Unraveling whole genome sequence of pesticide degrading and eggplant growth promoting endophytic strain *Klebsiella* sp. HSTU-Sny5

A THESIS

BY

SHAMIMA YESMIN

Student ID: 1805352

Session: 2018-2019

MASTER OF SCIENCE (M.S.)
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HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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

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DECEMBER, 2019

Certificate

This is to certify that the research work entitled "**Unraveling whole genome sequence of pesticide degrading and eggplant growth promoting endophytic strain *Klebsiella* sp. HSTU-Sny5"** is an original research work and submitted in partial fulfillment of the requirements for the degree of MS in Biochemistry and Molecular Biology. This research work done by **SHAMIMA YESMIN** bearing student ID No. **1805352** since 2018-2019, under my supervision, as a fellow for the degree of Master of sciences.

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Dedicated
To
My Beloved Parents

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All praises are for the almighty Allah for whom the author was able to complete this research work and prepare this manuscript in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry and Molecular Biology from Hajee Mohammad Danesh Science and Technology University (HSTU), Dinajpur within the appointed time.

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The Author

ABSTRACT

The study is aimed to characterize a pesticide degrading robust endophytic strain on eggplant growth promotion and to revealing their mechanism through genome wide investigation. To this end, ten different endophytic bacteria were isolated from tomato plant root by screening on the chlorpyrifos pesticide containing agar media.The strain was able to degrade the pesticide in liquid culture that is confirmed by FTIR analysis. The isolate was named as *Klebsiella* sp. HSTU-Sny5. The HSTU-Sny5 showed MR positive, VP negative, catalase positive, oxidase negative, TSI, CIU, sucrose, dextrose,MIU test, lactose, Urease and maltose positivein test. Indole-3 acetic acid (IAA), ACC deaminase activity, nitrogen fixing and phosphate solubilizing test were observed in positive. Further confirmation of growth promotion activity, these isolates was applied on eggplant.The eggplant germination, root length and shoot length was significantlyenhanced by after treatment of the HSTU-Sny5 strain, as consequence, theVigor Indexobtained508.30±8.20 for the untreatedand 734.40±15.11 for the HSTU-Sny5treated egg plants.The whole genome shot gun sequence of the strain showed one single 5,432,112bp chromosome, with an average 57.8% GC content and 5,231 predicted protein coding DNA sequences (CDS). Plant growth promoting such as phosphate metabolism genes (*pstC, pstA, phoU, phoR (sphS), phoB (sphR), pstB, pstS*), IAA production gene (Tryptophan synthase beta (trpB), Tryptophan synthase alpha (trpA), Anthranilate synthase, aminase component, Indole-3-glycerol phosphate synthase) and nitrogen fixation genes (*nifE,nifH, nifA, nifB, nifM, nifZ,nifS*, *nifU, nifN, nifT*) were found in the CDS of genome. Also siderophore production genes(*fhuB, tonB, fepG, fepB, fhuD, fhuC, fhuA, fntS* etc.), chitinase, H_2S production and sulfur assimilation, heat shock proteins, cold shock proteins, superoxide dismutase, and heavy metal (Arsenic resistance, Cadmium, Zinc, Cobalt). Pesticides degrading genes especially organophosphorus gene (*opdE*), and total 27 esterase proteins were found. The analysis of the HSTU-Sny5 genome revealed a large number of genes involved in uptake and exchange of nutrients, chemotaxis, mobilization, plant colonization, stress tolerance, growth promotion, and pesticide metabolism, which suggested the strains capabilityof providing probiotic roles in nature.

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LIST OF ABBREVIATIONS

g	:	Gram
ml	:	Millilitre
µl	:	Microlitre
g/L	:	Gram per litre
mg/ml	:	Milligram per millilitre
ng/µl	:	Nanogram per microlitre
pH	:	Negative logarithm of Hydrogen ion concentration
rpm	:	Revolutions per minute
RNA	:	Ribonucleic acid
DNA	:	Deoxyribonucleic acid
bp	:	Base pair
kb	:	Kilobase pair
NCBI	:	National Centre for Biotechnology Information
BLAST	:	Basic Local Alignment Search Tool
°C	:	Degree Celsius
$(NH_4)_2SO_4$	:	Ammonium sulphate
$MgSO_4 \cdot 7H_2O$	:	Magnesium sulphate heptahydrate
KH_2PO_4	:	Monopotassium phosphate
$CaCl_2 .2H_2O$	:	Calcium chloride dihydrate
NaH_2PO_4		Monosodium phosphate
Na_2HPO_4	:	Disodium phosphate
NaOH	:	Sodium hydroxide
HCl	:	Hydrochloric acid
NaCl	:	Sodium chloride
DNS	:	3,5- Dinitrosalicylic acid
nm	:	Nanometre

LIST OF ABBREVIATIONS

Alk. $CuSO_4$	:	Alkaline copper sulphate
Psi	:	Per square inch
dNTP	:	Deoxynucleotide triphosphate
M	:	Molar
mM	:	Millimolar
N	:	Normal
EDTA	:	Ethylenediaminetetraacetic acid
UV	:	Ultra violet
ND	:	Nano Drop
PCR	:	Polymerase Chain Reaction
RFP	:	Restriction Fragment Length Polymorphism
TAE	:	Tris Acetate EDTA
TBE	:	Tris Borate EDTA
KDa	:	Killo Daltone
NA	:	Nutrient Agar media
cm^{-1}	:	Per centimeter
FTIR	:	Fourier-transform infrared spectroscopy
RAST	:	Rapid Annotation using Subsystem Technology
ANI	:	Average Nucleotide Identity
NGS	:	Next Generation Sequencing

Chapter I

INTRODUCTION

Using fertilizers and pesticides have been enormously increased in modern time to meet the huge population. Bio-fertilizers being essential parts of organic farming play very important role in maintaining future soil fertility and sustainability by fixing atmospheric di-nitrogen (N=N) through colonization into the roots of the plants for the formation of tumor like nodules which acts as the factories of ammonia production, mobilizing fastened macro and small nutrients or convert insoluble phosphate within the soil into forms on the market to plants, thereby will increase their potency and accessibility. Presently there's a spot of 10 million tons of plant nutrients between removal of crops and supply through chemical fertilizers. In context of each the value and environmental impact of chemical fertilizers, excessive reliance on the chemical fertilizers isn't viable strategy in long run as a result of the value, each in domestic resources and foreign exchange, concerned in fixing of plant food plants and sustaining the assembly. During this context, organic manures (bio-fertilizers) would be the viable choice for farmers to extend productivity per unit space [1].

