Test

The potassium hydroxide (KOH) string test used to identify gram-negative bacteria. All the selected isolates except P1, P10 and TR2 were gram-negative, KOH dissolves the thin layer of peptidoglycan of the cell walls of gram-negative and they showed viscously and stuck in the loop within 30 seconds after adding KOH to the 24 hours cultured bacteria [99]. The P1, P10 and TR2 were gram-positive, gram-positive bacteria are not be affected by KOH, because they have thicker peptidoglycan layer in the cell wall. The cells are not lysed and they didn't show any viscous nature. The results are in figure 3a and 3b.

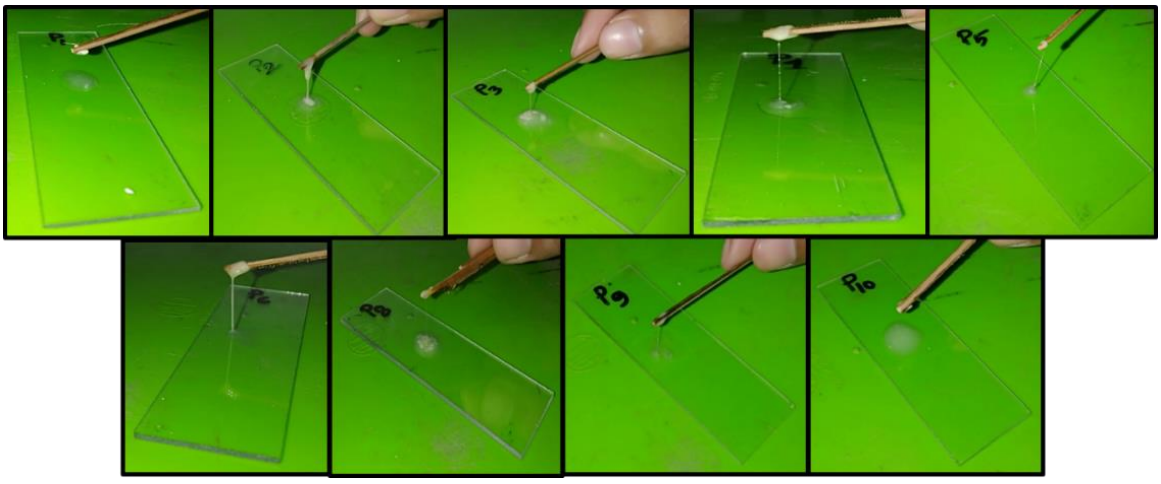


Fig 3 (a): KOH String Test

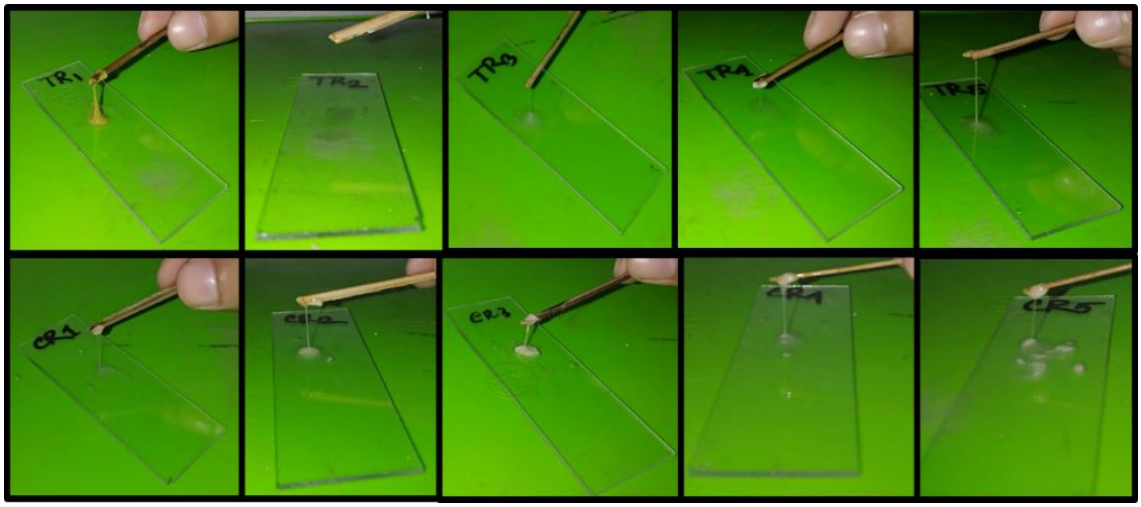


Fig 3 (b): KOH String Test

4.2 Biochemical characterization

Bacterial strains were collected from culture media according to their growth pattern, morphology, and appearance. The isolates were then sub-cultured and some specific biochemical tests were done for identification. The biochemical tests that were performed are described precisely in materials and method chapter 3.

The following biochemical tests were performed for all isolates except P7 and the results of the isolates are given below in table 8.

Table 8: List of performed biochemical tests

Performed Biochemical tests	
KOH staining	Methyl Red (MR) Test
Voges-Proskauer (VP) Test	Oxidase test
Catalase test	Motility Indole Urease (MIU) Test
Triple Sugar Iron Agar (TSI) test	Citrate utilization test
Urease test	Carbohydrate utilization Test: lactose, dextrose, maltose and sucrose
Extracellular hydrolytic enzyme assay CMC, Casein, Xylan, Amylase	

The results for the performed biochemical tests done on 19 isolates are presented in Table 9. In the KOH string test among the 19 endophytic bacterial isolates, 15.79% were gram-positive bacteria and 84.21% were gram-negative bacteria. On the basis of biochemical characterization 100%, 100%, 84.21%, 84.21%, 73.68%, 52.63%, 47.67% and 15.79% were positive for Catalase, Triple Sugar Iron Agar (TSI), MIU, Urease, Citrate utilization, Oxidase, VogesProskauer (VP), and Methyl Red (MR), respectively. The diversity of endophytic bacteria was also selected on the basis of carbohydrate utilization tests with different carbon sources (Table 8). Out of 19 endophytic bacterial isolates the maximum acid production was observed in dextrose (100%), sucrose (94.74%), maltose (78.95%) and lactose (47.37%), respectively. All the isolates showed 100% positive activity on CMC, Casein, Xylan and Amylase media.

4.2.2 Methyl Red-Voges-Proskauer (MR-VP) Test

Methyl Red-Voges-Proskauer (MR-VP) test was done for identifying an organism's metabolic pathway. Methyl Red (MR) test is used to determine which fermentation pathway is used to utilize glucose. In the mixed acid fermentation pathway, glucose is fermented and produces several organic acids (lactic, acetic, succinic, and formic acids). The development of red color indicates sufficient acid production which lowers the pH to 4.4 and conformed a positive test. Some bacteria utilize the butylene pathway for the fermentation of glucose. The 2,3 butanediol is the end and neutral product of glucose fermentation instead of organic acid production. After the addition of a-naphthol and KOH in MR/VP culture medium acetoin is oxidized to diacetyl. If the culture is positive for acetoin, it turns the medium “copper-red complex” [100, 101].

Following the principle of MR-VP test as stated by P1, P2 and P9 showed positive MR results as in those cases the MR-VP broth sustained the red colors by indicating acidic environment was established and others 16 isolates showed negative results by forming yellow colors, indicating no acidic environment was established. The MR results are shown in figure 4.

Table 9: Biochemical characterization of 19 endophytic isolates

Biochemical Test		Endophytic Bacteria	
		Positive	Negative
KOH string test		15.79%	84.21%
Methyl Red (MR) Test		15.79%	84.21%
Voges-Proskauer (VP) Test		47.37%	52.63%
Oxidase test		52.63%	47.37%
Catalase test		100%	-
Motility Indole Urease (MIU) Test		84.21%	15.79%
Triple Sugar Iron Agar (TSI) test		100%	-
Citrate utilization test		73.68%	26.32%
Urease test		84.21%	15.79%
Carbohydrate utilization Test	Different source of sugar		
	Lactose	47.37%	52.63%
	Dextrose	100%	-
	Maltose	78.95%	21.05%
	Sucrose	94.74%	5.26%
Extracellular hydrolytic enzyme assay	CMC	100%	-
	Casein	100%	-
	Xylan	100%	-
	Amylase	100%	-

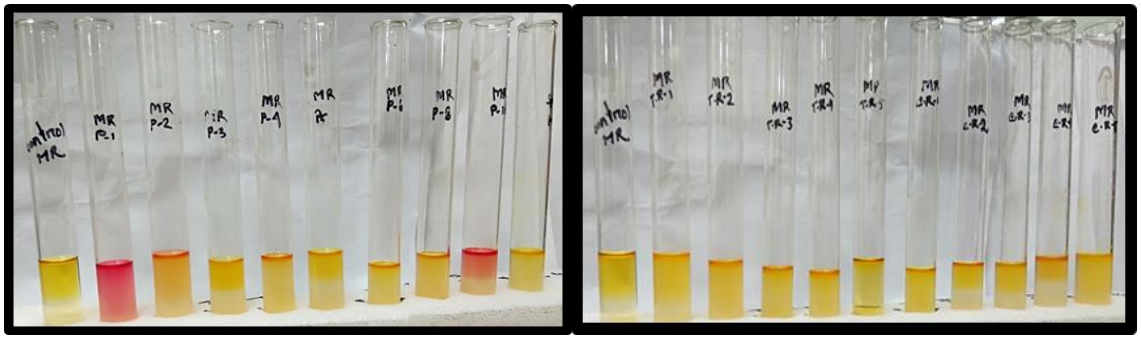


Fig 4: MR (Methyl Red) test result

In Voges-Proskauer (VP) Test the P2, P3, P4, P5, TR1, TR2, TR4, CR3 and CR5 showed positive results in the presence of alpha naphthol, 40% KOH and atmospheric oxygen the MR-VP broth changed into copper-red complex as shown in figure 5.

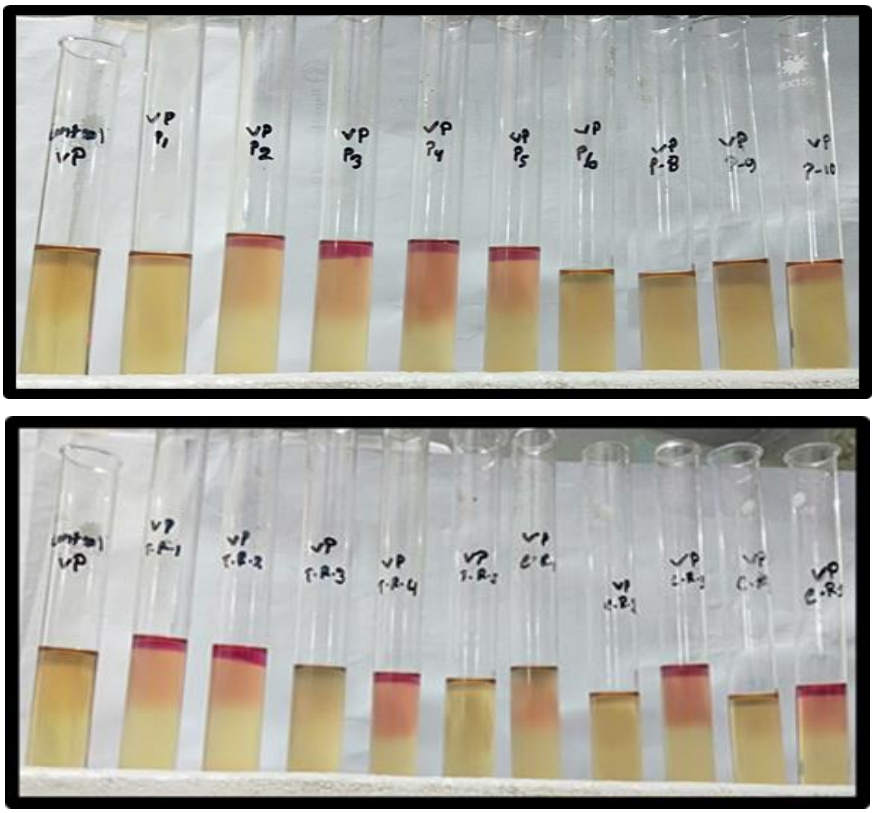


Fig 5: VP (Voges-Proskauer) Test result

4.2.3 Oxidase Test

The oxidase test is used to identify the presence of a cytochrome oxidase system that will catalyze the transport of electrons between electron donors in the bacteria and a redox dye- tetramethyl-p-phenylene-diamine. The dye is reduced to deep purple color. This test is used to assist in the identification of *Pseudomonas*, *Neisseria*, *Alcaligens*,

Aeromonas, *Campylobacter*, *Vibrio*, *Brucella* and *Pasteurella*, all of which produce the enzyme cytochrome oxidase [97].

After adding a drop of oxidase reagent (1% tetramethyl-p-phenylenediaminedi hydrochloride) up onto the filter paper, bacterial strains was smeared into the reagent. After few seconds P1, P5, P6, P10, CR2, CR3, CR4, CR5, TR1 and TR2 showed dark purple color formation whereas the other strains showed no color formation even after 30 seconds. This indicates that the rest of the strains doesn't utilize cytochrome c. oxidase but P1, P5, P6, P10, CR2, CR3, CR4, CR5, TR1 and TR2 does. The results are shown in figure 6.

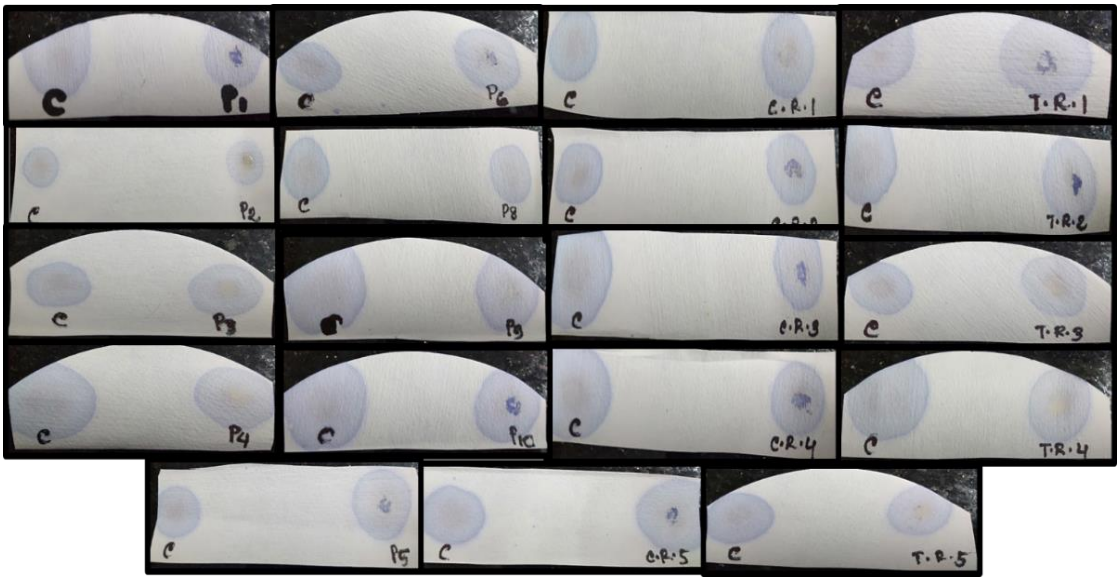


Fig 6: Oxidase Test result

4.2.4 Catalase test

Catalase test is done to determine the presence of catalase in microorganisms. The test can aid in the identification and differentiation of different microorganisms such as it differentiates the *Staphylococcus* species (catalase-positive) from *Streptococcus* species and *Enterococcus* species (catalase-negative), the aerotolerant *Clostridium* species (catalase-negative) from *Bacillus* species (catalase-positive), aerobes (catalase-positive) from obligate anaerobes (catalase-negative) and also identify bacteria in the *Enterobacteriaceae*, *Mycobacterium tuberculosis* family. The enzyme catalase mediates the breakdown of hydrogen peroxide into oxygen and water. The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into the hydrogen peroxide and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is

evident by a lack of or weak bubble production. Anaerobes generally lack the catalase enzyme [97].

All the 19 bacterial strains showed strong catalase activity by producing bubbles in the presence of 3% H_2O_2 as shown in figure 7.

Fig 7: Catalase Test result

4.2.5 Motility Indole Urease (MIU) Test

MIU test is useful for the identification of gram-negative *bacilli* especially bacteria of *Enterobacteriaceae*. This test helps to differentiate the organisms on the basis of motility, urease and indole production. Peptones of the medium provide carbon and nitrogen required for the growth of bacteria. Urea is responsible for a source of nitrogen for those organisms possess enzyme, urease. The phenol red is the pH indicator that turns pink-red in alkaline conditions. Urease is indicated by a color change of the pH indicator, Phenol Red, from yellow-orange, (pH 6.8) to red-pink, (pH 8.4) [97].

All the isolates have been tested for their motility, production of urease and indole. Except TR2, CR1 and CR2 all the other 16 isolates were shown MIU positive results (fig 8). The isolates P1, TR1, TR2, CR1, CR2 and CR4 clearly show no sign of motility but for urease and indole except TR2, CR2 and CR4 all the strains showed positive results.

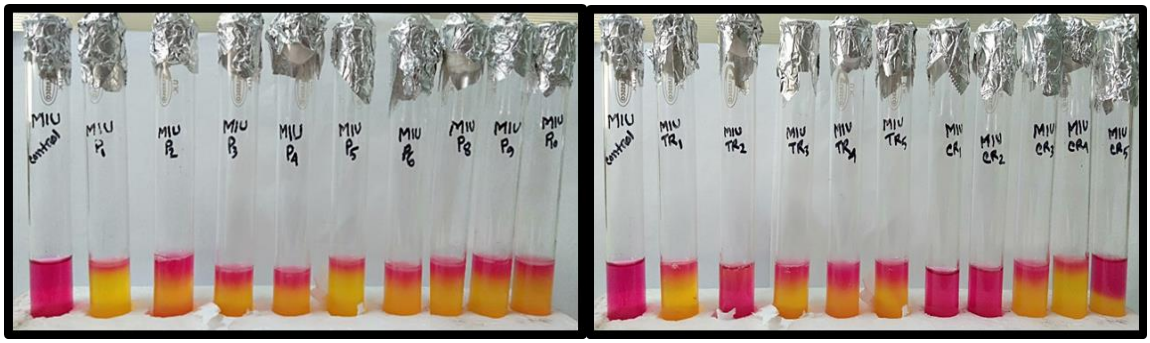


Fig 8: MIU Test result

4.2.6 Triple Sugar Iron (TSI) test

Triple Sugar Iron test determines the ability of an organism to ferment glucose, lactose, and sucrose, and their ability to produce hydrogen sulfide. Carbohydrate fermentation is indicated by the production of gas and a change in the color of the pH indicator from red to yellow. When lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in the butt and in the slant. Some organisms generate gases, which produces bubbles/cracks on the medium.

If lactose is not fermented but the small amount of glucose is, the oxygen-deficient butt will be yellow (remember that butt has comparatively more glucose than slant i.e. more media more glucose), but on the slant the acid produced (less acid produces in slant as media in slant is less) will be oxidized to carbon dioxide and water by the organism and the slant will be red (alkaline or neutral pH). If neither lactose/sucrose nor glucose is fermented, both the butt and the slant will be red. The slant can become a deeper red-purple (more alkaline) as a result of the production of ammonia from the oxidative deamination of amino acids (remember peoptone is a major constituent of TSI agar). If H_2S is produced, the black color of ferrous sulfide is seen [102].