Pesticides are used to control or to manage pest populations at a tolerable level. The suffix "-cide" literally means "kill", that means the term pesticide refers to a chemical substance that kills pests where It is incorrect to assume that the term pesticide refers only to insecticides. In modern agriculture practices, millions of tons of pesticides are applied annually all over the globe which covers the billions of dollars' market. The expenditures on pesticides were 35.8 billion in 2006 which rose up to 39.4 billion US dollars in 2007 [2]. The classification of pesticides is designed according to their toxicity, chemical group, environmental persistence, target organism, or other features. According to the Stockholm Convention on Persistent Organic Pollutants, 9 of the 12 persistent organic chemicals are pesticides. Classes of organic pesticides (consisting of organic molecules) include organochlorine, organophosphate, organometallic, pyrethroids, and carbamates among others [3, 4]. The common organophosphate pesticides used in the agriculture are chlorpyrifos, diazinon, cypermethrin and profenofos.

Chlorpyrifos is one of the common organophosphorus chemical substances used as insecticide in agriculture. The major degradation product 3, 5, 6-trichloro-2-pyridinol (TCP) is produced from CP depending on its half-life in soil that varies from 10 to 120 days [5]. It generates diethyl thiophosphoric acid (DETP) and 3,5,6-Trichloro-2-pyridinol (TCP) upon hydrolysis. Endophytic bacteria reside in the Numerous reports are available in literature where exposure of chlorpyrifos to humans has caused death by respiratory failure or cardiac arrest, mild exposure may lead to headache, dizziness [6].

Cypermethrin is a synthetic pyrethroid organophosphorus pesticide used as an insecticide in large-scale commercial agricultural applications as well as in consumer products for domestic purposes. It behaves as a fast-acting neurotoxin in insects which is easily degraded on soil and plants but can be effective for weeks when applied to indoor inert surfaces. Farmers use cypermethrin in agriculture to control ectoparasites which infest cattle, sheep, and poultry. In veterinary medicine, it is effective at controlling ticks on dogs [7]. Profenofos is a liquid with a pale yellow to amber color and a garlic-like odor which is also an organophosphate insecticide. It was first registered in the United States in 1982 [8]. Profenofos can be used on a variety of crops including cotton and vegetables such as maize, potato, soybean, and sugar beet [9].

Endophytic microorganisms, specifically bacteria or fungi, are known to inhabit plant tissues without causing disease symptoms in the host plant [10, 11, 12]. Endophytic microbial communities have vital roles in the development and growth of various host plants under favorable and various stress conditions, such as heat, salinity, heavy metal contamination, and drought [13]. Among endophytes, bacteria have a knack for inhabiting internal plant tissues and imparting beneficial effects for host growth. Such traits have been shown to improve growth and developmental processes [14, 15] of the host through the ability of endophytes to perform a range of functions, including assisting both primary and secondary nutrient uptake via atmospheric nitrogen fixation [16] synthesizing iron siderophores [17], and solubilizing minerals such as phosphate, potassium, and zinc [18, 19, 20]. Facilitation of plant growth promotion by endophytic bacteria occurs through several mechanisms; these include mineralization of inorganic substances from the soil into host roots and production of enzymes, phytohormones, and defense-related constituents within the host environment [21, 22]. In addition, these endophytic microbes can support the plant by providing nitrogen sources (by fixing atmospheric nitrogen into ammonia) and other nutrients, such as sulfur, iron, and phosphate with provides pesticide degrading activity.

The pesticide-degrading bacterial isolates obtained from plant root is able to degrade the ecotoxic strong chlorpyrifos, diazinon, cypermethrin and profenofos and able to bioremediate soil with high chlorpyrifos, diazinon, cypermethrin and profenofos concentration and finally the people will feel safe during up taking that crops with no health hazardous. These microbes possess not only pesticide degrading capacities and lower the toxic effects of these pesticides on plants but also various other traits that help in plant growth promotion and involved in cell wall degradation by producing enzymes with dye degrading capability.

Although plant growth-promoting bacteria use a number of different mechanisms to promote the growth of plants [23], arguably, the bacterial trait that is key in facilitating plant growth is the possession of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme is responsible for the cleavage of the plant ethylene precursor, ACC, into ammonia and alpha-ketobutyrate [24]. By decreasing ACC levels in plants, ACC deaminase-producing organisms decrease plant ethylene levels which when presenting high concentrations can lead to plant growth inhibition or even death. These endophytes can produce phytohormone indole-3-acetic acid (IAA) that can enhance cell growth. The endophytes play a role in root and shoot elongation of plants through phosphate solubilizing and nitrogen fixation. Main mechanism of phosphate solubilizing is to produce organic acid and acid phosphatase. This acid is produced in the periplasm of gram negative bacteria by a direct oxidation pathway of glucose. The organic acid diffuses freely outside the cells releasing high quantities of soluble phosphate from mineral phosphate [25]. Biological nitrogen fixation (BNF) is an important process from an ecological point of view as it can maintain a quantity of nitrogen to balance the lack of this element. The endophytic bacteria can fix atmospheric di-nitrogen (N_2) in plants through making association or without association with plants. There are two main classes of nitrogen fixing genes are found in endophytes ,these are nif and fix genes which are involved in BNF by forming a symbiotic association with leguminous plants [26]. The nif genes encode nitrogenase and show structural and functional resemblance with the nitrogen-fixing genes present in *Klebsiella pneumoniae* and other microbial groups [27]. Besides of nif genes, there are several other microbial genes which are significantly involved in the symbiotic N_2-fixation, these are known as fix genes. The enzyme nitrogenase is very sensitive to oxygen, hence anaerobic condition is required for BNF. BNF is an energy-requiring process and energy comes from the cellular metabolic process in the form of ATP. All the nitrogen-fixing microorgaisms carry enzyme hydrogenases which catalyze the transfer of electrons from pyruvate or hydrogen to ferredoxin or flavoprotein. The ferredoxin (the iron_sulfur electron carrier) is involved in the reversible oxidation and reduction reaction when dinitrogen binds with nitrogenase enzyme complexes [28]. In a sentence, the use of plant growth promoting endophytic bacteria as bio-fertilizers provides a promising alternative to chemical fertilizer and plays a role in pesticides biodegradation to promote sustainable development.

Previous studies have suggested the potential of HSTU-Sny5 as a plant growth-promoting bacterium; however, this strain has not been fully investigated for these characteristics. Therefore, the current study aimed to elucidate the whole HSTU-Sny5 genome and its plant

growth-promoting activity. Sequencing the complete genome of HSTU-Sny5 will aid in resolving the complex biological mechanisms of this microorganism that promote plant growth and induce hardiness against salinity and heavy metal stress. These genomic analyses will provide a foundation towards fully understanding the characteristics of this microorganism and its potential for broader application against environmental stressors. Furthermore, comparisons with other completely sequenced *Klebsiella* genomes will help delineating the unique and shared traits among different *Klebsiella* species, offering insights into the evolutionary changes that have occurred within this genus.

Objectives of this thesis

1. Isolation of pesticides mineralizing endophytic bacteria from tomato root.
2. Biochemical screening of isolated endophytic bacteria by: Biochemical tests analysis and morphological characteristic.
3. Growth promoting activity test of these pesticide degradable isolates by N_2-Fixation, phosphate solubilizing, IAA production and ACC deaminase activity test.
4. Pot experiment on eggplant to confirm growth promoting capability of isolate by conducting seed germination, seedlings stage and vegetative in the pot.
5. Pesticides biodegradation by FT-IR analysis.
6. Molecular identification using whole genome sequencing and analysis.
7. To identify pesticide degrading and growth promoting genes from the complete genome sequences.
8. To identify stress tolerance genes.

Chapter II
LITERATURE REVIEW

Current situation in agriculture are focused on the use of chemical pesticides and inorganic fertilizers, compelling the search for alternative ways to improve soil fertility and crop production to meet the increasing people. Recently natural agricultural practices have been got the most priority in expectation of moving towards environmentally sustainable development. Sustainable agriculture is destined as an integrated system of plant production that will make the most use of nonrenewable sources, integrate natural biological cycles and over the long term, satisfy human food and film needs, enhance environmental quality, sustain the economic viability of farm operations and increase the quality of life for farmers and ranchers and society as a whole [29].

Endophytic bacteria have been used to stimulate plant growth in agriculture since ancient time. Nevertheless, it was through the mixing of different soils and adding as inoculants to seeds [30]. Later on, the first patent ("Nitragin") was registered for plant inoculation with *Rluzobum* sp [31]. However, inoculation with *Bacillus sp*. for phosphate solubilizing and *Azotobacter* [32], had inconclusive results and was later abandoned. Major outbreaks in plant inoculation technology happened in late 1970s when *Azosprrillum* sp.for plant growth [33], and *Psedomonas* sp. for biocontrol ability were intensively investigated [34]. In recent years, several bacterial genera, such as *Bacillus, Flavobacterium, Acetobacter, Serratia* and *Delftia* mostly isolated from rhizosphere and non-rhizosphere soil, rhizoplane etc. were evaluated as plant growth promoting bacteria [35]. These microorganisms have the potential to reduce the application of agro-chemicals and maintain biotic diversity in the plant-associated biotommunity.

2.1 Pesticides

A pesticide can be defined as any substance or mixture of substances intended for preventing, destroying, repelling, or mitigating any pest (insects, mites, nematodes, weeds, rats, etc.).

Pesticides are used to control or to manage pest populations at a tolerable level. The suffix "-cide" literally means "kill", that means the term pesticide refers to a chemical substance that kills pests where It is incorrect to assume that the term pesticide refers only to insecticides. In modern agriculture practices, millions of tons of pesticides are applied annually all over the globe which covers the billions of dollars' market. The expenditures on pesticides were 35.8 billion in 2006 which rose up to 39.4 billion US dollars in 2007 [2]. Prior to World War II, the pesticides that we use now did not yet exist. Some pesticides currently in use were in fact developed during the World War II for use in warfare. The organophosphate insecticides were

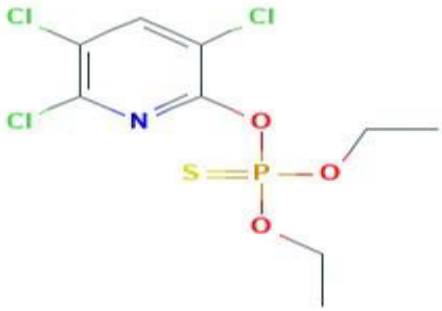
developed as nerve gases, and the phenoxy herbicides, including 2,4-D (the most commonly used herbicide in Canada), were created to eradicate the Japanese rice crop, and later used as a component of Agent Orange to defoliate large areas in jungle warfare. After World War II, these chemicals began to be used as pesticides in agricultural production, for environmental spraying of neighborhoods for mosquito eradication, and for individual home and garden use. Virtually all molecules of biogenic origin are readily degraded by microorganisms, given favorable growth conditions. Pesticides often contain functional groups such as chlorine substituents and novel arrangements rarely found in nature which is responsible to increase recalcitrance, thus leading to greater persistence. DDT, for example, proved to be recalcitrant yet its non-chlorinated analogue diphenylmethane was readily degraded [36, 37]. Due to the presence of chlorine substitutions include dieldrin and aldrin have proven to be recalcitrant. The location and number of chlorine substituents on a pesticide molecule will affect recalcitrance; for example, 2,4,5-T [2,4,5-trich1orophenoxy)acetic acid] is more recalcitrant than 2,4-D [(2,4- dichlorophenoxy)acetic acid] [38]. With the realization that chlorinated pesticides are recalcitrant, moves were made in the 1970s to use more readily degraded organophosphate pesticides.

The classification of pesticides is designed according to their toxicity, chemical group, environmental persistence, target organism, or other features. According to the Stockholm Convention on Persistent Organic Pollutants, 9 of the 12 persistent organic chemicals are pesticides. Classes of organic pesticides (consisting of organic molecules) include organochlorine, organophosphate, organometallic, pyrethroids, and carbamates among others [24, 25]. The common organophosphate pesticides used in the agriculture are chlorpyrifos, diazinon, cypermethrin and profenofos.