Isolates P2, TR1, TR5, CR2 and CR4 showed yellow slant and butt (acidic) as indicated by the color of the media after incubation at 37° C for 24 hours. So, it is evident that those strains are capable of fermenting glucose, lactose and sucrose. The red slant and yellow butt (alkaline slant and acidic butt) of the isolated strains P1, P3, P4, P5, P6, P8, P10, TR2, TR3 and TR4 indicate that they are capable of only the fermenting of glucose. The red slant and butt (alkaline) of P9, CR1, CR3 and CR5 evident, that all this strain is incapable of fermenting glucose, lactose and sucrose. All the strains can produce gas by

forming bubbles except P2, P4, TR1, TR3 and TR5 and the production of black colored in CR1 and CR5 indicates that these two isolates can utilize iron and produced H_2S. The results are shown in fig 9.

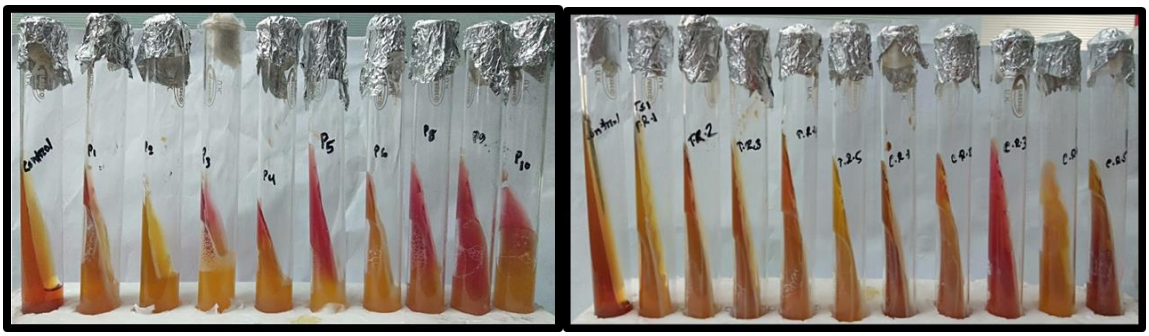


Fig 9: Triple Sugar Iron (TSI) Test result

4.2.7 Citrate Utilization (CIU) Test

CIU test identify an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) as the sole source of nitrogen. Bacteria that can grow on the medium produce an enzyme, citrate-permease, capable of converting citrate to pyruvate. Pyruvate can then enter the organism's metabolic cycle for the production of energy. Growth is indicative of utilization of citrate, an intermediate metabolite in the Krebs cycle. When the bacteria metabolize citrate, the ammonium salts are broken down into ammonia, which increases alkalinity. The shift in pH turns the bromthymol blue indicator in the medium from green to blue above pH 7.6 [103].

The selected strains P1, P2, P3, P4, P6, P8, P9, P10, TR1, TR3, TR5, CR2, CR3 and CR4 showed positive results after incubation at 37° C for 24 hours. All these strains turned the media from deep green to intense Prussian blue indicating the isolates used citrate as a carbon source of energy, thereby producing alkaline as by-products that can subsequently raise the pH of the media. The P5, TR2, TR4, CR1 and CR5 showed negative results and the deep green color sustained (Fig 10).

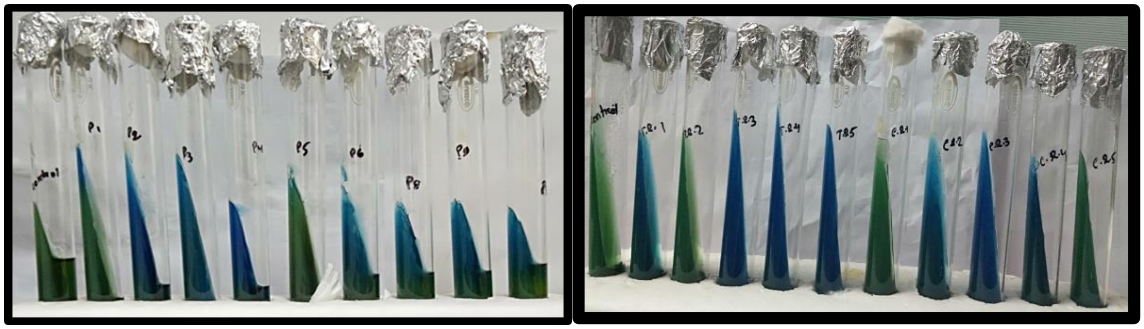


Fig 10: Citrate Utilization Test (CIU) result

4.2.8 Urease Test

The urease test identifies those organisms that are capable of hydrolyzing urea to produce ammonia and carbon dioxide. It is primarily used to distinguish urease-positive *Proteeae* from other *Enterobacteriaceae*. Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the color change of phenol red from light orange at pH 6.8 to magenta (pink) at pH of 8.1. Rapid urease-positive organisms turn the entire medium pink [104, 105].

After 24 hours of incubation all the isolates except P1, P10 and CR1 have produced ammonia from urea by using urease enzyme by developing light orange to magenta pink. The urease enzyme hydrolyzes urea into ammonia and CO_2 and changed the media color (fig 11a and 11b).

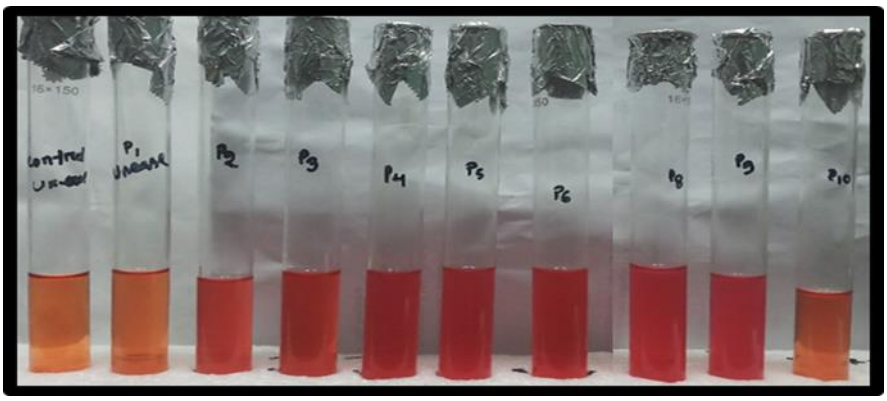


Fig 11 (a): Urease Test result

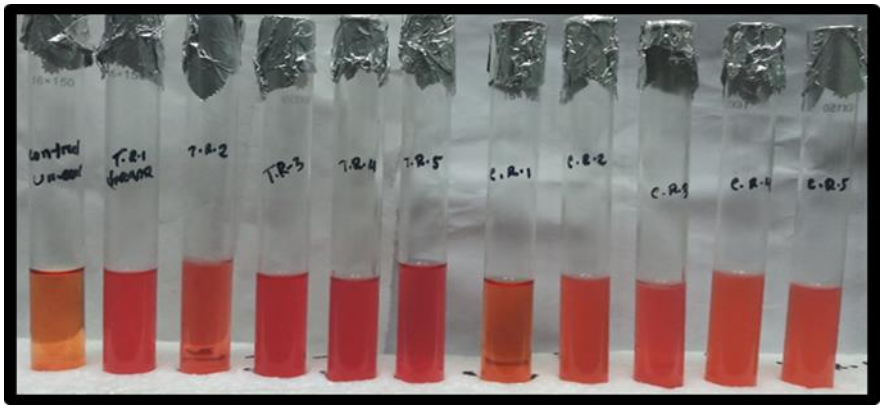


Fig 11 (b): Urease Test result

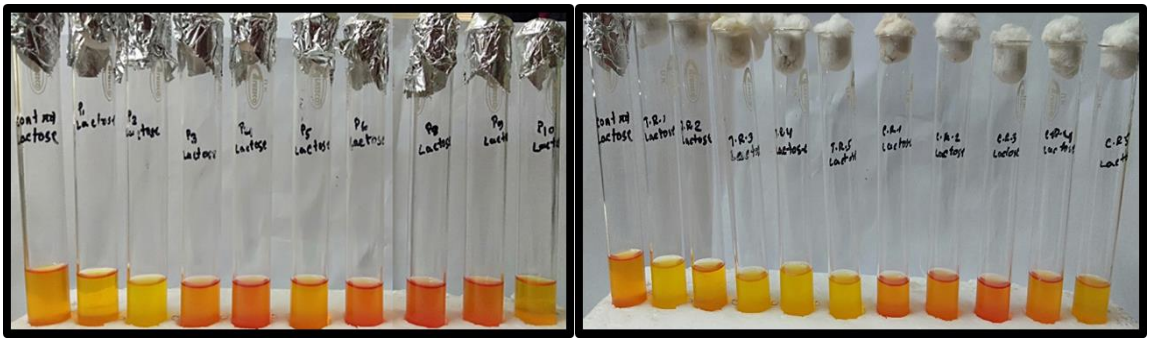
Table 10: Biochemical Characterization results of 19 isolates

Name of isolates	MR	VP	Oxidase	Catalase	MIU	TSI	Citrate	Urease
P1	P	N	P	P	P	P	P	N
P2	P	P	N	P	P	P	P	P
P3	N	P	N	P	P	P	P	P
P4	N	P	N	P	P	P	P	P
P5	N	P	P	P	P	P	N	P
P6	N	N	P	P	P	P	P	P
P8	N	N	N	P	P	P	N	P
P9	P	N	N	P	P	N	P	P
P10	N	P	P	P	P	P	P	P
TR1	N	P	P	P	N	P	P	N
TR2	N	N	P	P	P	P	N	P
TR3	N	N	N	P	P	P	P	P
TR4	N	P	N	P	P	P	N	P
TR5	N	N	N	P	P	P	P	P
CR1	N	N	N	P	N	N	N	N
CR2	N	N	P	P	N	P	P	P
CR3	N	P	P	P	P	N	P	P
CR4	N	N	P	P	P	P	P	P
CR5	N	P	P	P	P	N	N	P

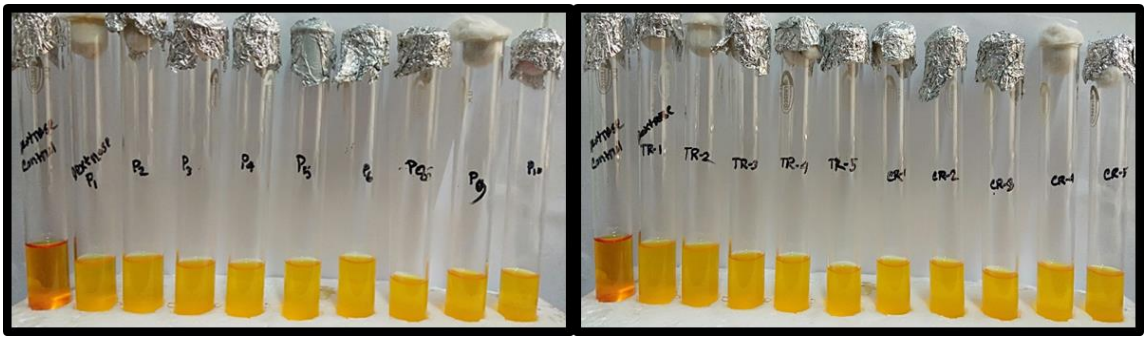
P= Positive, N= Negative

4.3 Carbohydrate Fermentation Test

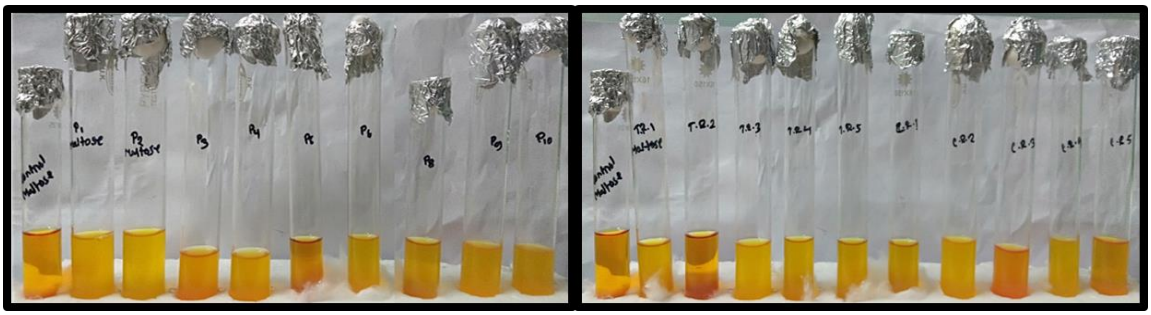
The carbohydrate fermentation test is used to determine whether or not a bacteria can utilize a certain carbohydrate. It tests for the presence of acid and/or gas produced from the fermentation of a single particular carbohydrate [102].



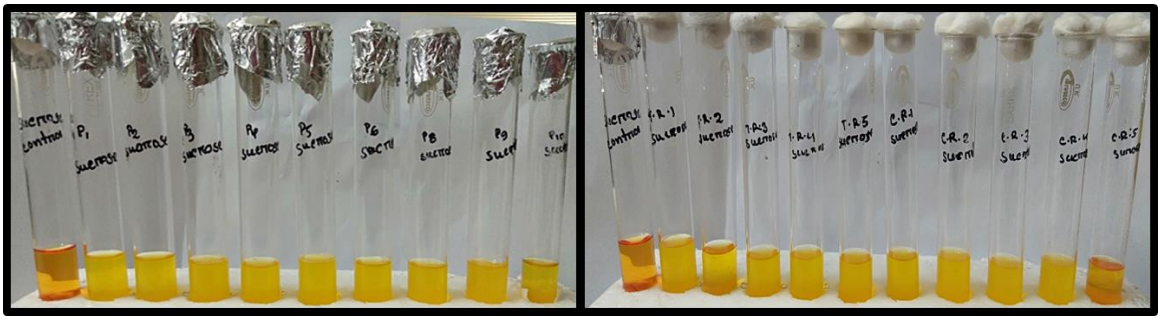
(a)



(b)



(c)



(d)

Fig 12: Carbohydrate utilization Test. (a) lactose, (b) dextrose, (c) maltose and (d) sucrose

The utilization of lactose, dextrose, maltose and sucrose was studied by suing fermentation broth in test tubes and the results are presented in table 11 and figure 12. Only the isolates P1, P2, TR1, TR2, TR3, TR4, TR5 and CR5 could utilize lactose as the sole carbon source in the broth and all the isolates could also use dextrose as their carbon source, but in maltose except P5, TR2, CR3 and CR5 could use as a sole carbon source. In sucrose, all the isolates except CR5 could utilize sucrose as a sole carbon source. In all cases, the media turned to yellow due to the acidic environment that was created by fermentation of sugars and phenol red indicator present in the medium.

4.4 Extracellular hydrolytic enzyme assay

All the bacterial strains were screened in vitro for having a different cell wall degrading enzyme activity such as cellulase, amylase, xylanase and casein [106, 107]. The majority of the bacterial strains showed good activity for these enzymes. Enzymes such as cellulase and pectinase are important for the intracellular root colonization of plant growth-promoting bacteria as these are hydrolytic enzymes with the ability to degrade cellulose/pectin material of the plant cell wall and to protect the host plant. All strains showed positive cellulase amylase, xylanase and casein activity and the results are in table 12 and fig 13.

Table 11: Carbohydrate utilization test results of 19 bacterial isolates

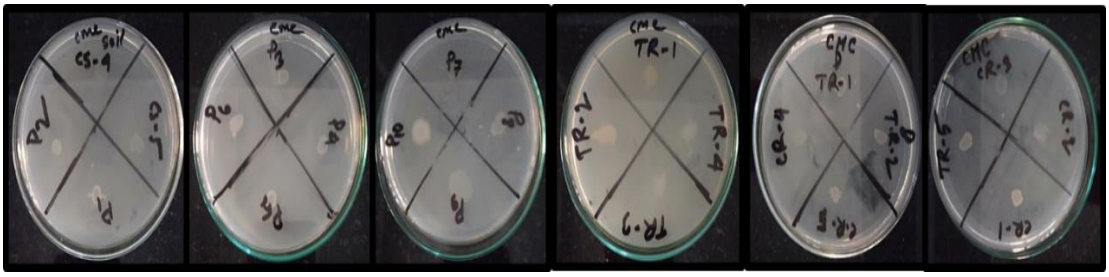
Name of isolate	Different utilized carbon sources			
	Lactose	Dextrose	Maltose	Sucrose
P1	++	+++	+++	+++
P2	+++	+++	+++	+++
P3	N	+++	+	+++
P4	N	+++	++	+++
P5	N	+++	N	+++
P6	N	+++	+++	+++
P8	N	+++	+	+++
P9	N	+++	+++	+++
P10	+	+++	+++	+++
TR1	+++	+++	+++	+++
TR2	+	+++	N	+++
TR3	+++	+++	+++	+++
TR4	+++	+++	+++	+++
TR5	+++	+++	+++	+++
CR1	N	+++	+++	+++
CR2	N	+++	+++	+++
CR3	N	+++	N	+++
CR4	N	+++	+++	+++
CR5	+	+++	N	N

Note: N= No utilization, + = weak utilization, ++ = utilization, +++ = utilization.

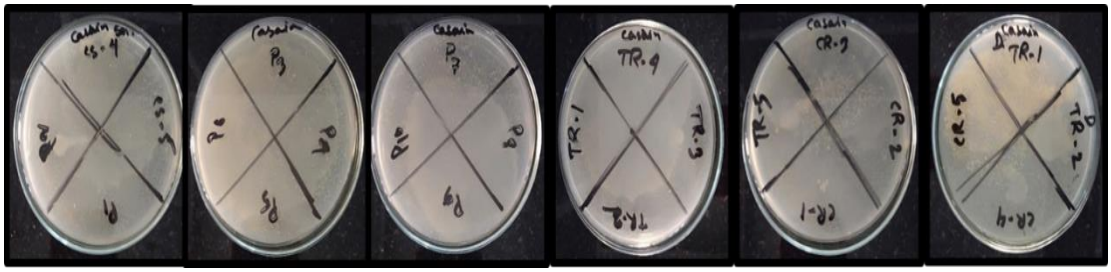
Table 12: Extracellular hydrolytic enzyme assay results

Name of isolate	Activity on different growth media			
	CMC	Casein	Xylan	Amylase
P1	+++	+++	++	+++
P2	+++	+++	+++	+++
P3	+++	+++	+++	+++
P4	+++	+++	+++	++
P5	+++	+++	+++	++
P6	+++	+++	++	+++
P8	+	+++	+	+
P9	+++	+++	+++	+++
P10	+++	+++	+++	+
TR1	++	++	+++	++
TR2	+++	+++	+++	+++
TR3	++	+++	+++	+++
TR4	++	+++	+++	+++
TR5	+++	+++	+++	+++
CR1	+++	+++	+++	+++
CR2	++	+++	+++	+
CR3	++	+++	+++	++
CR4	++	+++	+	+
CR5	+++	++	+++	+++

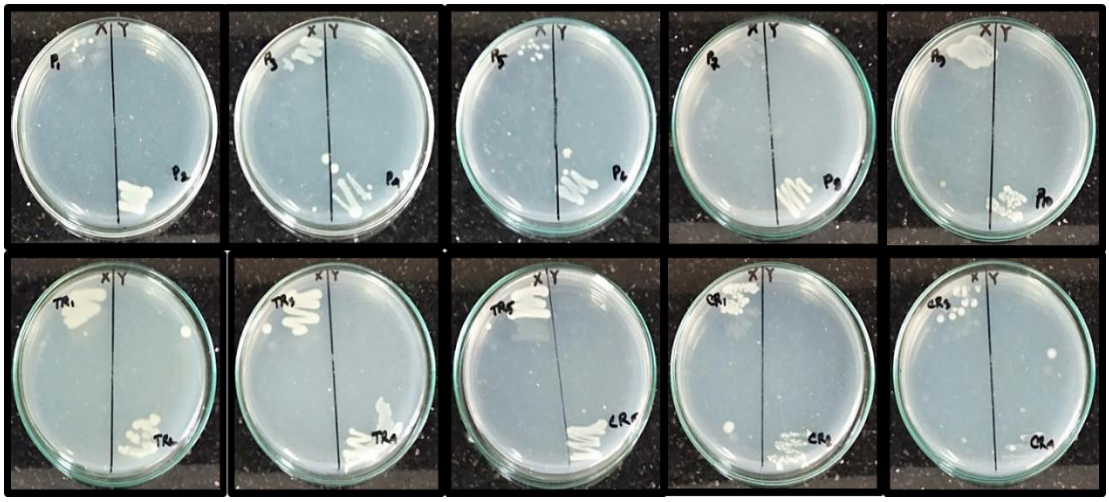
Note: + = weak growth, ++ = medium growth, +++ = high growth.



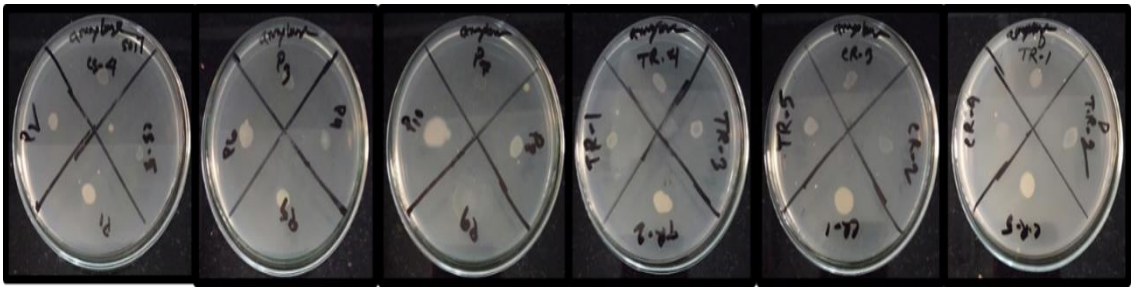
(a)



(b)



(c)



(d)

Fig 13: Extracellular hydrolytic enzyme assay. (a) CMC, (b) Casein, (c) Xylan and (d) Amylase

4.5 Molecular Characterization

The molecular characterization of the isolated strain P1, P2, P3, P4, P5, P6, P8, P9, TR1, TR2, TR3, TR4, TR5, CR1, CR2, CR3 and CR5 were carried out to get the clear picture of the diversity and composition of the endophytic bacteria by 16s rRNA gene sequencing [108, 109]. In this section the results of DNA sequencing analysis using 16s primer mentioned in the previous chapter as well as the bioinformatics analysis.

4.5.1 Agarose gel electrophoresis

After the amplification of the extracted genomic DNA sample with the help of universal 16s primer, the samples are then run into the agarose gel electrophoresis which gave specified band data within 1500bp confirming a successful PCR [114] (Fig 14). The 16s RNA PCR product were purified using PCR purification kit and purified PCR product sent for sequence analysis.

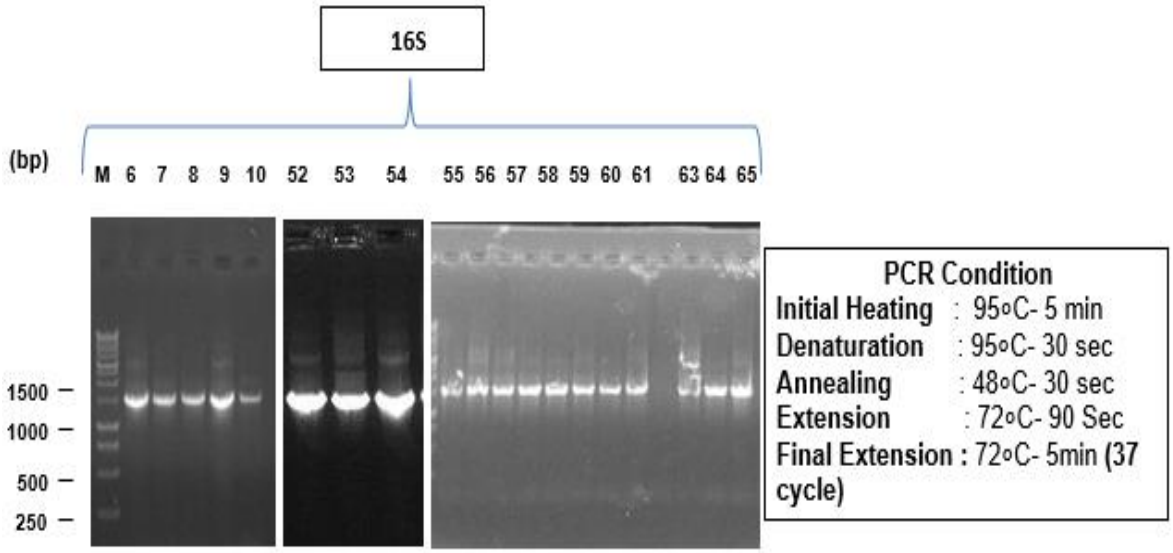


Fig 14: Gel documentation image showing P1, P2, P3, P4, P5, P6, P8, P9, TR1, TR2, TR3, TR4, TR5, CR1, CR2, CR3, CR4 and CR5 band size

4.5.2 DNA sequencing

The identified sequence homology of isolated strains HSTU–ASh55, HSTU–Sh56, HSTU–ASh57, HSTU–Sh58, HSTU–ASh59, HSTU–Sh60, HSTU–Sh61, HSTU–Sh7, HSTU–ASh52, HSTU–ASh53, HSTU–Sh66, HSTU–Sh9, HSTU–ASh54, HSTU–

Sh8, HSTU–Sh63, HSTU–ASh10, HSTU–Sh64 and HSTU–Sh65 with reported sequence in NCBI were given below.

Table 13: Identified nucleotide sequences of Bacterial Isolates P1 (HSTU –ASh55)

Sequence Name	16S rRNA Gene sequence
P1 (HSTU –ASh55)NCBI Accession number (**MN538297**)	AGCTATGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAA CACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGG AAACCGGGGCTAATACCGGATAACATTTTGAACCGCATGG TTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATG GACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCA CCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCG GCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGA GGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA CGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGT AAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATA AGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCT AACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG CGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGG TTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGG AGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGG AAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGAT ATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGT AACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGG ATTAGATACCCTGGTAGTCCACGCCGTAAAACGATGAGTG CTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAGTTAA CGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCT GAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTG GAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTAC CAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTT CTCCTTCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCG TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG AGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGGC ACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTG GGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCT ACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGA CCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTC GGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCG CTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCC CGGGCCTTGTACACATCGCCCGTCACACCACGAGAGTTTG TAACACCCGAATGATGCAGGTGG
Total bases	**1378 bp**

Table 14: Identified nucleotide sequences of Bacterial Isolates P2 (HSTU –Sh56)

Sequence Name	16S rRNA Gene sequence
P2 (HSTU – Sh56)NCBI Accession number (**MN611122**)	CGGGTGAGTAATGTCTAGGAAACTGCATGATGGAGG GGGATAACTACTGGAAACGGTAGCTAATACCGCATA ACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTC ATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAG GTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCT GGTCTGAGAGGATGACCAGCCACACTGGAACTGAGA CACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA ATATTGCACAATGGGCGCAAGCCTGATGCAGCCATG CCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTAC TTTCAGCGGGGAGGAAGGCGATAAGGTTAATAACCT TGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCT AACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTG CAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCAC GCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGG GCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCT AGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAG CGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTG GCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAG GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC CTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGG TTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGT TAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTA AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG TGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA CCTTACCTGGTCTTGACATCCACAGAACTTACCAGAG ATGCTTTGGTGCCTTCGGGAACTGTGAGACAGGTGCT GCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGG TTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTT GCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCC AGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAG TCATCATGGCCCTTACGACCAGGGCTACACACGTGCT ACAATGGCATATACAAAGGAGAAGCGACCTCGCGAG AGCAAGCGGACCTCATAAAGTATGTCGTAGTC
Total bases	**1194 bp**

Table 15: Identified nucleotide sequences of Bacterial Isolates P3 (HSTU –ASh57)

Sequence Name	16S rRNA Gene sequence
P3 (HSTU – ASh57) NCBI Accession number (**MN538298**)	CAAGTCGAGCGGCAGCGGAAAGTAGCATTGCTACTTTGCC GGCGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCC TGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACC GCATAATGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCT CTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGT GGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCT GAGAGGATGACCAGCCACACTGGAACTGAGACACGGTCC AGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT GGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAA GGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGT GCTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAGA AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAG CGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCG GGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGA GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAA ATGCGTAGAGATCTGGAGGAATACCGGTGGGGAAGGCGG CCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGG GGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCT TCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACG GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA AGAACCTTACCTACTCTTGACATCCAGAGAACTTACCAGA GATGCTTTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGC ATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGT CCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTC CGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGG AGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACG AGTAGGGCTACACACGTGCTACAATGGCGCATACAAAGAG AAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGT CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGT CGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAA TACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGG GAGTGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGA GGGCGACTTACCTTAGAGTTTC
Total bases	**1422 bp**

Table 16: Identified nucleotide sequences of Bacterial Isolates P4 (HSTU –Sh58)

Sequence Name	16S rRNA Gene sequence
P4 (HSTU – Sh58) NCBI Accession number (**MN611123**)	CTAACACATGCAAGTCGAACGGTAGCTCAGAGAGCTTGCT CTCGGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGG AAACTGCCTTGATGGAGGGGGATAACTACTGGAAACGGT AGCTAATACCGCATAATGTCGCAAGACCAAAGAGGGGGA CCTTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATT AGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATC CCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACT GAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGG AATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCC GCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCA GCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATT GACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCC AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGG AATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAG TCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATT CGAAACTGGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAA TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGAC GCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT ACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGAGG TTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAA ATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTC AAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG TGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCT TGACATCCACAGAACTTTCCAGAGATGGATTGGTGCCTTC GGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTC GTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAA CCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAA AGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATG ACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACAC GTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGA GAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCGGATT GGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGT AATCGTAGATCAGAATGCTACGGTGAATACGTTTCCCGGG CCTTGTA
Total bases	**1347 bp**

Table 17: Identified nucleotide sequences of Bacterial Isolates P5 (HSTU –ASh59)

Sequence Name	16S rRNA Gene sequence
P5 (HSTU – ASh59) NCBI Accession number (**MN538299**)	CAGGGAGCTATGCTCTCGGGTGACGAGTGGCGGACGGGT GAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAAC TACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGAC CAAAGAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGC CCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCT AGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCC ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGG CAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTA AAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAAC CGCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTA ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAG CGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCG GTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGG GAAATGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGG GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA AAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACA GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCG ATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTA ACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGG TTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGG TGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTT ACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATT GGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGT CGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAA CGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCG GGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAG GTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGG GCTACACACGTGCTACAATGGCGCATACAAAGAGAAGCG ACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTA GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA ATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACG TTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG TGGGTTGCAAAAGATAGTAGGTAGCTTAACCTTCGGGAGG GCGACTTC
Total bases	**1392 bp**

Table 18: Identified nucleotide sequences of Bacterial Isolates P6 (HSTU –Sh60)

Sequence Name	16S rRNA Gene sequence
P6 (HSTU–Sh60) NCBI Accession number (**MK611124**)	CGTGTGAGTAATGTCGGGGAAAATGCCTGATGGAGGGG GATAACTACTGGAAACGGTAGCTAATACCGCATAATGT CGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCA TCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGT AACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAG AGGATGACCAGCCACACTGGAACTGAGACACGGTCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAAT GGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAG AAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGA AGGTGTTGTGGTTAATAACCGCAGCAATTGACGTTACC CGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCG CGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACT GGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGAT GTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAA ACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTC CAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAAT ACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACG CTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGAT ACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGA GGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCG TTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAA AACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGG AGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA CCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATT GGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCT GTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCG GCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGA GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTAC GACCAGGGCTACACACGTGCTACAATGGCGCATACAAA GAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAA GTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCC ATGAAGTCGGAATTCGCTAGTAATCGTAGTTCAAATGC TTCTGTGAACACGTTCCCGGGCC
Total bases	**1280 bp**

Table 19: Identified nucleotide sequences of Bacterial Isolates P8 (HSTU –Sh61)

Sequence Name	16S rRNA Gene sequence
P8 (HSTU–Sh61) NCBI Accession number (**MN611125**)	ACGATCCCTAGCTGGTCGGAGAGGATGGCCAGCCACACT AGATGTGAGACACGGTCCAGACTCCTGCGGGAGGCAGCA GTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGC CATGCCGCGTGTATGAAGAAGGCATTCGGGTTGTAAAGT ACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCTC AGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGT TAATCGGAATTACTGGGGCGTAAAGCGCACGCAGGCGGT CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGG AACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGG GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACA AAGACTGACGCTCAGGTGCGAA A
Total bases	**491 bp**

Table 20: Identified nucleotide sequences of Bacterial Isolates TR3 (HSTU –Sh66)

Sequence Name	16S rRNA Gene sequence
TR3 (HSTU–Sh66) NCBI Accession number (**MN611128**)	GGATTACTGGGCGTAAGCGCACGCAGGCGGTCTGTCAAGT CGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTC GAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATT CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATA CCGGTGGTTGAAGGCGGCCCCCTGGACA
Total bases	**188 bp**

Table 21: Identified nucleotide sequences of Bacterial Isolates P9 (HSTU–Sh7)

Sequence Name	16S rRNA Gene sequence
P9 (HSTU–Sh7) NCBI Accession number (**MK656944**)	TACACATGCAGTCGAACGGTAGCACAGAGAGCTTGCTCTC GGGTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAAC TGCCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAA TACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGG GCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTA GGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGG TCTGAGAGGATGACCAGCCACACTGGAACTGAGACACGGT CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACA ATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAG AAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAG GCGTTGAGGTTAATAACCACAGCGATTGACGTTACCCGCA GAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAA TACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAA AGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCC CCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGGCT AAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTG AAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCG GCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTG GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT AAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCT TCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACG GCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGC ACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGA AGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAG ATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCAT GGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCC CGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAG GCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGG AAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGT AGGGCTACACACGTGCTACAATGGCGCATACAAAGAGAAG CGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGT AGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGG AATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAATAC GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG TGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGG CGCT
Total bases	**1412 bp**

Table 22: Identified nucleotide sequences of Bacterial Isolates TR1 (HSTU–ASh52)

Sequence Name	16S rRNA Gene sequence
TR1 (HSTU–ASh52) NCBI Accession number (**MK691484**)	GCAGTCGAACGGTAGCACAGAGAGCTTGCTCTCGGGTGAC GAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGA TGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCA TAACGTCGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTG CCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGT AACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGG ATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCCT ACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAA GCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGG GTTGTAAAGTACTTTCAGCGGGGAGGAAGGCGTTGAGGTTA ATAACCACAGCGATTGACGTTACCCGCAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG CGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTG GGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGG GGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT CTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAA GACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGA TTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTT GGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCG TTAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAA CTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCA TGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTC TTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTC GGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCG TGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACC CTTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGG AGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGT CAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCT ACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAA GCGGACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTG CAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGA TCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACA CCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAG GTAGCTTAACCTTCGGGAGGGCGCT
Total bases	**1404 bp**

Table 23: Identified nucleotide sequences of Bacterial Isolates TR2 (HSTU–ASh53)

Sequence Name	16S rRNA Gene sequence
TR2 (HSTU–ASh53) NCBI Accession number (**MK691485**)	TGCAGTCGAGCGGTGAGCACAGAGAGCTTGCTCTCGGGTGA CGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGC ATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCAT GCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGG TAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAG GATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTCC TACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCA AGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCG GGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGATAAGGTT AATAACCTTGTCGATTGACGTTACCCGCAGAAGAAGCACCG GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCA AGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCG GTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGG AACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGG GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTG GAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGAC TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA GATACCCTGGTAGTCCACGCCGTAAACGATGTCGATTTGGA GGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTA AATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCA AATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG GTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGA CATCCACAGAACTTACCAGAGATGCTTTGGTGCCTTCGGGA ACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTT GTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTA TCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGAC TGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAG TCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAA TGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGG ACCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAAC TCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAG AATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC CCGTCACACCATGGGAGTGGGTTGCAAAAGAAGTAGGTAG CTTAACCTTCGGGAGGGCGCTACC
Total bases	**1409 bp**

Table 24: Identified nucleotide sequences of Bacterial Isolates TR4 (HSTU–Sh9)

Sequence Name	16S rRNA Gene sequence
TR4 (HSTU–Sh9) NCBI Accession number (**MK656946**)	ACACATGCAGTCGAGCGGATGAGGAGAGCTTGCTCTCTGA TTCAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCT GGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCG CATACGTCCTACGGGAGAAAGTGGGGGATCTTCGGACCTC ACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTG GGGTAACGGCTCACCAAGGCGACGATCCGTAACTGGTCTG AGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG GCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG CCCTCGGGTCGTAAAGCACTTTAAGCTGGGAGGAAGGGTT GTAACCTAATACGTTGCAGCTTTGACGTTACCAGCAGAAT AAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACA GAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCG CGCGTAGGTGGCTTGGTAAAGTTGAATGTGAAATCCCCGG GCTCAACCTGGGAACTGCATCCAAAACTGCCTGGCTAGAG TACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAA TGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGAC CACCTGGACTGATACTGACACTGAGGTGCGAAAGCGTGGG GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA ACGATGTCAACTAGCCGTTGGGGTCCTTGAGACTTTAGTGG CGCAGCTAACGCAATAAGTTGACCGCCTGGGGAGTACGGC CGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCAC AAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAG AACCTTACCAGGCCTTGACATGCAGAGAACTTTCCAGAGA TGGATTGGTGCCTTCGGGAACTCTGACACAGGTGCTGCAT GGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC CGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACATCA TGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAG GAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGC CTGGGCTACACACGTGCACAATGGTCGGTACAAAGGGTTG CCAAACCGCGAGGTGGAGCTAATCCCATAAAACCGATCGT AGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGG AATCGCTAGTAATCGTGAATCAGAACGTCACGGTGAATAC GTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGA GTGGGTTGCACCAGAAGTAGCTAGTCTAACCTTCGGGAGG ACGGTAC
Total bases	**1410 bp**

Table 25: Identified nucleotide sequences of Bacterial Isolates TR5 (HSTU–ASh54)

Sequence Name	16S rRNA Gene sequence
TR5 (HSTU–ASh54) NCBI Accession number (**MK691486**)	TGCAGTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGGTGA CGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTG ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGC ATAACGTCGCAAGACCAAAGTGGGGGACCTTCGGGCCTCA TGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGG GGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGA GAGGATGACCAGCCACACTGGAACTGAGACACGGTCCAGA CTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG CGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGC CTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGAT AAGGTTAATAACCTTGTCGATTGACGTTACCCGCAGAAGA AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGG AGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGC TCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTC TTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCC CTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGA GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC GATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCG GAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCG CAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAA GCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA CCTTACCTGGTCTTGACATCCACAGAACTTAGCAGAGATGC TTTGGTGCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCT GTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCA ACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTAGGCCG GGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAG GTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGG GCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGA CCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGT CCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAAT CGCTAGTAATCGTAGATCAGAATGCTACGGTGAATACGTT CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTG GGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCG CT
Total bases	**1405 bp**