2.1.1 Chlorpyrifos

Organophosphorus pesticides are one of the major groups of pesticides accounting 38 per cent of total global pesticide consumption that replaced organochlorines to a greater extent against crop loss by pest attack and improving crop yield. Chlorpyrifos (O,diethyl O3,5,6-trichloro-2-pyridyl phosphorothioate) is one of the broad spectrum organophosphate pesticide used against various agriculture and household pests which is used for crop protection in cotton, rice, sugarcane, peanuts, tobacco, vegetables, fruits and ornamental plants against sucking, chewing and boring insects. It was the fourth largest consumed organophosphate pesticide next to monocrotophos, acephate and endosulfan [39]. According to WHO classification, chlorpyrifos

belongs to class II pesticides with moderate toxicity [40]. The excessive and injudicious application of pesticides led to environmental deterioration and human health concerns [41]. The Chlorpyrifos enter in to the human body via inhalation, ingestion and through skin contact and inhibits acetylcholine esterase enzyme in the central nervous system [42]. Chemical structure of chlorpyrifos is given below.



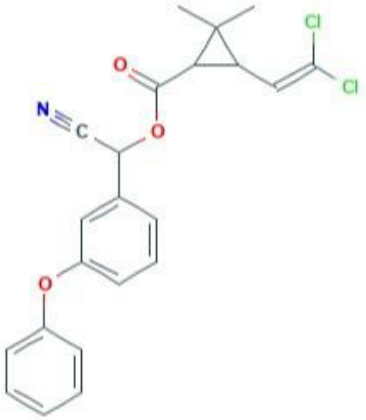
The residue of both CP and TCP has been detected in ecosystems and imparts numerous toxicological effects on the various life forms particularly in humans [43]. These may include headaches, agitation, inability to concentrate, weakness, tiredness, nausea, diarrhea and blurred vision. Respiratory paralysis and death can be happened due to long term use of chlorpyrifos and it is a great threat to pregnant women according to the Agency for Toxic Substances and Disease Registry (ATSDR). For that reason chlorpyrifos was prohibited for indoor home use in 2001 in the U.S but it continues to be used in agricultural Fields, with an estimated 8 million pounds applied annually. According to its manufacturer Dow, chlorpyrifos has been registered for use in 100 countries for over 50 crops [44]. It was reported that the residual CP and TCP ratios in Korean cabbage were 0.93-6.01 and 0.57-2.61%, respectively [45]. The CP-degrading fungal strain *Vertcillium* sp. was used in the detoxification of the insecticides on vegetables [5].

2.1.2 Cypermethrin

Cypermethrin is a synthetic pyrethroid insecticide that has high insecticidal activity, low avian and mammalian toxicity, and adequate stability in air and light which is used to control many pests including lepidopterous pests of cotton, fruit and vegetable crops and is available as an emulsifiable concentrate or wettable powder. The product which contains 2.5 pounds of cypermethrin per gallon according to the label for an Ammo®2.5 E.C insecticide should not be applied directly to water to areas where surface water is present. Also, cypermethrin should not be applied when wind may cause beyond the intended treatment area. The technical grade

Cypermethrin-25EC, Bavistin and 2, 4-D were selected for the degradation because these pesticides are widely used for controlling the different pest on crop in India.

Chemical structure of cypermethrin is given below.



In cypermethrin-25 EC belonging to pyrethroide family used as insecticide in large scale commercial agriculture applications as well as consumer products for domestic purpose that was synthesized in 1974 and firstly marketed in the year 1977. Pyrethroide have four major a generation among which Cypermethrin belongs to fourth generation [46]. The synthetic pyrethroid insecticides are analogs of naturally occurring pyrethrins as per botanical origin [47], and derived from dry flower of Chrysanthemum cinerariaefolium plant which have been known since the nineteenth century. It is unlikely to contaminate the ground water because it binds tightly to soil particles.

2.2 Biological Availability

In soils, biodegradation of pesticides is restricted by the poor accessibility of the chemical. Variety of things influence accessibility, although effects are primarily because of either low solubility and/or sturdy surface assimilation to the soil matrix [38]. The chemical structure of a pesti-cidal, its size and useful teams all confirm the water solubility of the compound. Some pesticides are extremely soluble, et al are applied as their soluble salts or amides to boost dispersion. Less soluble pesticides are applied as associate degree emulsion or suspension of fine solids in a binary compound carrier. Strategies of application aim to supply a good dispersion of the pesticide; this may aid biodegradation. For the growth at the expense of pesticides that have low solubilities in water, microorganisms could need some physiological variations. In soils, chemical molecules could bind tightly to soil organic matter, thus reducing biological accessibility. Chemical natural process will either enhance or decrease

microorganism degradation rates in soil. The concentration of chemical applied to the soil is also deadly, however once absolute to soil an understandable reduction in toxicity is seen permitting biodegradation to proceed.

2.3 Nutrient Availability

Availability of essential nutrients like carbon, nitrogen, phosphorus and element may additionally limit the speed at that microorganism degrades pesticides. The concentration of the chemical should not be thus high on be noxious, nor thus low such biodegradation doesn't proceed because of an absence of induction of applicable degradative enzymes and uptake mechanisms, failure to induce enough accelerator activities, or issues in providing enough energy even for cell maintenance [48, 49]. Some pesticides, such as 2,PD and carbofuran, function a carbon supply for microorganism growth, however, within the presence of different promptly degradable carbon sources, microorganism are less doubtless to degrade the chemical.

Other pesticides, as an example DDT, aren't utilised as a carbon supply and are biodegraded co-metabolically, thus an alternate carbon supply is needed because the growth substrate. Once pesticides are applied in a very mixture, varied effects are discovered. Addition of 1 compound could shorten or lengthen the acclimatization amount required before another compound is degraded. Insecticide change to soils treated with EPTC and add reserved the degradation of the 2 herbicides, and the degradation of EPTC by Rhodococcus TE1 was reserved by insecticide [50]. Nevertheless the synchronous degradation of two, 4-D and mecoprop by mixed microorganism cultures has been discovered [51], till recently, degradation of the many pesticides has solely been studied beneath aerobic conditions. The importance of anaerobic degradation, and of chlorinated compounds particularly, has become additional apparent. Degradation of two 4, 5-T beneath anaerobic conditions proceeded promptly within the presence of different lepton acceptors. Associate in nursing anaerobic isolate, Desulfomonile tiedjei, derives energy from anaerobic dechlorination reactions. It seems that dechlorination of additional extremely chlorinated compounds is favoured beneath anaerobic conditions. Degradation of some pesticides could proceed beneath alternating aerobic-anaerobic conditions, ought to different lepton acceptors be obtainable. Different pesticides could need aerobic conditions for aromatic ring cleavage reactions to proceed. Chemical reaction of diazinon, as an example, will occur beneath each aerobic and anaerobic

conditions however succeeding ring cleavage of the chemical reaction product IHMP (2-isopropyl-6-methyl-4-hydroxy-pyrimidine) needs aerobic conditions [52].