Table 26: Identified nucleotide sequences of Bacterial Isolates CR1 (HSTU–Sh8)

Sequence Name	16S rRNA Gene sequence
CR1 (HSTU–Sh8) NCBI Accession number (**MK656945**)	ATACATGCAGTCGAGCGAACTGATTAGAAGCTTGCTTCTA TGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACC TGCCTGTAAGACTGGGATAACTTCGGGAAACCGAAGCTAA TACCGGATAGGATCTTCTCCTTCATGGGAGATGATTGAAA GATGGTTTCGGCTATCACTTACAGATGGGCCCGCGGTGCA TTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGAT GCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGG AATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCG CGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTT AGGGAAGAACAAGTACAAGAGTAACTGCTTGTACCTTGAC GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTA TTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGAT GTGAAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAAC TGGGGAACTTGAGTGCAGAAGAGAAAAGCGGAATTCCAC GTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCA GTGGCGAAGGCGGCTTTTTGGTCTGTAACTGACGCTGAGG CGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGT AGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGT TTCCGCCCTTTAGTGCTGCAGCTAACGCATTAAGCACTCCG CCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATT GACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT CGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTC TGACAACTCTAGAGATAGAGCGTTCCCCTTCGGGGGACAG AGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTG AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGAT CTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGTGACTGC CGGTGACAAACCGGAGGAAGTGGGGATGACGTCAAATCA TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGG ATGGTACAAAGGGCTGCAAGACCGCGAGGTCAAGCCAATC CCATAAAACCATTCTCAGTTCGGATTGTAGGCTGCAACTCG CCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCA TGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC CGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGA GTAACCGTAAGGAGCTAG CCGC
Total bases	**1424 bp**

Table 27: Identified nucleotide sequences of Bacterial Isolates CR2 (HSTU –Sh63)

Sequence Name	16S rRNA Gene sequence
CR2 (HSTU–Sh63) NCBI Accession number (**MN611126**)	GGATAACTACTGGAATCGGTAGCTGATACCGCATAACGT CGCAAGACCAAAGAGGGGGACCTTCGGGCCTCTTGCCAT CAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAA CGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGG ATGACCAGCCACACTGGAACTGAGACACGGTCCAGACTC CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGG CCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGTG TTGTGGTTAATAACCACAGCAATTGACGTTACCCGCAGA AGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTA AAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATC CCCGGGCTCAACCTGGGAACTGCATTCGAAACTGGCAGG CTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGC GGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCG AAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCG AAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG TCCACGCCGTAAACGATGTCGACTTGGAGGTTGTGCCCT TGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCG CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAA TTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA ATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACAT CCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAA CTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGT TGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC TTATCCTTTGTTGCCAGCGGTTAGGCCGGGAACTCAAAG GAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGA CGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACAC GTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCG AGAGCAAGCGGACCTCATAAAGTGCGTC GAAGTCCGG
Total bases	**1159 bp**

Table 28: Identified nucleotide sequences of Bacterial Isolates CR3 (HSTU–ASh10)

Sequence Name	16S rRNA Gene sequence
TR5 (HSTU–ASh55) NCBI Accession number (**MN559051**)	ATGCAAGCTCGAGCGGTAGCACAGAGAGCTTGCTCTCGGG TGACGAGCGGCGGACGGGTGAGTAATGTCTGGGAAACTG CCTGATGGAGGGGGATAACTACTGGAAACGGTAGCTAAT ACCGCATAACGTCGCAAGACCAAAGTGGGGGACCTTCGG GCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTGGT AGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTG GTCTGAGAGGATGACCAGCCACACTGGAACTGAGACACG GTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC ACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTG AAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAG GAAGGCGGTGAGGTTAATAACCTCGTCGATTGACGTTACC CGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGC GGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGG GCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTG AAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGG CAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGT AGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGG CGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGC GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG TCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTT GAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCC TGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTG ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC GATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACA GAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTGTGA GACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAA TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTT TGTTGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCC AGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA TCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATG GCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGA CCTCATAAAGTATGTCGTAGTCCGGATTGGAGTCTGCAAC TCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCA GAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACC GCCCGTCACACCATGGGAGTGGGTTGCAAAAGATAGTAG GTAGCTTAACCTTCGGGAGGGC
Total bases	**1406 bp**

Table 29: Identified nucleotide sequences of Bacterial Isolates CR5 (HSTU–ASh65)

Sequence Name	16S rRNA Gene sequence
CR5 (HSTU–ASh65) NCBI Accession number (**MN538300**)	ACAGTCTACTAATAGCATGCTCGAACGTGTAGCACAGAG AGCTTGCTCTCGGGTGACGAGTGGCGGACGGGTGAGTAA TGTCTGGGAAACTGCCTGATGGAGGGGGATAACTACTGG AAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAA GAGGGGGACCTTCGGGCCTCTTGCCATCAGATGTGCCCA GATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAG GCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCAC ACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGAT GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGT AAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAATA ACCACAGCGATTGACGTTACCCGCAGAAGAAGCACCGG CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGC AAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCA GGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAA CCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTG TAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGC GTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCC CCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGG GAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA AACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGC TTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTA CGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGC CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAA CGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTT TCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAG GTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTT GGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGT TGCCAGCGGTTAGGCCGGGAACTCAAAGGAGACTGCCA GTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCA TCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAAT GGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCG GACCTCATAAAGTGCGTCGTAGTCCGGATTGGAGTCTGC AACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAG ATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTAC ACTCCGCCCGTCACACCATGGGAGTGGGTTGCAAAAGAT AGTAGGTAGCCTAGCCTTCAGGGAGGTCCGTACTTCCAC CGTAGAG
Total bases	**1438 bp**

Table 30: Identified nucleotide sequences of Bacterial Isolates CR4 (HSTU –Sh64)

Sequence Name	16S rRNA Gene sequence
CR4 (HSTU–Sh64) NCBI Accession number (**MN626360**)	TTAGGAATTTTGAGCGAGGGGAGAAAGCCTGATCCAGCA GATGCGAGCGAGTGGGAAGGAGTCCTTGAGGTTGGAAGA GACTGTTGTCCGGAAAGGAATCCTGGGCTCTAATGCAGC CGGGGGAAGACGGTACCGGAAGAATCAGCACCGGCTAA CTGCGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGC GTTAATCGGAATTACTGGGCGTAAAGCGTGCGCAGGCGG TTTGCTAAGACCGATGTGAAATCCCCGGGCTCAACCTGG GAACTGCATTGGTGACTGGCAGGCTAGAGTATGGCAGAG GGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGAG ATGTGGAGGAATACCGATGGCTGAAGGCAGCCCCCTGGG CCAATACTGACGCTCATGCACGAAAGCGTGGGGAGCAAA CAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATG TCAACTAGTTGTTGGGGATTCATTTCCTTAGTAACGTAGC TAACGCGTGAAGTTGACCGC
Total bases	**527 bp**

4.6 Bioinformatics analysis

4.6.1 Conversion

The isolated strains came out as .ab1 files from the genetic analyzer which had to be converted to 'FASTA' format which is a universal readable text format. For this purpose DNA sequence assembler v4 (2013), HeracleBioSoft was used.

4.6.2 Identification

All the 18 identified strains (*Bacillus sp.* HSTU–ASh55, *Klebsiella sp.* HSTU –Sh56, *Enterobacter sp.* HSTU-ASh57, *Enterobacter sp.* HSTU –Sh58, *Enterobacter sp.* HSTU-ASh59, *Enterobacter sp.* HSTU –Sh60, *Enterobacter sp.* HSTU –Sh61, *Pantoea sp.* HSTU–Sh7, *Pantoea sp.* HSTU–ASh52, *Klebsiella sp.* HSTU–ASh53, *Enterobacter sp.* HSTU –Sh66, *Pseudomonas sp.* HSTU–Sh9, *Klebsiella sp.* HSTU–ASh54, *Bacillus sp.* HSTU–Sh8, *Enterobacter sp.* HSTU –Sh63, *Klebsiella sp.* HSTU–ASh10, *Burkholderia sp.* HSTU –Sh64 and *Pantoea sp.* HSTU-Sh65) were identified by using 'NCBI megablast'.

4.6.3 NCBI BLAST analysis

The identification of nucleotide sequences of the 18 isolates were identified by using NCBI nucleotide 'BLAST' (basic local alignment search tool) analysis. This analysis was done by the nucleotide collection database of NCBI. This method was done by the default optimization tool 'Megablast' which is an intra-species comparison tool for the identification of unknown nucleotide sequences by aligning and comparing with a known similar sequence. After analysis the isolates HSTU–ASh55, HSTU–Sh56, HSTU–ASh57, HSTU–Sh58, HSTU–ASh59, HSTU–Sh60, HSTU–Sh61, HSTU–Sh7, HSTU–ASh52, HSTU–ASh53, HSTU–Sh66, HSTU–Sh9, HSTU–ASh54, HSTU–Sh8, HSTU–Sh63, HSTU–ASh10, HSTU–Sh64 and HSTU–Sh65 were identified as *Bacillus sp., Klebsiella sp., Enterobacter sp., Enterobacter sp., Enterobacter sp., Enterobacter sp., Enterobacter sp., Pantoea sp., Pantoea sp., Klebsiella sp., Enterobacter sp., Pseudomonas sp., Klebsiella sp., Bacillus sp., Enterobacter sp., Klebsiella sp., Burkhloderia sp.* and *Pantoea sp.*, respectively. All the identified sequences are given in table 31 with their ID, bp, species and gene bank accession no.

4.6.4 Phylogenetic analysis

The phylogenetic analysis considered a highly reliable and important bioinformatics tool. The importance of phylogenetic analysis lies in its simple manifestation and easy handling of data. The simple tree representation of the evolution makes the phylogenetic analysis easier to comprehend and represent as well. The varied applications of phylogenetics in different fields of biology make this analysis an absolute necessity.

The evolutionary history of all 18 identified bacterial isolates was inferred using the Neighbor-Joining method [114]. Phylogenetic tree of HSTU–ASh55, HSTU–Sh56, HSTU-ASh57, HSTU-Sh58, HSTU–ASh59, HSTU-Sh60, HSTU–Sh61 and HSTU–Sh7 (fig 15) was conducted by using 29 nucleotide sequences and the optimal tree with the sum of branch length = 3.80841158 is shown. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site [115]. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed

for each sequence pair (pairwise deletion option). There were a total of 3111 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [116].

Table 31: Summary of all identified endophytic bacterial isolates by partial sequencing of 16s rRNA with bp and accession no.

Strains (Bacterial IDs)	Total sequence bp	Database NCBI	GenBank accession no.
P1 (HSTU–ASh55)	1378bp	*Bacillus sp.*	MN538297
P2 (HSTU-Sh56)	1194bp	*Klebsiella sp.*	MN611122
P3 (HSTU-ASh57)	1422bp	*Enterobacter sp.*	MN538298
P4 (HSTU-Sh58)	1347bp	*Enterobacter sp.*	MN611123
P5 (HSTU-ASh59)	1392bp	*Enterobacter sp.*	MN538299
P6 (HSTU-Sh60)	1280bp	*Enterobacter sp.*	MN611124
P8 (HSTU-Sh61)	491bp	*Enterobacter sp.*	MN611125
P9 (HSTU–Sh9)	1412bp	*Pantoea sp.*	MK656944
TR1 (HSTU–ASh52)	1404bp	*Pantoea sp.*	MK691484
TR2 (HSTU–ASh53)	1409bp	*Klebsiella sp.*	MK691485
TR3 (HSTU-Sh66)	188bp	*Enterobacter sp.*	MN611128
TR4 (HSTU–Sh9)	1410bp	*Pseudomonas sp.*	MK656946
TR5 (HSTU–ASh54)	1405bp	*Klebsiella sp.*	MK691486
CR1 (HSTU–Sh8)	1424bp	*Bacillus sp.*	MK656945
CR2 (HSTU-Sh63)	1159bp	*Enterobacter sp.*	MN611126
CR3 (HSTU-ASh10)	1406 bp	*Klebsiella sp.*	MN559051
CR4 (HSTU- Sh64)	527bp	*Bulkholderia sp.*	MN626360
CR5 (HSTU-Sh65)	1438bp	*Pantoea sp.*	MN538300

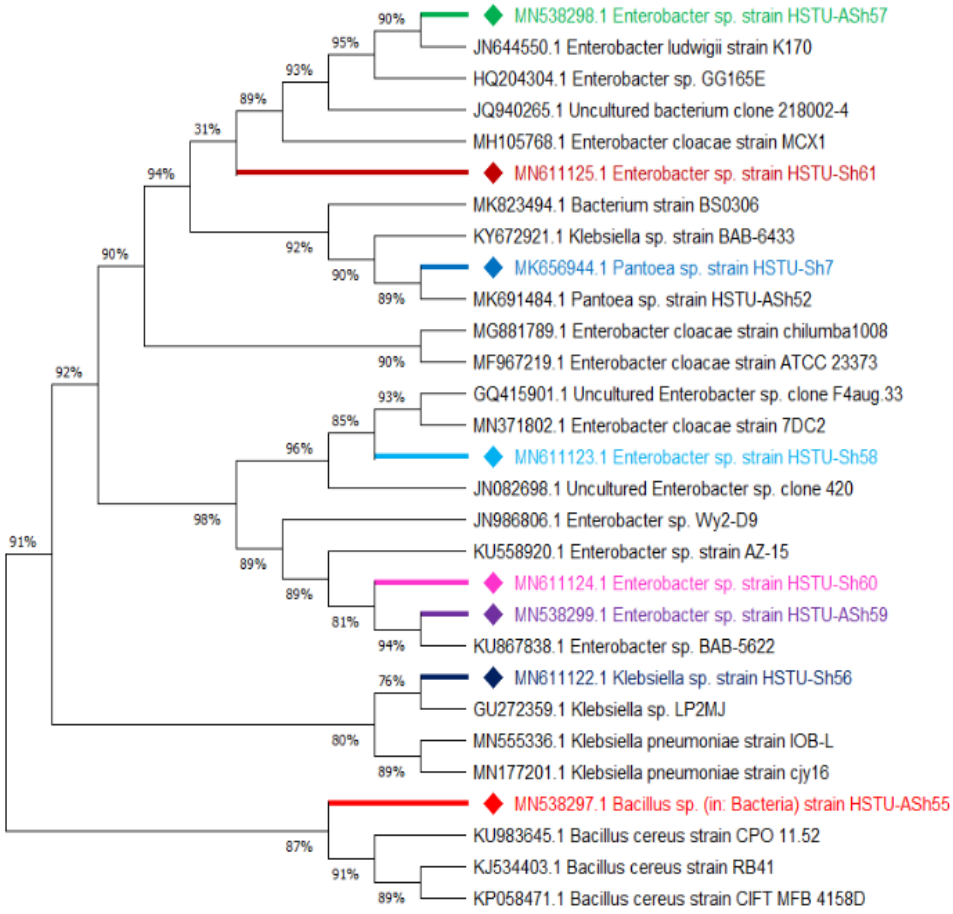


Fig 15: Phylogenetic tree of HSTU–ASh55, HSTU–Sh56, HSTU-ASh57, HSTU-Sh58, HSTU–ASh59, HSTU-Sh60, HSTU–Sh61 and HSTU–Sh7

Phylogenetic tree of HSTU–ASh52, HSTU–ASh53, HSTU-Sh66, HSTU-Sh9, HSTU–ASh54 (fig 16) was conducted by using 28 nucleotide sequences and the optimal tree with the sum of branch length = 3.86782430 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [115] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 3104 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [116].

Phylogenetic tree of HSTU–Sh8, HSTU–Sh63, HSTU-ASh10, HSTU-Sh64, HSTU–ASh65 (fig 17) was conducted by using 30 nucleotide sequences and the optimal tree with the sum of branch length = 4.144097 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method [115] and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 3117 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [116].

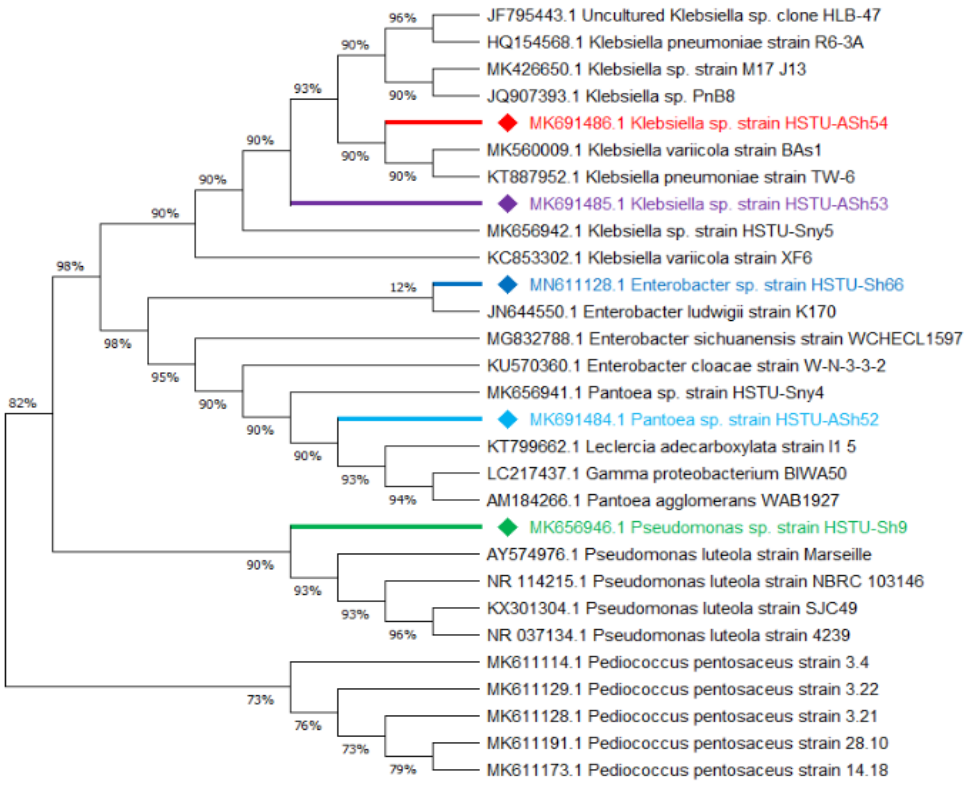


Fig 16: Phylogenetic tree of HSTU–ASh52, HSTU–ASh53, HSTU-Sh66, HSTU-Sh9 and HSTU–ASh54

4.7 Screening of endophytic bacterial isolates as PGP traits

Bacterial plant growth promotion is a deep-rooted and multifaceted phenomenon that is often proficient via the activities of more than one PGP trait showed by the associated bacteria [122]. In this study, the isolated endophytic bacteria were screened for different PGP traits such as phosphate solubilization, IAA production, N_2 fixation and

ACC production. Among these, 100% of bacterial isolates were found positive for phosphate solubilization, N_2 fixation, IAA production and ACC production.