2.4 Bacterial degradation of pesticides

The application of microorganisms for the biodegradation of synthetic dyes is an attractive and alternative method for the wastewater treatments. The use of microorganisms for the removal of synthetic dyes from industrial effluents offers considerable advantages. The process is relatively inexpensive; running costs are low and end products of complete mineralization are generally not toxic. The degradation of pesticides results in the production of carbon dioxide (CO_2) and water (H_2O) by the oxidation of parent compounds. The bacterium involves in the degradation process energy intake from these degradation products. The efficiency of degradation process depends upon optimum atmospheric conditions, that is, temperature, pH of soil, moisture contents, and so forth. The modifications of different bacterial specimens via genetic mutations also enhance effectiveness of applied microbes. The biodegradable pesticides have positive effects on the fertility of agricultural soil. There is a vital advantage of microorganism usage for degradation of pesticides. This is due to the diversity, wide distribution, and adaptation of variable metabolic pathways. The gene clusters are involved in microbial degradation. The genetic manipulation and construction of gene engineering bacteria are also used for degradation of pesticides [53].

The degradation of pesticides in place is typically achieved by a syndicate of microbes instead of one species. Pure culture studies do, however, enable the mechanisms by that the chemical is metabolized to be elucidated. The mechanisms for transport of the chemical into the cell, degradation pathways and induction and regulation of degradative pathways may be studied. Pure culture studies additionally enable the situation of cistrons concerned in degradation of the chemical; this may result in the location of the genes involved in pesticide degradation and also the development of specific gene probes for detection of those organisms in place, precluding the necessity for previous cultivation [54].

Traditional isolation ways, involving enrichment culture and plating techniques, are accustomed isolate several of the microbes that are ready to degrade pesticides in pure culture. Sources of isolates for the enrichment cultures are usually soils that have a previous history of treatment with pesticides. One in all the main difficulties in analytic microorganism that are ready to degrade chemicals is that the chemical structure of the pesticide which might typically

limit biodegradability. Microorganism utilize offered organic compounds. Therefore, by increasing the solubility of hydrophobic substrates by the employment of surfactants, or solvents in biphasic cultures or by dissolving the chemical as its salt (for carboxyl acids and phenols), the isolation of microorganism ready to degrade these hydrophobic compounds may be increased. Once isolated in pure culture, the power of the microorganism is to utilize the chemical as sole carbon supply.

To understand the fate of pesticides in the environment it is important to know the biochemical pathways utilized during microbial attack on the pesticide [55]. This information allows prediction of the fate of not only the pesticide but also the metabolites generated. This information can be crucial as microbial attack on a pesticide may result in metabolites that are more toxic and more mobile than the parent molecule. Some intermediates may accumulate in the environment, for example DDE a residue of DDT, others may affect the rate of degradation of the parent molecule [55]. Genetic studies of pesticide-degrading bacteria have centered on cloning and characterization of specific enzymes involved in pesticide degradation. The involvement of plasmid-encoded catabolic sequences in pesticide degradation has been widely documented. The potential for the spread of plasmid-encoded catabolic genes has long been recognized and this phenomenon may be linked to the poor efficacies of some readily degraded pesticides in certain soils [56].

2.5 Endophytic bacteria

Plants are thought-about as complicated ecosystems wherever completely different niches are populous by Brobdingnagian diversity of microorganism. Such niches embrace the interior tissues wherever endophytic microorganism live within while not inflicting any harmful effects to the host [57]. The plant associated microorganisms play vital role in sport of nutrients and forestall negative impact of phytopathogens. In general, the plant germ interactions could surface at the rhizosphere and among the plant tissues. The microorganisms that complete their one part of life cycle within the plant tissues while not inflicting apparent symptoms within the host plant are thought-about as microorganism endophytes [58]. The endophytic communities were primarily comprised of 5 phyla viz. *Proteobacteria* (90%), *Actinobacteria* (1.5%), *Planctomycetes* (1.4%), *Verrucomicrobia* (1.1%) and *Acidobacteria* (0.5%). The usually found microorganism endophytic genera are *Pseudomonas, Bacillus, Burkholderia, Stenotrophomonas, bacteria genus, Pantoea* and *Microbacterium* etc. All of those genera also are common inhabitants of the rhizosphere.

2.6 Bacterial entry and plant-microbial interaction

Of the many different microorganisms that are commonly found in soil (bacteria, fungi, protozoa, and algae), bacteria are by far the most common. In fact, it has been estimated that there are often around 10^8 to 10^9 bacterial cells per gram of soil [59]. Plants are continuously in contact with bacteria throughout their lifetime. Different bacteria can affect plants differently. Based upon their mode of action and the plant's response, bacteria can be generally divided into three classes:

- Plant pathogens; those bacteria that inhibit plant growth and cause different disease symptoms in various parts of the plant.
- Plant asymptomatic bacteria; those that do not produce any observable effects on plant growth, and
- Plant growth-promoting bacteria (PGPB); those that support plant growth to significant levels. In addition, the effect that a particular bacterium has on a plant may vary according to the environmental conditions. Thus, a bacterial strain that stimulates plant growth by providing fixed nitrogen, a compound that is typically present in limited amounts in many soils, will not provide any benefit to plants when a large amount of chemical nitrogen fertilizer is added to the soil.

The preferred sites of microorganism attachment and ulterior entry are the top root zone with the thin-walled surface root layer like the cell elongation and therefore the plant organ zone.At these sites bacterium are usually organized in microcolonies comprising many many cells [60]. For active penetration, endophytic bacterium need to be well-equipped with cellulolytic enzymes that hydrolyse the plant's exodermal cell walls. In vitro production of those enzymes has been according for several endophytes. The expression of endoglucanase, the most cellulase answerable for reaction of β (1→4) linkage in polysaccharide, was detected as planta at the first sites of entry of *Azoarcus sp*. BH72 [61].