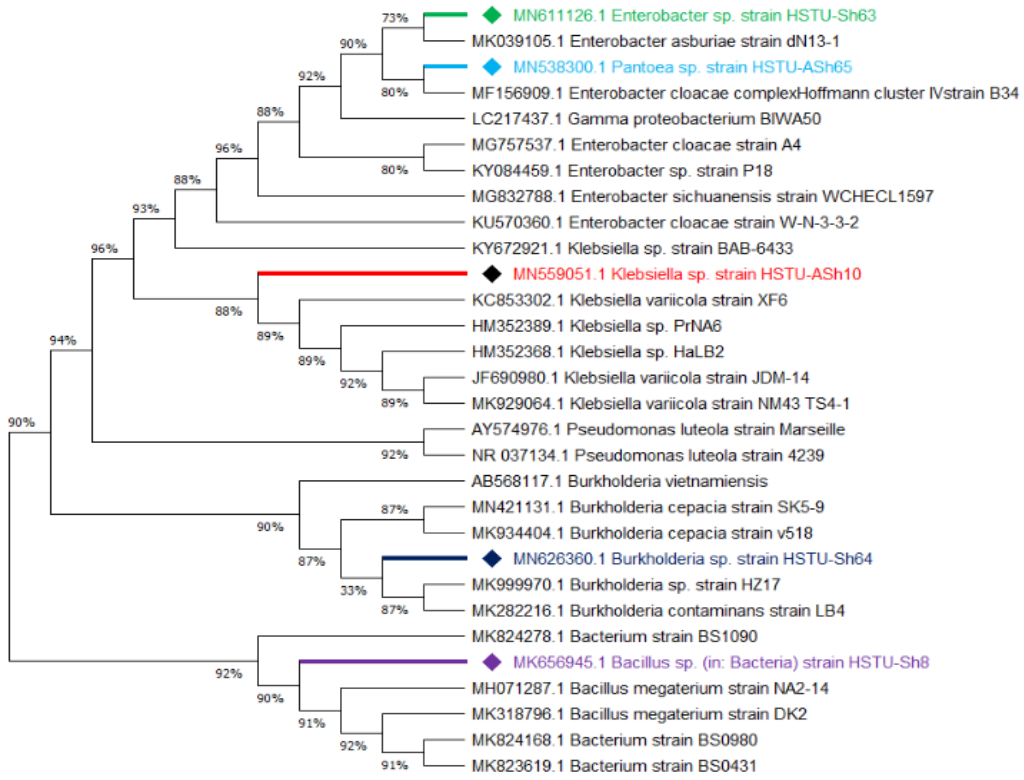


Fig 17: Phylogenetic tree of HSTU–Sh8, HSTU–Sh63, HSTU-ASh10, HSTU-Sh64 and HSTU–ASh65

4.7.1 Qualitative analysis of Phosphate solubilization by the isolates

Phosphorus (P) is the second important macronutrient that promotes plant growth, after nitrogen (N) and the major part of the reservoirs are not available for them. Phosphorus helps in many physiological activities such as cell division, photosynthesis and development of the root system. This element is quite insoluble in soils and accordingly, this element was applied exogenously in traditional agriculture as chemical P fertilizers. Nevertheless, when applied as fertilizer to crop fields, P passes rapidly to become insoluble. Plants take P from the soil in the form of phosphate anions (H2PO4 and HPO4-) and the potentiality of endophytic bacteria to solubilize insoluble phosphate thereby, enhancing the P availability for plant growth [96].

The total of 19 isolates was cultured in Pikovskayas Agar medium to check their phosphate solubilizing ability where the main energy source is dextrose. All the bacterial

isolates showed activity and form zone around the colony on the Pikovskayas Agar medium indicating the positive test for P-solubilization (Fig 18).

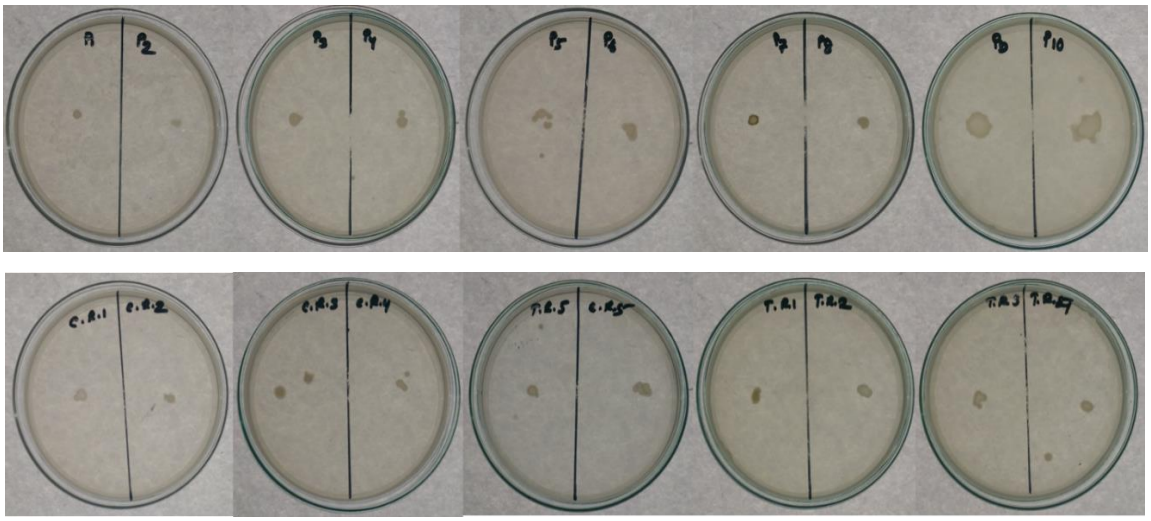


Fig 18: Qualitative Phosphate solubilization activity

4.7.2 Qualitative analysis of Nitrogen fixation by the isolates

Nitrogen (N) is the most limiting nutrient for plants that require it for the formation of amino acids and subsequently, proteins. Endophytes have the exclusive of managing the process of combination or conversion of atmospheric nitrogen into organic forms, which can be finally assimilated by plants All the isolated endophytic bacterial strains were tested qualitatively to check the ability for nitrogen fixation using both N-free (*Azotobacter* agar medium) and N-containing (yeast extract mannitol agar) medium [117, 118]. In N-free medium, all the isolates were showed activity by conforming the ability to fix nitrogen (Fig 19).

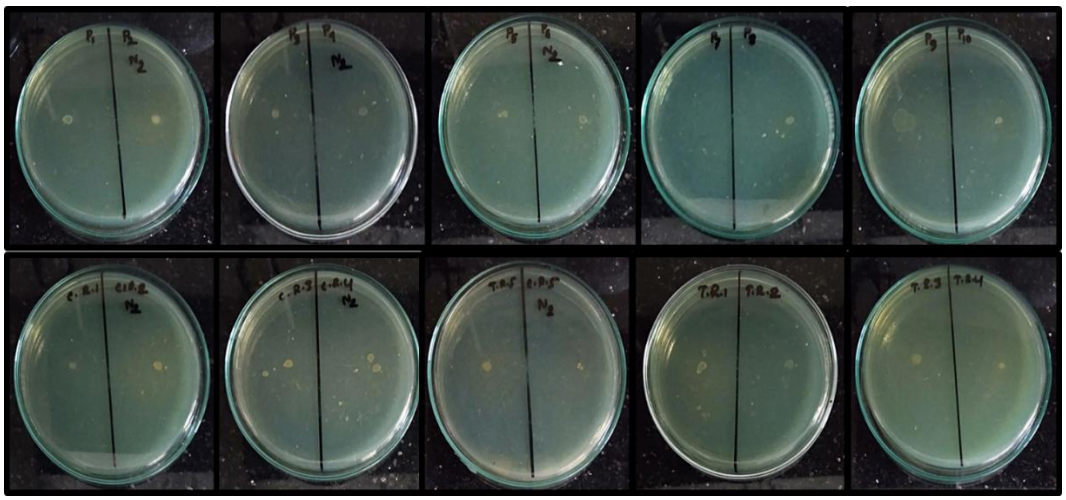


Fig 19: Qualitative analysis of Nitrogen fixation in N-free (*Azotobacter* agar) medium

All the isolates are able to fix atmospheric nitrogen in the N-containing medium (fig 20). Eight of the isolates, P4, P5, P8, P9, TR3, TR4, CR1 and CR2 showed the highest growth and formed a clear zone on the medium. The diameter of the halo zone was recorded in cm (Table 32).

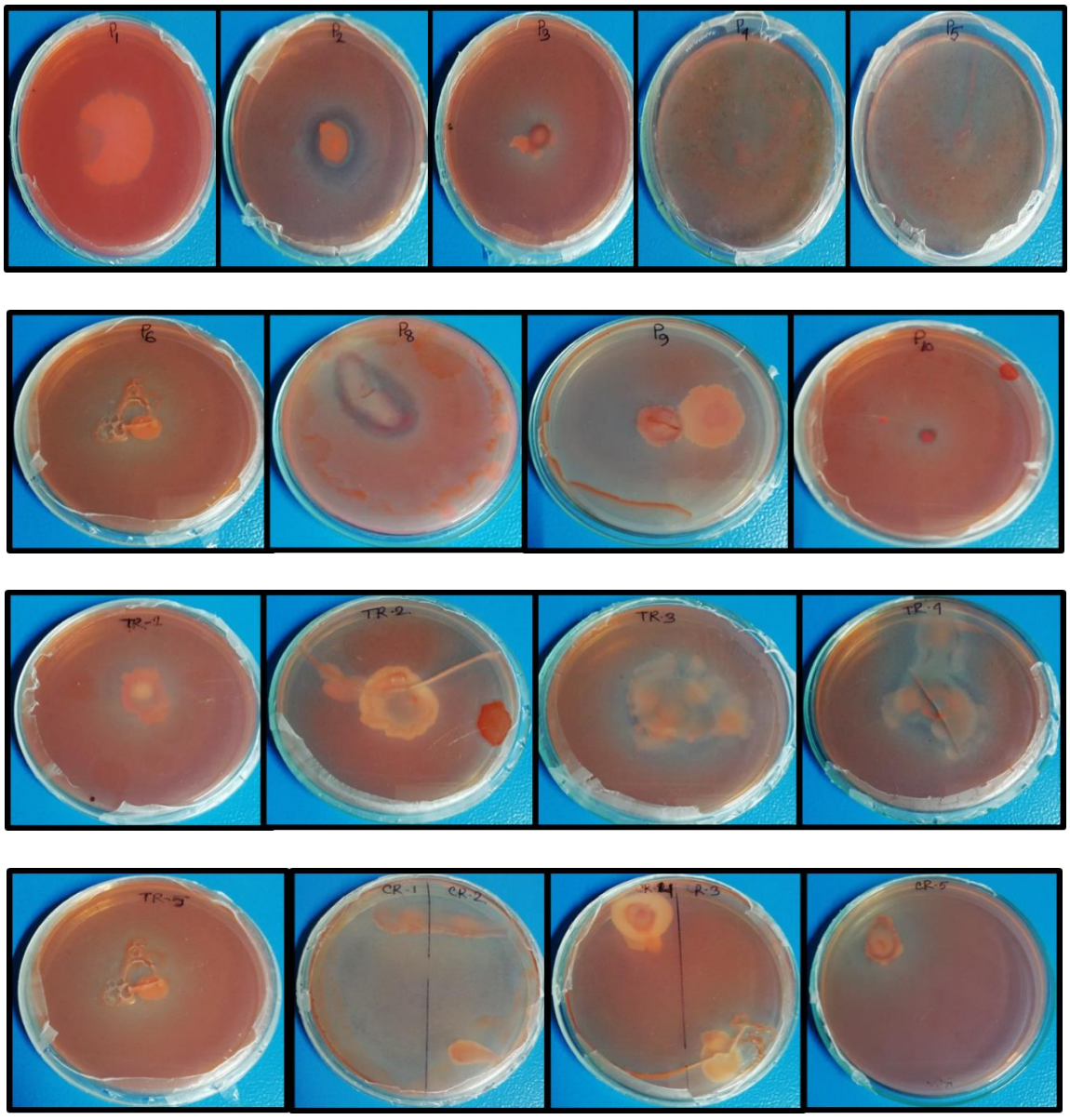


Fig 20: Qualitative analysis of Nitrogen fixation in N-containing medium

Table 32: Growth rate and zone formation by the isolates on yeast extract mannitol agar medium after 7 days of incubation

Name of isolate	Growth on N-free medium	Diameter of Halo Zone (cm)
P1	++	3.3
P2	++	3.0
P3	+	2.5
P4	+++	8.0
P5	+++	8.0
P6	+	2.7
P8	+++	8.0
P9	+++	8.0
P10	+	1.0
TR1	+	2.3
TR2	++	3.4
TR3	+++	5.0
TR4	+++	6.5
TR5	+	2.0
CR1	+++	8.0
CR2	+++	8.0
CR3	++	3.4
CR4	+	2.3
CR5	++	2.3

4.7.3 Quantitative analysis of Indole Acetic Acid (IAA) production

IAA is an important physiological active auxin that helps to increase the root surface and length by providing greater access to soil nutrients and soil uptake. Production of IAA allows the bacteria in a diverse way to colonize the plant tissues and it also support to loosen plant cell wall and promotes root exudation providing additional nutrients. IAA also functions as an important signal molecule that regulates the host plant root development and promotes the plant-microbe interaction [119].

All the endophytic bacterial isolates were tested for qualitative IAA production [142]. When the Salkowaski's reagent added to the Luria broth supplemented with 0.01%

tryptophan all the endophytic bacterial isolates produced pink color which indicates the production of IAA (Fig 21). The amount IAA in 48 h grown liquid cultures supplemented with tryptophan ranged from 0.499 to 7.002 µg/ml which is more than the control amount (Table 33). The isolates P2, P5, TR1, TR3, TR4 and TR5 produced more than 3 µg IAA ml^{-1} culture.

Table 33: Quantitative Indole acetic acid (IAA) production by endophytic bacterial isolates

Name of isolate	Absorbance at 540nm	Amount of IAA(µg/ ml) with Tryptophan
Control	0.269	0.42
P1	0.334	0.6206
P2	1.289	3.514
P3	0.633	1.003
P4	0.650	1.578
P5	1.462	4.038
P6	0.980	2.578
P8	1.117	2.993
P9	0.797	2.02
P10	0.395	0.8
TR1	2.140	6.093
TR2	0.294	0.499
TR3	2.44	7.002
TR4	1.334	3.65
TR5	2.35	6.729
CR1	0.57	1.33
CR2	0.884	2.28
CR3	0.798	2.026
CR4	0.395	0.8
CR5	0.409	0.9

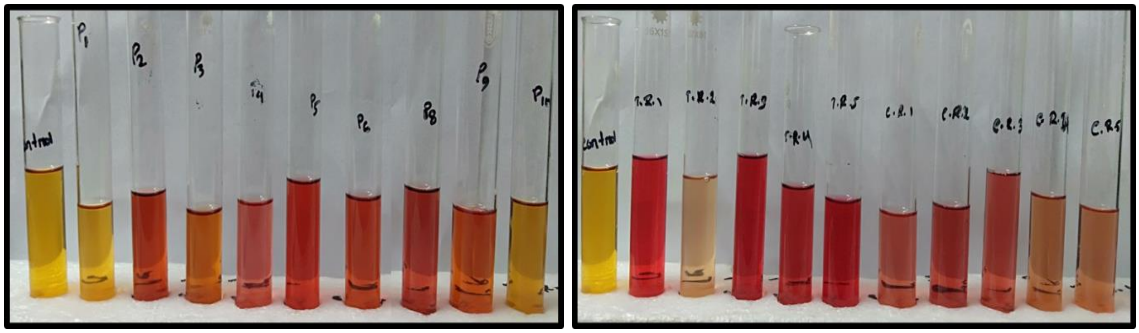


Fig 21: Quantitative Indole acetic acid (IAA) production in Luria broth

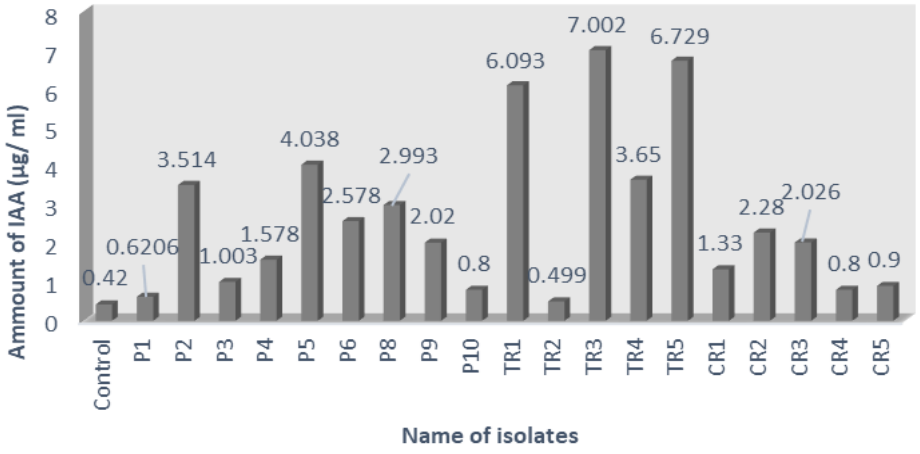


Fig 22: Quantitative indole acetic acid (IAA) production by 19 isolates

4.7.4 Quantitative estimation of ACC-deaminase activity

The enzyme 1-Aminocyclopropane-1-carboxylate (ACC) deaminase catalyzes the degradation of ACC into α-ketobutyrate and ammonia. ACC, which is the immediate precursor of the plant hormone ethylene. ACC helps plants in sustaining growth and development under stress (biotic and abiotic) conditions by reducing stress and helps in ethylene production in plants. This enzyme plays a significant role in sustaining plant growth and development under biotic and abiotic stress conditions by reducing stress-induced ethylene production in plants [120].

All the endophytic bacterial isolates were subjected for ACC deaminase activity on Dworkin and Foster (DF) minimal medium. The ability of isolates to utilize ACC as the sole source of N was assessed on the basis of bacterial growth on the liquid medium containing substrate ACC and (NH4)2SO4 separately on DF minimal medium and the absorbance were taken at 600nm (Table 34 and Fig 23).