2.7 Plant-growth promoting bacteria (PGPB)

Beneficial free-living soil bacteria in the rhizosphere are generally referred to as plant growthpromoting bacteria (PGPB) and are found in association with the roots of various plants [62]. In the era of sustainable agricultural production, the interactions in the rhizosphere by soil microorganisms play a pivotal role in transformation, mobilization and solubilizing from a limited nutrient pool in the soil and ensuing uptake of essential nutrients by the crop plants.

The beneficial effects of biocontrol PGPB towards various phytopathogens have been extensively studied. Soilborne plant pathogenic fungi infect several crops like cereals, legumes, oilseeds, vegetables and fruits. The use of PGPB-inoculants as biofertilizers and/or antagonists of plant pathogens provide a promising alternative to chemical fertilizers and pesticides. Seed treatment with plant growth promoting rhizobacteria (PGPR) increased the growth of several crops [63, 64].

2.8.1 Plant growth promotional (PGP) traits in endophytic bacteria

2.8.1.1 IAA production

Organic substances capable of regulating plant growth produced either cndogeneously or applied exogeneously are called plant growth regulators. They regulate growth by affecting physiological and morphological processes at very low concentrations [65]. Sevcral microorganisms arc capable of producing auxins, cytokinins, gibberilins, ethylene or abscisic acid. Bacteria synthesize indole acetic acid (IAA) predominantly by an alternate tryptophan-dependant pathway, through indole pyruvic acid. However, the role of bacterial IAA in plant growth remains undetermined. Promotion of root growth is one of the major markers by which the beneficial effect of plant growth promoting bacteria is measured [66]. The establishment of roots, whether by elongation of primary roots or by proliferation of lateral and adventitious roots, is advantageous for young seedlings as it increases their ability to anchor themselves to the soil and to obtain water and nutrients from the environment, thus enhancing their survival. Most PGPB synthesize IAA and their effect on plants mimics that of exogeneous IAA.

2.8.1.2 ACC deaminase

A number of bacteria contain the enzyme I-amino-cyclopropane-l-carboxylate (ACC) 1 deaminase and this enzyme could cleave ACC, the immediate precursor to ethylene in the biosynthetic pathway for ethylene in plants [67, 68]. ACC deaminase activity plays a role in root lengthening by decreasing ethylene production in the roots of host plants. For most of the plants, ethylene is required to break seed dormancy [69], but after germination, high-level ethylene would inhibit root elongation [70]. PGPB that contain the enzyme ACC deaminase, when bound to the seed coat of a developing seedling, act as a mechanism for ensuring that the ethylene level does not become elevated to the point where initial root growth is impaired. By facilitation the formation of longer roots, these bacteria may enhance the survival of some

seedling, especially during the first few days after the seeds are planted. The plants treated with ACC deaminase producing bacteria are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as flooding, heavy metals, the presence of phytopathogens, drought and high-salt [71]. These bacteria are beneficial to plant growth since in the natural environment plants are often subjected to stress due to ethylene production.

2.8.1.3 Phosphate solubilizing bacteria (PSB)

Most agricultural soils contain large reserves of phosphorous, a considerable part of which is accumulated and fixed as a consequence of regular application of chemical (P) fertilizers. However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants [71]. The phenomenon of fixation and precipitation of P in soil is generally dependant on pH and soil type. Phosphorous is particularly essential for rice growth and development. Phosphorus accounts 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 $H_2PO_4^-$ [72]. Soil microorganisms are capable in releasing P from inorganic and organic pools of total soil P through solubilizing and mineralization. Solubilizing can be accomplished by a range of 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 solubilizing [73].

Phosphate solubilizing by PSB strains is associated with the release of low molecular weight organic acids mainly gluconic and ketogluconic acid 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 9 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 solubilizing capability of PSB has direct correlation with pH of the medium. The use of PSB in agricultural practice would not only offset the high cost of

manufacturing phosphate fertilizers but would also mobilize insoluble phosphorus in the fertilizers and soils to which they are applied.

Phosphate solubilizing bacteria (PSB) constitute 1 to 50%, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5% in P solubilizing potential among the whole microbial population in soil. Among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas, Bacilli* and *endosymbiotic* Rhizobia have been described as effective phosphate solubilizers. 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 [74].

High proportion of PSM is accumulated 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 solubilizing as well as can enhance plant growth by enhancing the availability of other trace element such as iron (Fe), zinc (Zn) etc.

2.8.1.4 Nitrogen fixing bacteria

Nitrogen is an essential component of amino acids and proteins. Nitrogen is abundant in the atmosphere but atmospheric nitrogen can only be used by certain organisms like microbes that supply inorganic form of nitrogen to their host, insects, or plants via symbiotic or non-symbiotic interactions [75]. Thus, nitrogen fixation process is vital in providing fixed N_2 to organisms.

Biological N_2 fixation (BNF) is the conversion of atmospheric nitrogen into ammonia [76]. About 90 genera of microbes are able to fix nitrogen by utilizing nitrogenase enzyme [77]. The endophytic bacteria can fix atmospheric dinitrogen (N_2) in plants through colonization into the roots of the plants for the formation of tumor like nodules which acts as the factories of ammonia production, mobilizing fastened macro and small nutrients or convert insoluble phosphate within the soil into forms on the market to plants, thereby will increase their potency and accessibility.

Table: Genes of involved in nitrogen fixation[78]

Locus Tag	Size/aa	Gene	Gene product
A6A40_02185	852	fixA	Electron transfer flavoprotein beta subunit
A6A40_02190	1080	fixB	Electron transfer flavoprotein alpha chain
A6A40_02195	1302	fixC	Flavoprotein-ubiquinone oxidoreductase
A6A40_09085	210	fixU	Nitrogen fixation protein
A6A40_02200	285	fixX	Ferredoxin-like protein
A6A40_09040	1866	nifA	Nif-specific transcriptional activator
A6A40_09050	1518	nifB	Nitrogenase FeMo cofactor biosynthesis protein
A6A40_02900	1440	nifD	Nitrogenase molybdenum-iron protein alpha chain
A6A40_02875	1407	nifE	Nitrogenase molybdenum-cofactor biosynthesis protein
A6A40_02905	897	nifH	Nitrogenase iron protein
A6A40_02895	1560	nifK	Nitrogenase molybdenum-iron protein subunit beta
A6A40_02870	1371	nifN	Nitrogenase molybdenum-cofactor biosynthesis protein
A6A40_02230	1206	nifS	Nitrogenase metalloclusters biosynthesis protein
A6A40_02235	924	nifU	Iron-sulfur cluster assembly scaffold protein
A6A40_02225	1122	nifV	Homocitrate synthase
A6A40_02215	336	nifW	Nitrogenase-stabilizing/protective protein
A6A40_02865	399	nifX	Nitrogenase molybdenum-iron protein
A6A40_09070	333	nifZ	Nitrogenase P-cluster assembly
A6A40_09075	306	nifZ	Nitrogenase P-cluster assembly
A6A40_02220	852	cysE	Serine acetyltransferase
A6A40_02925	909	draG	ADP-ribosyl-[dinitrogen reductase] hydrolase
A6A40_02920	891	draT	ADP-ribosyl-[dinitrogenase reductase] transferase
A6A40_07245	2847	glnD	[Protein-PII] uridylyltransferase
A6A40_07685	339	glnB	Nitrogen regulatory protein P-II
A6A40_05220	1200	ntrB	Nitrogen regulation sensor histidine kinase
A6A40_05215	1146	ntrC	Nitrogen regulation response regulator
A6A40_05205	1401	ntrX	Sigma-54-dependent transcriptional regulator
A6A40_05210	2319	ntrY	Nitrogen regulation sensor histidine kinase

There are two main classes of nitrogen fixing genes are found in endophyes, these are *nif* and *fix* genes which are involved in BNF by forming a symbiotic association with leguminous plants [26]. The *nif* genes encode nitrogenase and show structural and functional resemblance with the nitrogen-fixing genes present in *Klebsiella pneumoniae* and other microbial groups [27]. Besides of *nif* genes, there are several other microbial genes which are significantly involved in the symbiotic N_2-fixation, these are known as fix genes. The enzyme nitrogenase is very sensitive to oxygen, hence anaerobic condition is required for BNF. BNF is an energy-requiring process and energy comes from the cellular metabolic process in the form of ATP. All the nitrogen-fixing microorgaisms carry enzyme hydrogenases which catalyze the transfer of electrons from pyruvate or hydrogen to ferredoxin or flavoprotein [28].

Chapter III

METHOD AND MATERIALS

3.1 Careful handling of laboratory apparatus and glassware

All the laboratory equipments that were used in this study including conical flasks, glass pipettes, beakers, glass rods, petri-dishes, micropipette tips, eppendorf tubes, falcon tubes, PCR tubes etc. had been autoclaved at 121°C in 15 psi for 15 minutes before usage to make sure they were in sterile condition.

3.2 Media, solutions and reagents

Different types of media were used for endophytic bacterial isolation, morphological analysis, nitrogen fixing and phosphate solubilizing capacity of endophytes and for identification of specific properties. The solutions and reagents for biochemical and molecular characterizations were freshly prepared and sterilized through autoclaving before use. All chemicals needed were acquired from the laboratory. None of the solutions or reagents were further purified since they were of a reagent grade.

3.3 Instruments

Electronic Balance, Shaking Incubator, Spectrophotometer, Digital pH meter Centrifuge, Magnetic strirrer hot plate, Autoclave, Laminar Air Flow Clean Bench, Vortex shaker, Refrigrater (LG) and Microscope etc

3.4 Pesticide (chlorpyrifos) collection:

One pesticide using this research purpose such as chlorpyrifos (CAS: 2921-88-2) + cypermethrin was purchased from Sigma Aldrich and local pesticide shop in Dinajpur.

3.5 Bacterial sample source collection:

Bacterial sample source was collected from tomato plant root in the Hajee Mohammad Danesh Science and Technology University, Basher hut, Dinajpur.

3.6 Isolation of pesticide degrading bacteria

3.6.1 Isolation of pesticide degrading endophytic bacteria from tomato root

Endophytic bacteria was isolated from tomato plant root. For the isolation of pesticide degradable endophytic bacteria from tomato root was done in following steps;

Surface sterilization using 75% (v/v) ethanol for 2 min

⬇

Washing the samples with autoclaved distilled water

⬇

Smashing the samples with mortar and pestle

⬇

Addition of distilled water into this paste and then the mixture was poured into test tubes

⬇

Finally inoculation of that sterilized sample into PVK agar (Pikovskayas Agar) media

⬇

And then the isolates were grown in pesticide containing media.

One pesticide was used for this test. These was Chlorpyrifos with Cypermethrin (where chlorpyrifos was 500g/1L with cypermethrin 50g/1L). The media was Normal growth media with minimal nutrient and also pesticides as sole carbon source in which the isolated bacteria from PVK agar media were inoculated. The normal growth media was prepared and autoclaved at 121°c in 15 psi for 15 minutes. After a while when media temperature was cooled down then pesticide was poured into these media with chlorpyrifos + cypermethrin 500 µl/150ml concentration. Then the mixer of media and pesticide was stirred perfectly. After that the diluted soil sample was inoculated by streaking method with a platinum loop in front of sprit lamp. The bacterial inoculated petri dishes were incubated at 37°c for 24 hours.

3.7 KOH string test

A loopful of growth from a bacterial colony was emulsified on the surface of a glass slide in a suspension of 3% KOH. The suspension was stirred continuously for 60 seconds after which the loop was gently pulled from the suspension. The test was considered positive if string occurred within the first 30 seconds after mixing the bacteria in KOH solution [79].

3.8 Biochemical characterization

The selected bacterial strains were differentiated based on their metabolic pattern by using different biochemical tests [80], including the following test :

- MR-VP test
- Catalase test,
- Oxidase test,
- Triple Sugar Iron (TSI) test,
- Citrate utilization test (CIU),
- Motility Indole Urease (MIU) Test
- Urease test and
- Individual sugar fermentation/carbohydrate tests (dextrose, lactose, maltose and sucrose) [81].

3.8.1 Methyl Red-Voges-Proskauer Test/ (MR-VP) Test

MR-VP Test was performed for identifying an organism's metabolic pathway. 4.25g MR-VP broth was prepared 250ml distilled water. The solution was autoclaved 121°c in 15 psi for 15 minutes. Then 10ml broth was transferred to 20 test tubes. After that the selected isolates were inoculated into the each tubes and incubated at 37°C for 24 hours. After incubation, 5ml of solution contained with bacteria separated from each test tube to another 20 test tube for VP test. To perform MR test five drops of methyl red reagent (0.04g/60ml of 100% ethanol) was added for detecting MR positive isolates by searching for red colors in the test tubes. For conducting VP test 1.2 ml of 5% alpha-naphthol was added to individual test tubes. Following after 0.4 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 copper-red color development which indicates a positive result [80].