Table 34: Absorbance of ACC deaminase activity by the isolates

Name of isolate	Negative control	Sample absorbance with ACC	Positive control - $(NH_4)_2SO_4$	Sample absorbance with $(NH_4)_2SO_4$
P1	0.067	0.076	0.109	0.069
P2	0.067	0.076	0.109	0.073
P3	0.067	0.089	0.109	0.070
P4	0.067	0.070	0.109	0.069
P5	0.067	0.079	0.109	0.081
P6	0.067	0.17	0.109	0.070
P7	0.067	0.123	0.109	0.068
P8	0.067	0.101	0.109	0.065
P9	0.067	0.089	0.109	0.062
P10	0.067	0.074	0.109	0.061
TR1	0.067	0.069	0.109	0.066
TR2	0.067	0.087	0.109	0.091
TR3	0.067	0.144	0.109	0.076
TR4	0.067	0.070	0.109	0.072
TR5	0.067	0.083	0.109	0.076
CR1	0.067	0.062	0.109	0.0101
CR2	0.067	0.088	0.109	0.078
CR3	0.067	0.099	0.109	0.074
CR4	0.067	0.111	0.109	0.112
CR5	0.067	0.076	0.109	0.120

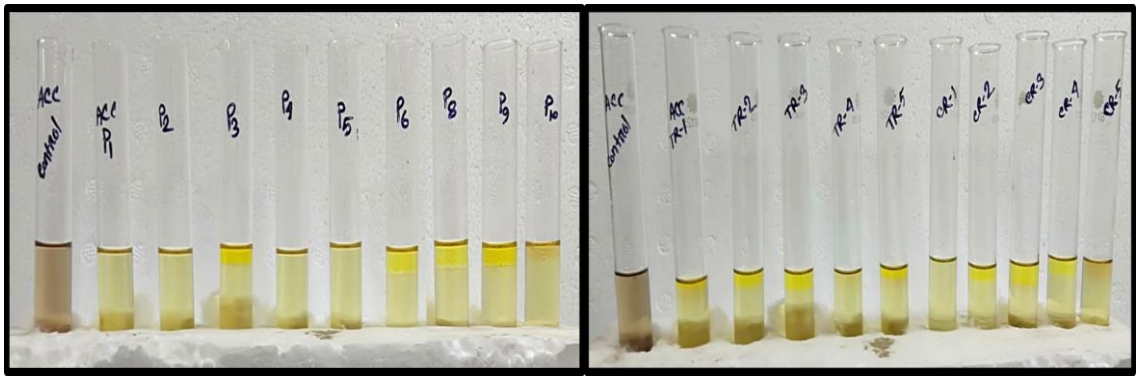


Fig 23: Quantitative estimation of ACC-deaminase activity of all 19 isolates

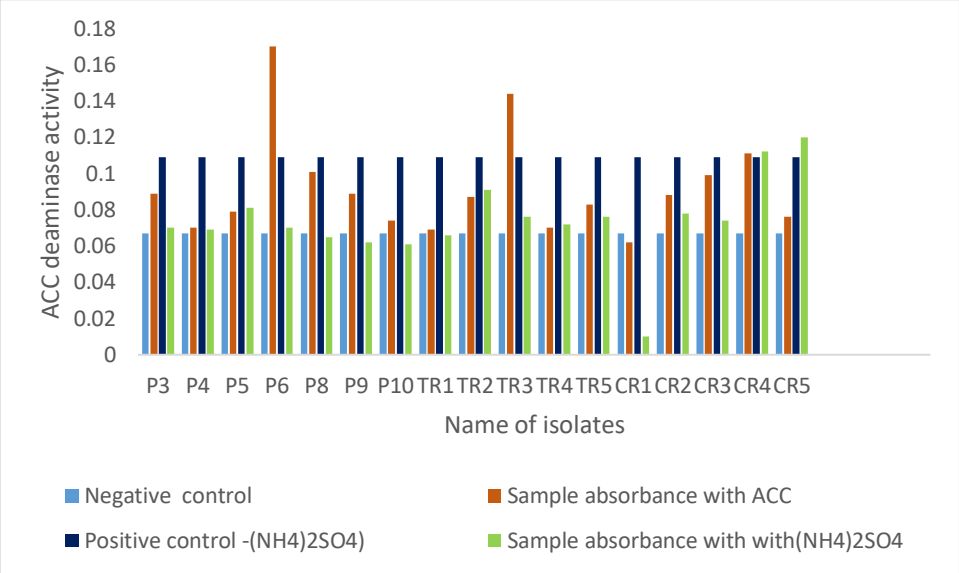


Fig 24: Quantitative estimation of ACC-deaminase activity of all 19 isolates with negative and positive control

4.8 Application of endophytic bacteria on tomato plant

Endophytic bacteria enhance the plant growth by direct mechanism or indirect mechanism, in the direct mechanism they provide nutrients and in the indirect mechanism, they promote growth by protecting the plants from other pathogens [19]. The application of endophytic bacteria to crop plants can be several ways which include seed treatment, soil application, bacterization of plant-pathogen and even foliar application. The growth-promoting ability of 19 isolates was applied to check the gemination effect and also to check the seedlings effect of tomato.

4.8.1 Application of endophytic bacterial isolates on germination and seedling growth of tomato (Dark condition)

The isolates were selected based on their molecular, cultural identification and multiple growth-promoting functions. All the isolates showed better plant growth-promoting traits such as IAA production, ACC deaminase activity, phosphate solubilization and nitrogen fixation. The application of the 19 isolated strains was carried out on the seeds of the tomato. The seeds were treated with bacterial suspension for 6 hours before including on a petri dish. After 9 days the effect of all isolates on germination percentage, seedling length and vigor index were recorded (Fig 25).

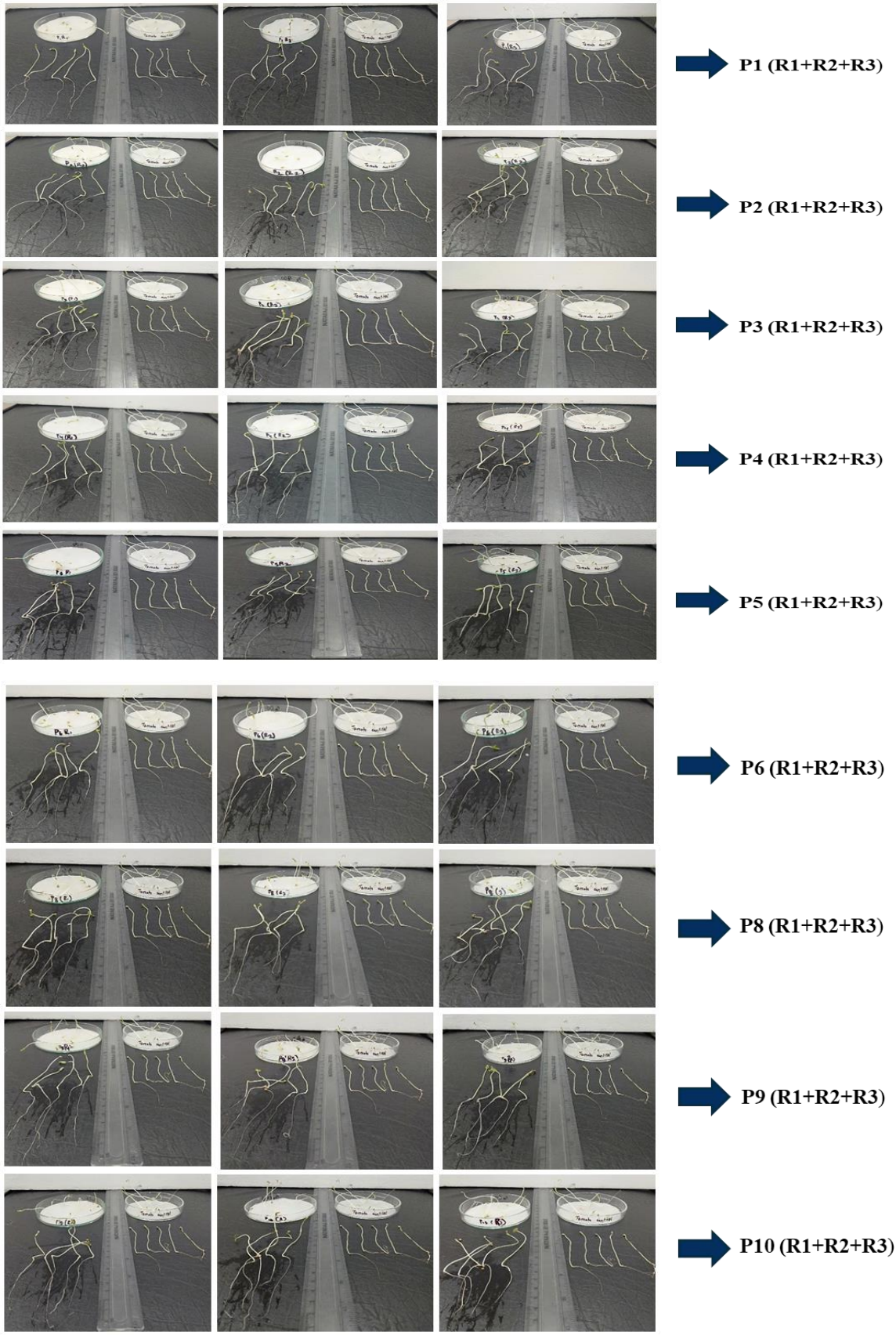


Fig 25 (a): Application of endophytic bacteria on germination test of tomato. Right side control and Left side treatment. (Data were collected after 9 days of germination)

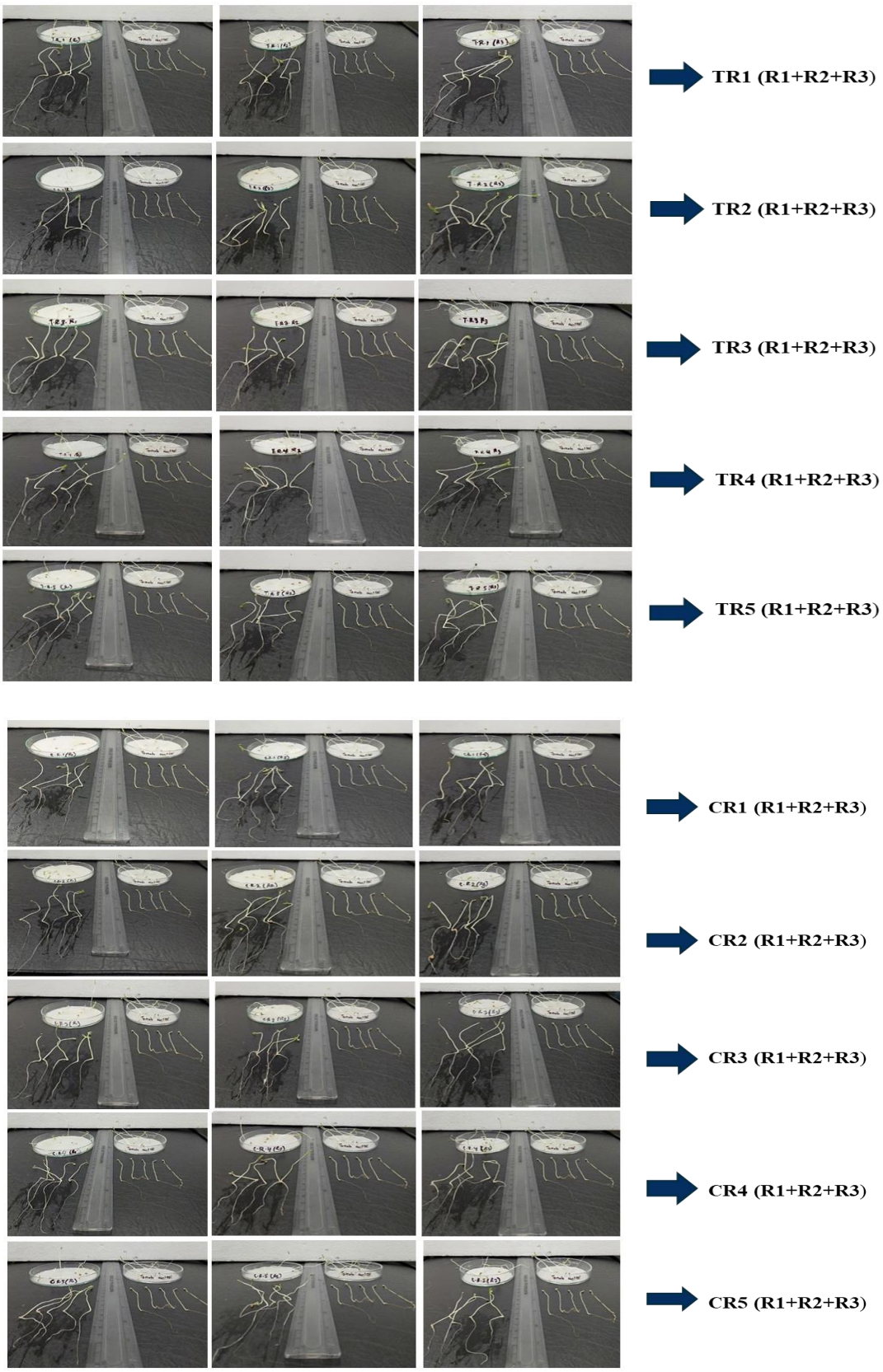


Fig 25 (b): Application of endophytic bacteria on germination test of tomato. Right side control and Left side treatment. (Data were collected after 9 days of germination)

Table 35: Root, shoot and seedling length, germination percentage and vigour index of tomato comparing with control after 9 days of germination

Name of isolates	Germination %	Root length (cm)	Shoot length (cm)	Seedling length (cm)	Vigour Index
Control	69.23±0.00^{b}	3.50±0.58^{c}	5.34±0.11^{b}	8.84±0.53^{b}	611.99±36.71^{b}
P1	87.18±4.44^{a}	6.06±1.09^{ab}	6.11±0.43^{ab}	12.17±1.44^{a}	1056.56±76.19^{a}
P2	94.87±4.44^{a}	5.31±0.9^{b}	6.03±0.38^{ab}	11.35±1.19^{a}	1074.07±84.69^{a}
P3	89.75±4.44^{a}	5.49±2.06^{ab}	5.95±0.65^{ab}	11.44±1.52^{a}	1032.34±264.51^{a}
P4	89.75±8.88^{a}	6.07±2.27^{ab}	6.27±0.39^{a}	12.34±1.00^{a}	1101.63±21.45^{a}
P5	87.18±8.89^{a}	5.81±0.68^{ab}	5.95±0.12^{ab}	12.77±0.63^{a}	1024.11±95.98^{a}
P6	94.87±8.88^{a}	6.47±0.17^{ab}	5.93±0.30^{ab}	12.40±0.20^{a}	1176.33±110.20^{a}
P8	87.18±4.44^{a}	5.89±0.24^{ab}	5.90±0.29^{ab}	11.79±0.42^{a}	1027.33±36.66^{a}
P9	87.18±4.44^{a}	7.19±0.32^{ab}	6.04±0.37^{ab}	13.23±0.46^{a}	1152.41±36.67^{a}
P10	97.44±4.44^{a}	6.63±0.63^{a}	6.27±0.39^{a}	12.91±0.46^{a}	1258.37±90.83^{a}
TR1	97.44±4.44 ^{a}	6.37±0.50^{ab}	6.49±0.38^{a}	12.86±0.86^{a}	1250.52±25.88^{a}
TR2	89.75±11.75^{a}	5.74±0.50^{ab}	6.25±0.55^{a}	11.99±1.04^{a}	1081.13±207.52^{a}
TR3	94.87±4.44^{a}	5.51±1.02^{ab}	6.17±0.50^{a}	11.68±0.58^{a}	1106.58±24.03^{a}
TR4	92.31±0.00^{a}	6.63±0.61^{ab}	6.19±0.31^{a}	12.81±0.90^{a}	1182.80±82.36^{a}
TR5	87.18±11.75^{a}	6.51±0.76^{ab}	6.31±0.72^{a}	12.82±0.11^{a}	1118.37±158.72^{a}
CR1	94.87±8.88^{a}	6.50±0.40^{ab}	6.03±0.41^{ab}	12.53±0.50^{a}	1187.51±97.02^{a}
CR2	92.31±7.69^{a}	6.77±1.08^{ab}	6.38±0.40^{a}	11.15±1.27^{a}	1220.64±218.99^{a}
CR3	97.44±4.44^{a}	6.12±0.33^{ab}	6.57±0.11^{a}	12.69±0.44^{a}	1237.14±79.34^{a}
CR4	100±0.00^{a}	6.61±1.02^{ab}	6.01±0.43^{ab}	12.63±1.05^{a}	1262.67±105.40^{a}
CR5	89.75±8.88^{a}	6.40±1.06^{ab}	6.22±0.42^{a}	12.62±1.42^{a}	1140.70±243.90^{a}

Note: Observation recorded after 9 days.

Each value is the mean of five replications.

Different letters in superscripts indicate significantly different values.

Germination percentage

In germination percentage all the isolates were shown significant results after treated with bacterial suspension comparing with control. The isolates CR4 (isolated from cauliflower root) was shown the highest significant percentage of germination rate which

was 100% and the least significantly germination percentage was with the control (69.23%), which was without any endophytic treatment. The isolates P10 (97.44%), TR1 (97.44%) and CR1 (all 94.87%) were also shown better results.

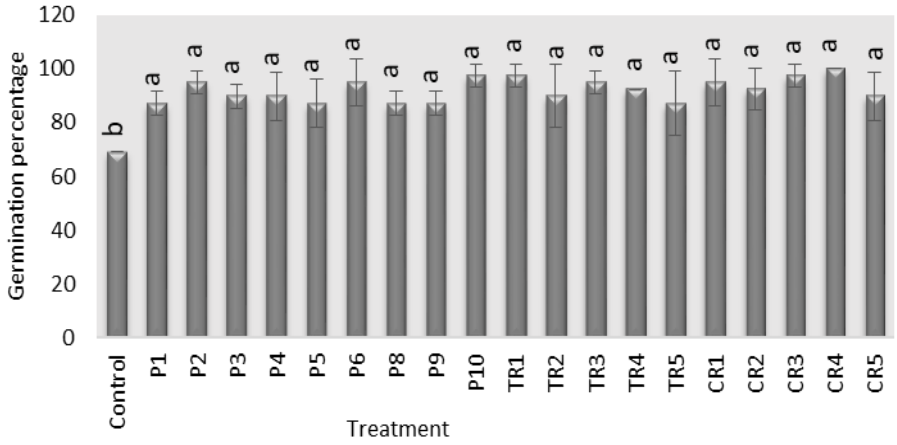


Fig 26: Effect of endophytic bacterial isolates on germination percentage of tomato seeds

Seedling length

The root and shoot length was significantly highest in P9 (7.19 cm) and CR3 (6.57 cm) thus they have the high capability of nitrogen fixation. The seedling length was significantly highest in the seeds treated with P9 (13.23 cm) and the lowest was found in the untreated control (8.84 cm). Besides this, the other endophytic treated individuals also showed significant changes compared with control.

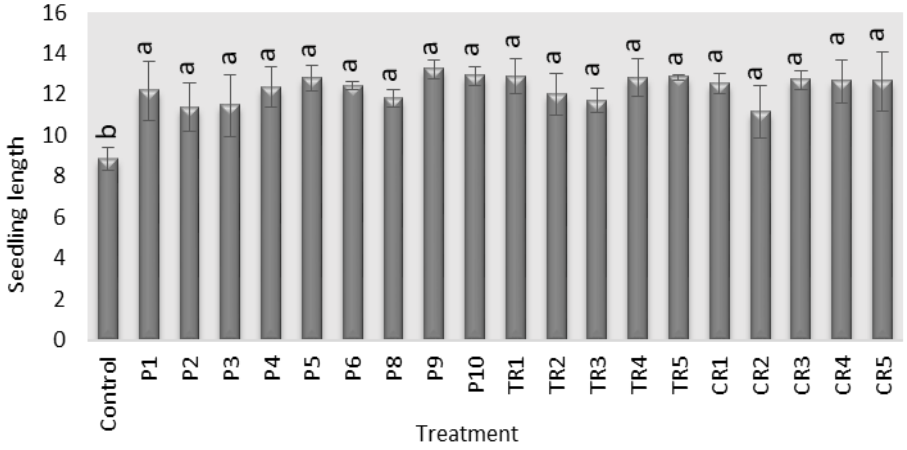


Fig 27: Effect of endophytic bacterial isolates on seedling length of tomato seeds

Vigour index

The highest significant vigour index [121] was calculated in CR4 (1262.67 cm) treated seeds and the lowest was in the untreated control (611.99 cm). The treatments CR4, P10, TR1 and CR3 also showed better significant results. All these results showed that the tomato seeds treated with endophytic bacteria increase the germination percentage as well as seedling length and the overall vigour index of the tomato plant.

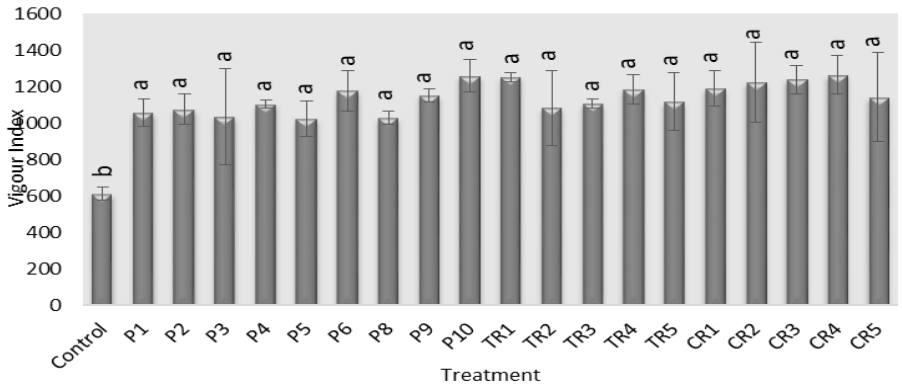


Fig 28: Effect of endophytic bacterial isolates on vigor index of tomato seeds

4.9 Application of endophytic bacterial isolates in field pot experiment on growth and development of tomato

After the results of the germination effect by endophytic isolates in dark conditions, the study was then conducted in pot culture to get a clear effect on tomato plant growth and development. All the 19 isolated bacteria were applied to the growing 10 days seedlings of tomato which germinated on the pot without using any biofertilizer. After 3 times of treatments, the 1st and 2nd data were recorded at the age of 27 days and 40 days, respectively. The root, shoot, seedling length and weight were recorded. The following image represents the tomato growth after 60 days of germination:

Fig 29: Effect of endophytic isolates on tomato after 60 days

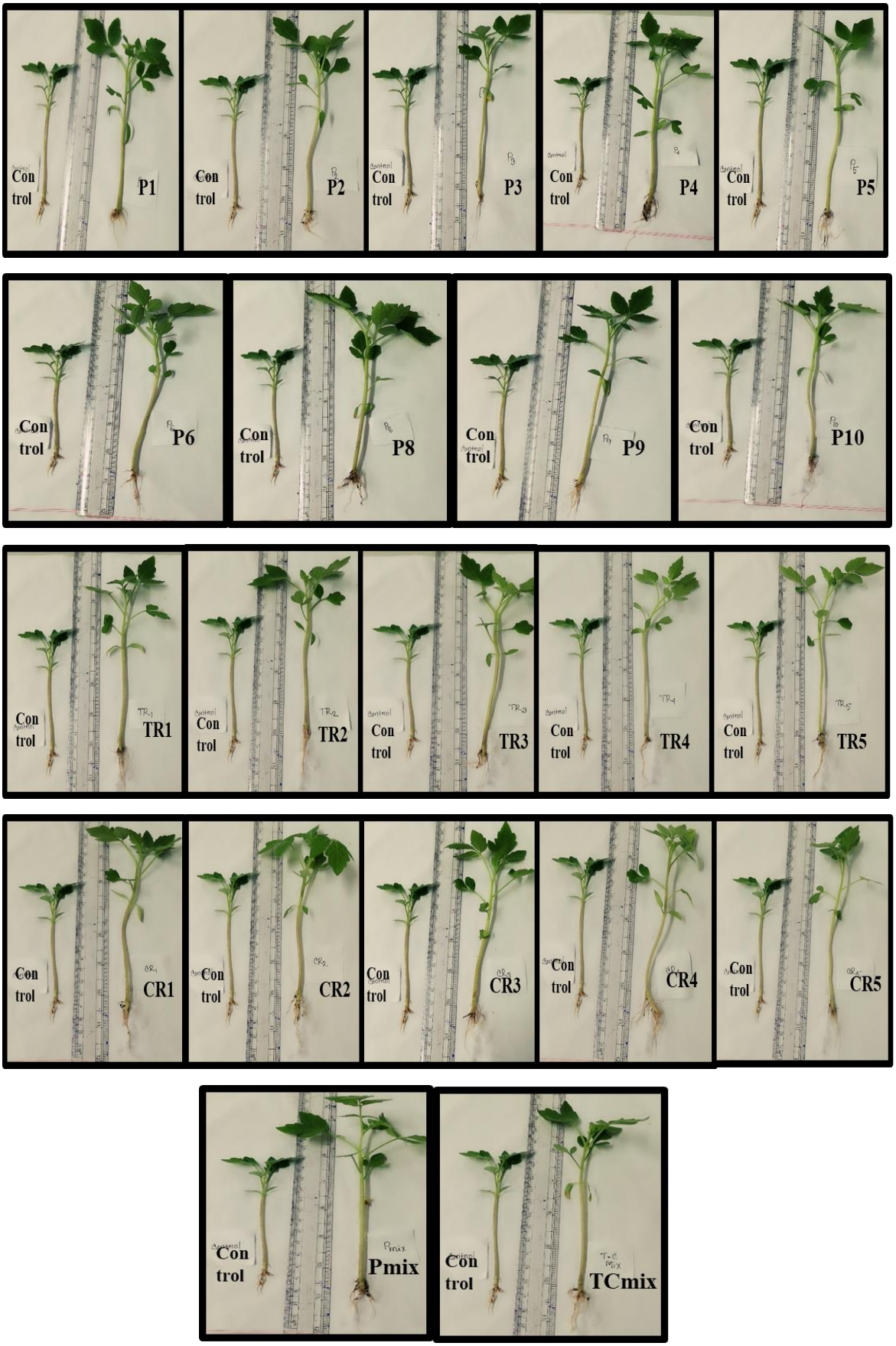


Fig 30: Effect of endophytic bacteria on tomato comparing with control in the left side at 27 days

Fig 31: Effect of endophytic bacteria on tomato comparing with control in the left side at 40 days

4.9.2 Effect of endophytic bacteria on root length of tomato plant (Pot field experiment)

The tomato root length was found to increase steadily with time. The root length of the treated plant was greater compared to the control. The root lengths were also varied significantly among the treatments. The length of the root was recorded after 27 days and 40 days and the results are presented in table 36.

Table 36: Effect of endophytic bacteria on root length of tomato plant

Treatment	Root Length 27 days	Root Length 40 dayss
Control	2.50 ± 0.20^{j}	2.43 ± 0.40^{e}
P1	3.40 ± 0.20^{i}	5.17 ± 0.96^{bcd}
P2	4.00 ± 0.20^{hi}	4.53 ± 0.70^{bcd}
P3	5.30 ± 0.70^{def}	5.10 ± 0.20^{bcd}
P4	7.50 ± 0.30^{a}	5.17 ± 1.02^{bcd}
P5	4.40 ± 0.80^{fgh}	4.60 ± 1.01^{bcd}
P6	4.40 ± 0.30^{fgh}	4.40 ± 1.01^{bcd}
P8	4.20 ± 0.30^{ghi}	4.97 ± 1.00^{bcd}
P9	5.00 ± 1.10^{efg}	4.63 ± 0.81^{bcd}
P10	6.00 ± 0.10^{cd}	4.67 ± 1.61^{bcd}
TR1	4.10 ± 0.30^{ghi}	4.10 ± 0.36^{cd}
TR2	6.30 ± 0.80^{bcd}	7.03 ± 0.47^{a}
TR3	4.30 ± 0.70^{ghi}	5.87 ± 1.00^{ab}
TR4	6.50 ± 0.70^{bc}	5.73 ± 1.57^{abc}
TR5	5.80 ± 0.20^{cde}	5.47 ± 0.90^{abcd}
CR1	6.10 ± 0.30^{bcd}	3.97 ± 0.40^{d}
CR2	6.10 ± 0.30^{bcd}	5.90 ± 0.53^{ab}
CR3	5.90 ± 0.30^{cde}	5.77 ± 1.42^{abc}
CR4	7.00 ± 0.70^{ab}	4.90 ± 0.36^{bcd}
CR5	5.60 ± 0.30^{cde}	5.53 ± 0.40^{abcd}
Pmix	5.60 ± 0.40^{cde}	3.90 ± 0.36^{de}
TCmix	4.20 ± 0.90^{ghi}	3.87 ± 0.35^{de}

Note: Observation recorded on 27^{th} and 40^{th} days.

Each value is the mean of three replications.

Different letters in superscripts indicates significantly different values.

Among all the intervals in day 27 the highest root length was recorded in P4 (7.50 cm). The treatments with CR4 (7.00 cm), TR4 (6.50 cm), TR2 (6.30 cm), CR1 (6.10) and CR2 (6.10 cm) also gave the best results. The least root length was recorded in the control (2.50 cm).

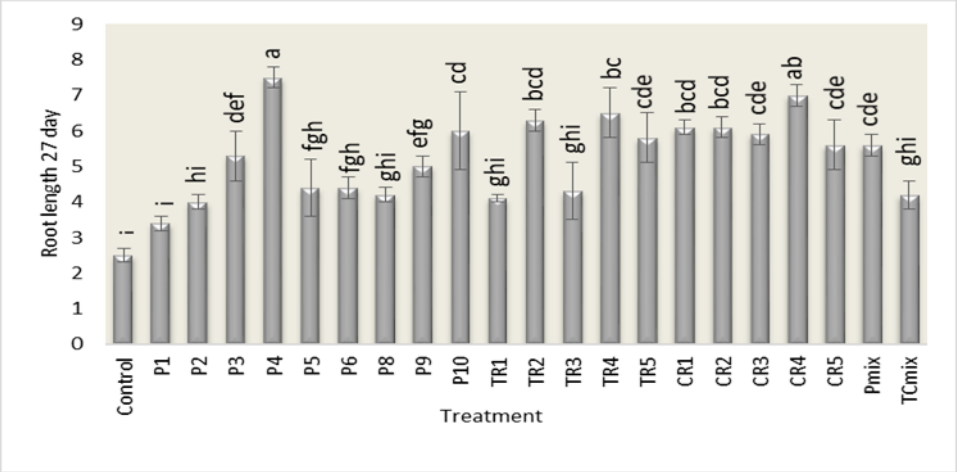


Fig 32: Effect of endophytic isolates on root length (27 day) and the highest significant root length showed by P4

In 40 days recorded intervals the treatment with TR2 (7.03 cm) showed the highest root length. Also the treatments with CR2 (5.90 cm), TR3 (5.87 cm), CR3 (5.77 cm), TR4 (5.73 cm), CR5 (5.53 cm), TR5 (5.47 cm), P1 (5.17 cm) and P4 (5.17 cm) showed the best results compared to control. The least length was recorded in the control that is (2.43 cm).

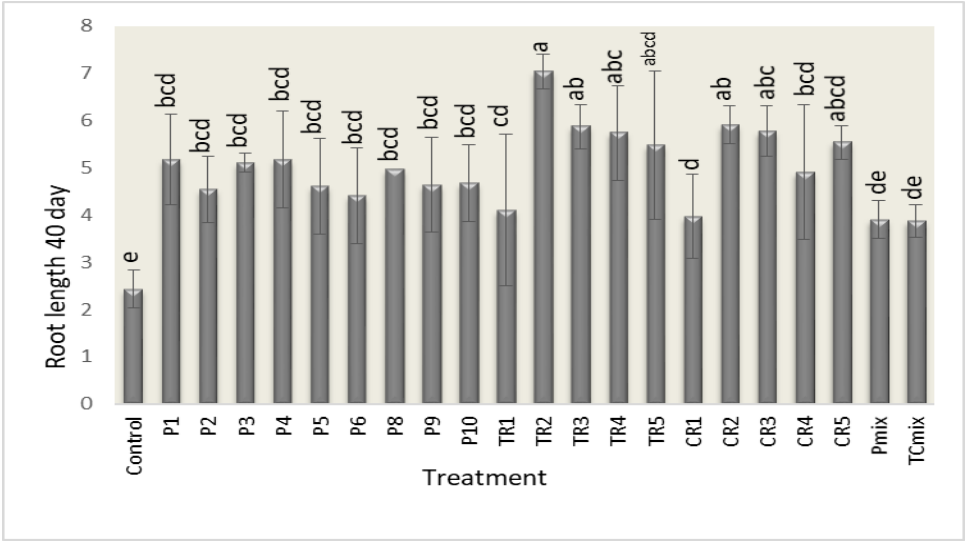


Fig 33: Effect of endophytic isolates on root length (40 day) and the highest significant root length showed by treatment TR1

4.9.3 Effect of endophytic isolates on shoot length of tomato (Pot field experiment

The plant shoot length was also found to increase steadily with time. After the treatment period, the collected data showed a variety of treatment compared to control. The root length was also recorded after 27 days and 24 days. The shoot length data are presented in table 37.

Table 37: Effect of endophytic bacteria on shoot length of tomato plant (Pot field experiment)

Treatment	Shoot Length 27 days	Shoot Length 40 days
Control	9.50 ± 0.90^{i}	11.00 ± 0.80^{i}
P1	11.00 ± 0.40^{fgh}	15.00 ± 1.20^{defgh}
P2	12.70 ± 0.80^{abcde}	15.60 ± 0.85^{cdef}
P3	11.00 ± 0.10^{fgh}	15.20 ± 1.25^{cdefgh}
P4	13.70 ± 0.30^{ab}	15.10 ± 0.82^{defgh}
P5	10.90 ± 0.70^{fgh}	15.23 ± 0.64^{cdefgh}
P6	13.40 ± 0.80^{ab}	16.67 ± 0.49^{bcd}
P8	12.50 ± 0.30^{abcde}	14.63 ± 0.70^{efgh}
P9	12.40 ± 0.90^{bcde}	16.40 ± 0.70^{cde}
P10	12.90 ± 0.30^{abcd}	14.17 ± 0.41^{fgh}
TR1	12.60 ± 0.70^{abcde}	18.77 ± 0.67^{a}
TR2	11.50 ± 0.90^{efgh}	14.00 ± 0.95^{gh}
TR3	13.80 ± 0.40^{a}	15.80 ± 0.79^{cdef}
TR4	12.90 ± 0.80^{abcd}	14.77 ± 0.25^{efgh}
TR5	11.70 ± 0.30^{defg}	15.30 ± 0.61^{cdef}
CR1	13.00 ± 0.90^{abc}	15.97 ± 1.00^{cde}
CR2	10.90 ± 1.10^{fgh}	15.30 ± 0.56^{cdefg}
CR3	10.60 ± 0.40^{ghi}	14.20 ± 0.30^{fgh}
CR4	10.20 ± 0.40^{hi}	14.63 ± 0.42^{efgh}
CR5	11.40 ± 0.60^{efgh}	17.70 ± 1.25^{ab}
Pmix	12.00 ± 1.86^{cdef}	13.77 ± 0.93^{h}
TCmix	10.93 ± 0.23^{fgh}	14.83 ± 0.47^{efgh}

Note: Observation recorded on 27 and 40 days.

Each value is the mean of three replications.

Different letters in superscripts indicate significantly different values.

In 27 days recorded intervals the highest shoot length was recorded in the treatment with TR3 that is 13.80 cm and the least shoot length was recorded in the control that is 9.50 cm. The treatment with P4 (13.70 cm), P6 (13.40 cm), CR1 (13.00 cm), P10 (12.90 cm), TR4 (12.90 cm), TR1 (12.60 cm) and P8 (12.50 cm) also showed the best results.

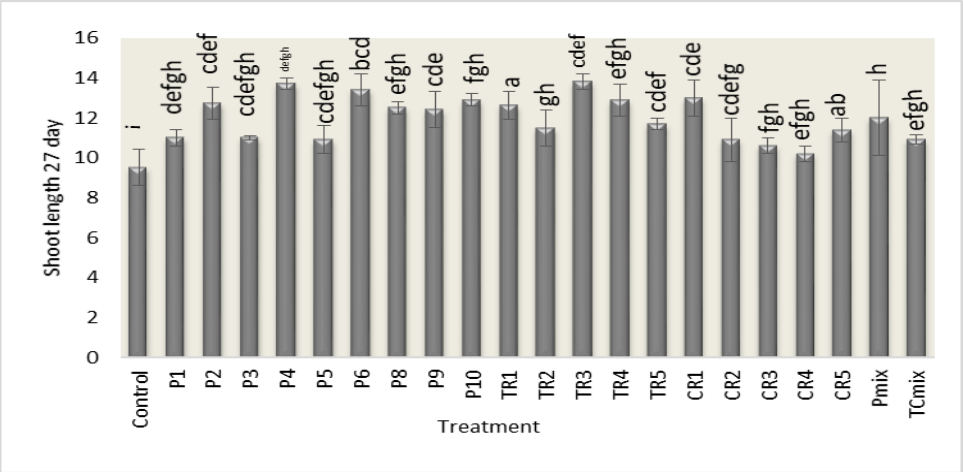


Fig 34: Effect of endophytic isolates on shoot length (27 day) and the highest significant shoot length showed by treatment TR3

In 40 day intervals, the highest shoot length was recorded in TR1 that is 18.77 cm and the least shoot length was in the control that is 11.00 cm. The treatment with selected isolates CR5 (17.70 cm), P6 (16.67 cm), P9 (16.40 cm), CR1 (15.97 cm), TR3 (15.80 cm) and P2 (15.60 cm) also showed the results of the best shoot length.

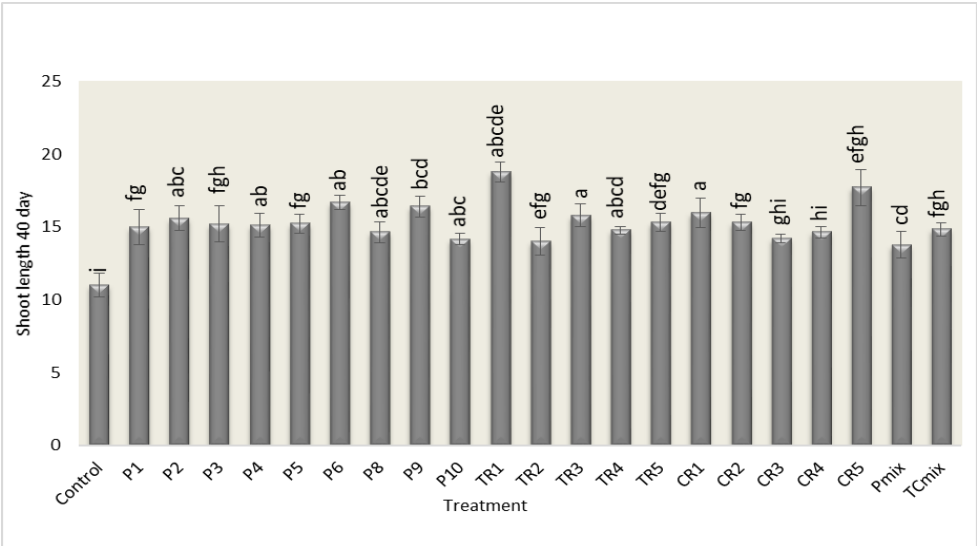


Fig 35: Effect of endophytic isolates on shoot length (40 day) and the highest significant shoot length showed by treatment TR1

4.9.4 Biomass

The fresh weight and dry weight of 27 days and 40 days of the harvested plant is presented in table 37 and 38. The total fresh weight and dry weight in the plant treated with endophytic bacteria were found to be higher than untreated control plant showing a significant impact on overall plant biomass.

4.9.4.1 Effect of endophytic isolates on the fresh weight of tomato (Pot field experiment)

The fresh weight of tomato was recorded on 27 days and 40 days and the recorded weight was significantly differenced among the treatment.

In day 27, the highest significant fresh weight was with the plant treated with P1 and the weight is 1.89 gm/plant. The least significant fresh weight was recorded in the control and the weight is 0.39 gm/plant. The P8 (1.43 gm/plant), P4 (1.26 gm/plant), TR3 (1.21 gm/plant), Pmix (1.21 gm/plant) and CR1 (1.11 gm/plant) treated plants also showed the best fresh weight.

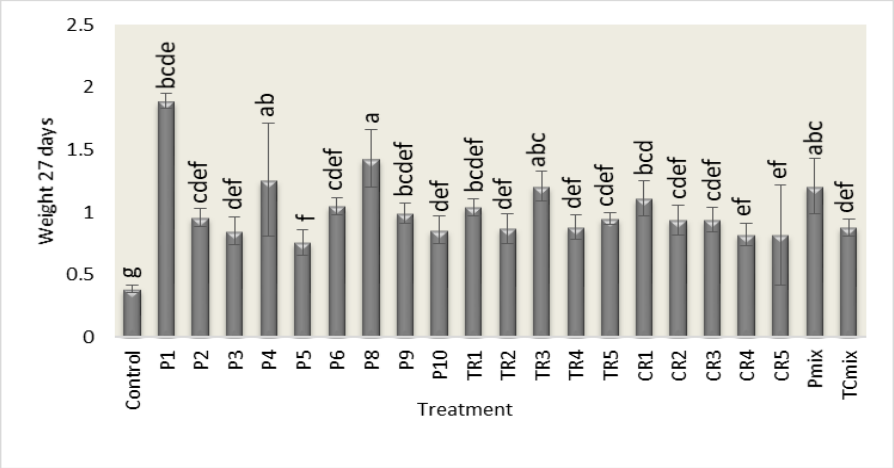


Fig 36: Effect of endophytic isolates on fresh weight (27 day) and the highest significant fresh weight showed by treatment P1

In day 40, the highest fresh weight was recorded in P9 (2.50 gm/plant) and the least fresh weight was observed in the control (0.99 gm/plant). The plants treated with P2 (2.46 gm/plant) TR1 (2.43 gm/plant), CR5 (2.42 gm/plant), P6 (2.40 gm/plant) and TR3 (2.25 gm/plant) also showed the best results.

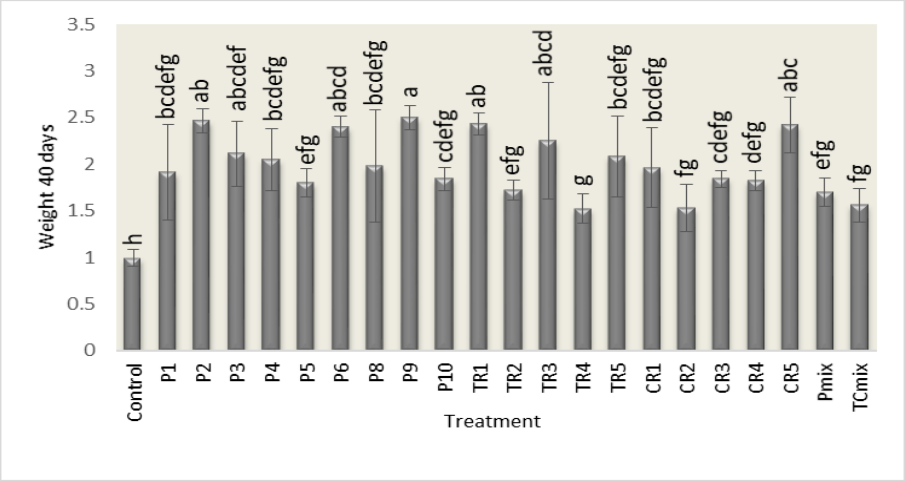


Fig 37: Effect of endophytic isolates on fresh weight (40 day) and the highest significant fresh weight showed by treatment P9

4.9.4.2 Effect of endophytic isolates on dry weight of tomato (Pot field experiment)

There was a significant difference among the treatments in dry weight and the highest dry weight was recorded in P4 and that is 0.31 gm/plant (27 days). In 27 days intervals the P8 (0.26 gm/plant), Pmix (0.25 gm/plant), TR3 (0.23 gm/plant), P1 (0.22 gm/plant) and CR1 (0.21 gm/plant). The least dry weight was observed with the control (0.09 gm/plant).

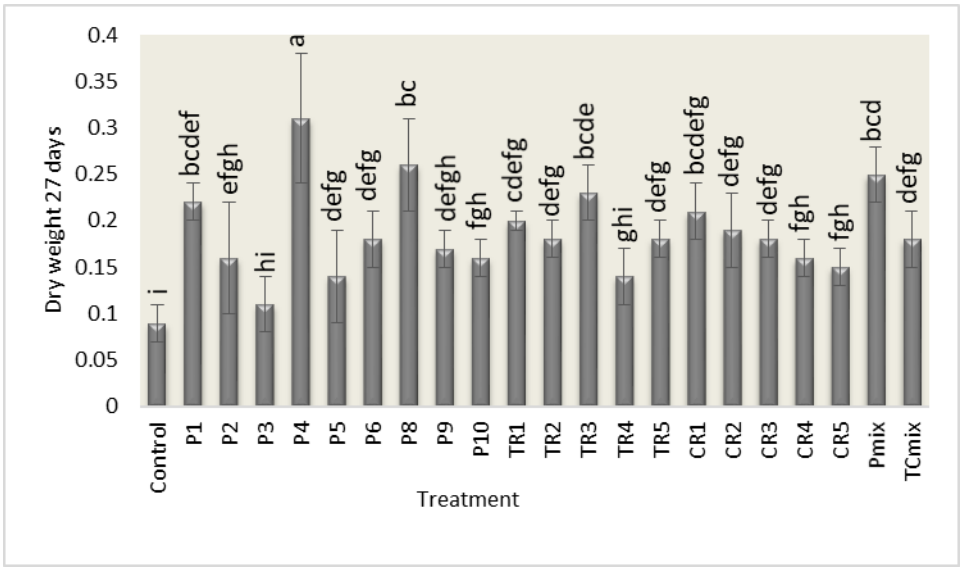


Fig 38: Effect of endophytic bacteria on dry weight (27 days) and the highest significant dry weight showed by treatment P4

In 40 days recorded intervals, the highest dry weight was recorded in TR5 and the treatment showed the weight 0.48 gm/plant. The least dry weight was recorded in the control (0.10 gm/plant). Here the treatment P2 (0.45 gm/plant), P9 (0.44 gm/plant), TR1 (0.44 gm/plant), CR1 (0.38 gm/plant), P4 (0.37 gm/plant), P6 (0.35 gm/plant) and CR5 (0.34 gm/plant) also showed the best results.

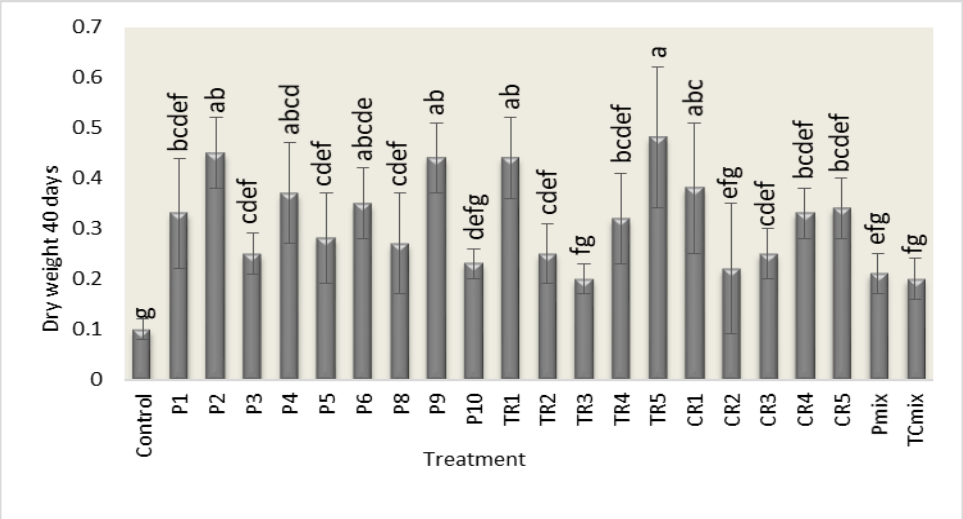


Fig 39: Effect of endophytic bacteria on dry weight (27 days) and the highest significant dry weight showed by treatment TR5

4.10 Effect of endophytic bacterial isolates on growth and development of tomato as consortia

All the isolated strains were treated to the tomato plant as a consortium to check the growth and development of tomato. The 20 isolated strains were divided into four groups;

Group 1	P1, P2, P3, P4 and P5
Group 2	P6, P7, P8, P9 and P10
Group 3	TR1, TR2, TR3, TR4 and TR5
Group 4	CR1, CR2, CR3, CR4 and CR5

For consortium effect study 25 days aged healthy tomato seedlings were bought from the market. The treatment was conducted in 3 times at the age of 10^{th}, 15^{th}, and 20^{th} days after the plantation.

(A)

(B)

(C)

Fig 40: Consortium effect: (A) Control 1+ Control 2+ Control 3

(B) Treatment 1+2+3+4 (Soil + Compost + Bacteria)

(C) Treatment 1+2+3+4 (Soil + Bacteria)

4.10.2 Effect of endophytic isolates on tomato as consortia

The root, shoot and seedling length was recorded after 25 days of the plantation. For root length, the soil+ bacteria group showed the most significant results than the soil+ bacteria+ compost group. The highest significant root length was recorded in T8 (13.60 cm) which was a treatment of soil+ bacteria and the least root length was recorded in T11

(8.13 cm) Control 1 containing only soil. The treatments T7 (13.27 cm), T5 (12.77 cm), T6 (12.60 cm) and T3 (12.33 cm) were shown the best root length results.

Table 38: Consortium effect of endophytic bacteria on tomato

No.	Treatment	Root length	Shoot length	Seedling length
T11	Control 1	8.13 ± 1.95^{b}	9.33 ± 3.21^{e}	17.47 ± 1.45^{d}
T1	Control 2	8.60 ± 1.39^{b}	12.67 ± 1.53^{d}	21.27 ± 2.89^{cd}
T2	Control 3	11.00 ± 2.00^{ab}	14.60 ± 0.53^{cd}	25.60 ± 1.97^{bc}
T3	Soil+ Bacteria+ Compost	12.33 ± 1.26^{a}	18.27 ± 1.10^{ab}	30.60 ± 1.49^{a}
T4	Soil+ Bacteria+ Compost	11.10 ± 1.01^{ab}	14.47 ± 1.75^{cd}	25.57 ± 2.70^{bc}
T5	Soil+ Bacteria+ Compost	12.77 ± 1.54^{a}	16.33 ± 1.04^{bc}	29.10 ± 0.66^{ab}
T6	Soil+ Bacteria+ Compost	12.60 ± 2.08^{a}	17.17 ± 2.06^{abc}	29.77 ± 0.25^{ab}
T7	Soil + Bacteria	13.27 ± 1.01^{a}	19.40 ± 0.36^{ab}	32.67 ± 1.44^{a}
T8	Soil + Bacteria	13.60 ± 1.97^{a}	18.90 ± 2.46^{ab}	32.50 ± 3.90^{a}
T9	Soil + Bacteria	12.27 ± 2.16^{a}	20.17 ± 1.76^{a}	32.43 ± 3.39^{a}
T10	Soil + Bacteria	12.17 ± 2.25^{a}	19.83 ± 2.31^{a}	32.00 ± 4.44^{a}

Note: Observation recorded after 25 days.

Each value is the mean of three replications.

Different letters in superscripts indicate significantly different values.

The significant shoot length was also recorded in soil+ bacteria group. The highest shoot length was recorded in T9 (20.19 cm) and the least was recorded in control 1; T11 (9.33 cm). The T10 (19.83 cm), T7 (19.40 cm), T8 (18.90 cm), T3 (18.27 cm) and T6 (17.17 cm) also showed the significant results compare to control.

The highest significant seedling length was recorded in T7 (Soil+ bacteria) and the length is 32.67 cm. Least significance length was recorded in T11 (17.47 cm) that was the control 1 containing only soil. The treatment T8 (32.50 cm), T9 (32.43 cm), T10 (32.00 cm) and T3 (30.60 cm) also showed the best results.

CHAPTER V

DISCUSSION

CHAPTER V

DISCUSSION

On the basis of KOH string test of 19 isolated endophytic bacteria 15.79% isolates were gram positive and the rest of 84.21% isolates were gram negative endophytes. Another report on isolation of endophytic bacteria from pigeon pea roots, leave tissue and stems shows that among the 40 isolates 25 of the endophytic bacterial isolates were gram negative while 15 were gram positive [123]. The biochemical characterization revels that among the endophytic isolates 84.21% were positive for MIU and Urease test, 73.68% were positive for CIU test, 52.63% were positive for Oxidase test, 47.37% were positive for Voges-Proskauer (VP), 15.79% were positive for Methyl Red (MR) test and all endophytic bacterial isolates were positive for Catalase and TSI test. For carbohydrate utilization test 47.37%, 78.95%, 94.77% and 100% were positive for Lactose, Maltose, Sucrose and Dextrose, respectively. All the isolates have extracellular enzyme (Cellulase, Xylanase, Protease and Amylase) producing capacity. Similarly, another report on 16 isolated salt tolerant endophytic and rhizospheric bacteria 10 out of 16 isolates were able to assimilate starch, glucose and fructose, while seven were able to assimilate nitrate and 8 were positive for indole production [124].

Among the 18 molecularly characterize endophytic bacteria 38.89% was *Enterobacter sp*., 22.22% was *Klebsiella sp.*, 16.67% was *Pantoae sp.*, 11.11% was *Bacillus sp.*, 5.56% was *Pseudomonas sp*., and 5.56% was *Burkholderia sp*. All the isolates were capable to promote plant growth.

Bacterial plant growth promotion is a deep-rooted and multifaceted phenomenon that is often proficient via the activities of more than one PGP trait showed by the associated bacteria [122]. Several studies also shows that the *Enterobacter sp*. can promote plant growth in both dicot and monocot plant. It also proved that this genera had the potential to improve maize seedling growth under axenic conditions. *Enterobacter sp.* strain showed both the highest growth-promoting activity under axenic conditions as well as colonization capacity [125]. Other studies also showed that *Enterobacter* and related Enterobacteriales are also good phytohormone producers and have PGP effects in sugarcane, wheat, pepper and soybean [126, 127].

Studies shows that the genera Bacillus is a free-living nitrogen fixers and have other PGP traits, which make them suitable candidates for application [50]. Auxins are

phytohormones produced by several bacteria, being these compounds key signaling molecules for bacterial communication to coordinate activities. Amongst these auxins, indole-3-acetic acid (IAA) is the best known and most active auxin in plants. Auxin-producing Bacillus spp. have been reported to exert a positive effect on the development of several crops, such as Solanun tuberosum (potato) or Oryza sativa (rice) [128, 129]. Moreover, members of the genus Bacillus were reported as cytokinin producers [129, 130]. Bacillus megaterium and also Azotobacter chroococcum strains were found to produce cytokinins and promote cucumber growth [131]. Liu et al. [132] reported that oriental thuja seedlings inoculated with cytokinin-producing Bacillus subtilis strains have better resistance to drought stress.

The genera *Pseudomonas*, *Bacillus*, *Klebsiella*, *Enterobacter* and *Pantoea* amongst others, have been reported to be efficient phosphate solubilizers [133]. The combination of *Serratia* and *Pseudomonas* ACC-deaminase-producing strains improved the yield of wheat plants in saline conditions [134].

All the isolates showed positive results and also formed clear zone in quantitative estimation of nitrogen fixation and phosphate solubilization. In case of IAA production, the isolates produced pink color with Salkowaski's regents and indicated the positive results. The maximum amount was produced by TR3 (7.002µg/ ml) belonging to the genera *Enterobacter* followed by TR1 (6.093µg/ ml) which is belongs to *Pantoea* genera. The ability of isolates to utilize ACC as a sole source of N was assessed on Dworkin and Foster (DF) minimal medium and the isolates TR3 gave the maximum absorbance at 600nm and which belongs to the genera *Enterobacter*.

The growth-promoting ability of all the isolates was checked on tomato plants individually and in the consortium. The plate assay for germination effect of isolated strains was carried out on tomato seeds and the highest significant amount of germination percentage, root length, shoot length, seedling length, vigour index was found in CR4 (100%), P9 (7.19 cm), CR3 (6.57 cm), P9 (13.23 cm) and CR4 (1262.67 cm), respectively. In pot (field) application, the highest significant root length, shoot length, fresh weight and dry weight were recorded in TR2 (7.03 cm), TR1 (18.77 cm), P9 (2.50 gm/ plant) and TR5 (0.48 gm/ plan), respectively. Similarly, the growth promotion of several crop plants in response to inoculation with growth-promoting bacteria has also been reported by others

[135]. For consortium effect, the significant result was recorded in the endophytic treated plant compared with endophytic+compost and with other controls.

CHAPTER VI

CONCULATION

CHAPTER VI
CONCULATION

- This study was carried out to isolate endophytic bacteria from several plant root associated with plant growth promoting ability to cultivate organic tomato.
- Total twenty endophytic bacteria were isolated, where ten bacteria from rice root (P1–P10), five bacteria from tomato root (TR1– TR5) and five bacteria from cauliflower root (CR1– CR5).
- On the basis of performed biochemical characterization test of 19 isolates, 84.21% were positive for MIU and Urease test, 73.68% were positive for CIU test, 52.63% were positive for Oxidase test, 47.37% were positive for Voges-Proskauer (VP), 15.79% were positive for Methyl Red (MR) test and all endophytic bacterial isolates were positive for Catalase and TSI test. For carbohydrate utilization test 47.37%, 78.95%, 94.77% and 100% were positive for Lactose, Maltose, Sucrose and Dextrose, respectively. All the isolates have extracellular enzyme (Cellulase, Xylanase, Protease and Amylase) producing capacity.
- After molecular characterization of 18 isolates it was found that the isolates P1 and CR1 belongs to *Bacillus* genera. The isolates P2, TR2, TR5 and CR3 belong to the *Klebsiella* genera. The isolates P3, P4, P5, P6, P8, TR3 and CR2 belong to *Enterobacter* genera. The isolates P9, TR1 and CR5 belongs to the *Pantoea* genera. The isolates TR4 and CR4 belong to the *Pseudomonas* and *Bulkholderia* genera.
- For plant growth promoting (PGP) ability all the 19 isolates were positive for nitrogen fixation, phosphate solubilization, IAA production and ACC deaminase activity.
- Application of these 19 isolates to promote tomato growth showed significant results on germination percentage, root length, shoot length, seedling length and weight comparing with control in both single and consortium treatments.
- In germination effect (dark condition), the significant germination percentage and vagur index was recorded in CR4 (100%) and (1262.67 cm) belong to *Bulkholderia* genera, root and seedling length was with P9 (7.19 cm) and (13.23 cm) belong to *Pantoae* genera, shoot length was with CR3 (6.57 cm) belong to *Klebsiella* genera.
- In pot (field) application of 40 days data, the significant root and dry weight was with TR2 (7.03 cm) and P9 (0.48 gm/ plant) belong to *Klebsiella* genera, the shoot

length and fresh weight was with TR1 (18.77 cm) and P9 (2.50 gm/ plant) belong to the *Pantoae* genera.

- For consortium effect of isolated strains, the significant results was recorded to the endophyte treated plant compared to the compost and fertilizer treated plant.
- The application of these endophytes individually or in consortium increase the germination effect, root length, shoot length, seedling length and vigour index of Tomato. Finally, it can be concluded that these isolated endophytic strains have a great potential on organic tomato growth and development without using chemical fertilizer.