3.8.2 Catalase test

This test was performed to detect the presence of catalase enzyme of the isolates. It was carried out by adding 3% H_2O_2 into a small amount of bacterial colony on a clean glass slide. A positive catalase test provides rapid production of bubbles indicating catalase utilization by the isolate. A negative result indicates the absence of catalase enzyme. This test identifies members of Enterobacteriaceae family which are catalase positive [82].

3.8.3 Oxidase test

Oxidase test was done on the selected isolates to see if they can utilize cytochrome c oxidase which is basically an enzyme of the electron transport chain [81]. 20 pieces of clean small sized filter paper were taken to carry out this test. One side the filter paper was marked as control and the other side was marked as sample name of each filter paper. A drop of oxidase reagent (1% tetramethyl-p-phenylenediaminedi hydrochloride) was added onto the filter paper on both side. Using an inoculating loop, a small amount of bacterial pure culture of the selected isolates were streaked onto the oxidase droplet in where sample name was marked. Formation of deep blue or dark purple color within 10-30 seconds of the reagent was considered as positive result [80]. Oxidase positive bacteria are basically aerobic and can use oxygen as a terminal electron acceptor.

3.8.4 Triple Sugar Iron (TSI) test

TSI media is a semi-solid media containing three sugar (Lactose, Sucrose, and Glucose) and also iron which is done to confirm the ability of the bacteria to utilize these sugars. 13g of TSI media was prepared with addition of 2.5g agar into 200ml of distilled water. The solution was mixed perfectly by using magnetic stir. After preparing the media solution 6 ml of the broth was distributed to each test tubes. Each test tubes were kept on sprit lamp to mix appropriately. The media was sterilized by autoclaving at 15 psi, 121°C for 15 min. 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 [83].

Table 1. TSI result evaluation parameters [83]

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.

3.8.5 Citrate Utilization Test (CIU)

The citrate test is utilized to identify the ability of the bacterial isolate to utilize citrate as a carbon and energy source. 4.5g of Simmon citrate agar media was prepared with addition of 2.5g agar into 200ml of distilled water. The solution was mixed perfectly by using magnetic stir. After preparing the media solution 6 ml of the broth was distributed to each test tubes. Each test tubes were kept on sprit lamp to mix appropriately. The media was sterilized by autoclaving at 15 psi, 121°C for 15 min. 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 in each test tubes and the tubes were subjected to Incubation 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 [80].

3.8.6 Motility Indole Urease (MIU) Test

The MIU test is done for multiple reasons including to detect if the selected isolate can utilize urea, if the organism is mobile or not and if the bacteria can produce indole.[84 and 87]The media was prepared using 3.78g MIU agar medium into 200ml distilled water. 5 ml sterile 40% urea solution per 95 ml was prepared simultaneously in basal medium. After that the media solution was autoclaved at 15 psi. pressure (121°C) for 15 minutes. The media was left to cool down around 50°C the urea solution was added completely and then 6ml of solution was distributed in each test tubes [80]. The selected isolates were inoculated and incubated at 37°C for 24 hours. The results were interpreted as per following table:

Table 2. MIU result evaluation parameters [80]

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

Note: Basically the results for motility and urease are observed first and then the medium is read for indole production. But to be absolutely sure indole and urease test has been done separately to identify the ability of the selected isolates to use urea and tryptophan.

3.8.7 Urease test

The media was formulated using 3.4g urea agar base into 200ml of distilled water and a pH of 6.8 was maintained. After formulation, 6 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 [80].

3.8.8 Fermentation of sugar: lactose, maltose, sucrose, dextrose

Specific sugar fermentation test was done using phenol red carbohydrate broth to observe if the selected isolates can utilize these sugars. Different sugars (Lactose, Maltose, Dextrose, and Sucrose) media were made according to protocol provided by the manufacturers and 6ml of the media were transferred into each test tubes. The final pH of 7.4 ± 0.2 was maintained. After that the selected isolates were inoculated and incubated at 37°C for 24 hours. The appearance and color of the media was observed after incubation. 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 was indicated as negative [83]

3.9 Characterization of endophytic bacteria isolate for Plant Growth Promotional (PGP) traits

3.9.1 Quantitative analysis of Indole acetic acid (IAA) production

IAA production in different isolates of endophytes were detected by inoculating bacterial suspension in 7 ml Luria Bertanni broth containing tryptophan 0.01% (L-Trp) separately and incubated at 37°C for 7days in a shaking incubator. Then 1ml of each culture was transferred into labeled eppendroff tubes and centrifuged at 10,000 rpm. After that 1ml of supernatant was transferred to the glass test tubes with by adding 2 ml of Salkowski‟s reagent (1 ml of 0.5ml FeCl3 in 50 ml of 35% HClO4). Then the mixture was kept in the dark for 120 minutes. Absorbance of reddish pink colour was measured spectroscopically (ELICO UV-VIS spectrophotometer) at 530 nm and quantified IAA concentration by standard curve [84].

3.9.2 Quantitative estimation of ACC-deaminase production

For determining ACC deaminase activity, endophytes and soil bacteria strains were grown in rich Tryptic Soy Broth medium (TSB) for 18 h at 28°C. The cells were then harvested by centrifugation, washed with 0.1 M Tris–HCl (pH 7.5), and incubated for another 18 h in minimal medium containing 3 mM ACC as the sole source of nitrogen. The bacterial cells were collected by centrifugation and suspended in 5 mL of 0.1 moL L- 1 of Tris–HCl, pH 7.6, and transferred to microcentrifuge tube. The contents of the tubes were centrifuged at 16,000

rpm for 5 min and supernatant was removed. The pellets were suspended in 2 mL 0.1 moL L-1 Tris HCl, pH 8.5.

Thirty microliters of toluene was added to the cell suspension and vortexed for 30 s. 200 µL of the toluenized cells were placed in a fresh microcentrifuge tube, 20 µL of 0.5 moL L-1 ACC was added to the suspension, vortexed, and then incubated at 30°C for 15 min, following the addition of 1 mL of 0.56 moL L-1 HCl, the mixture was vortexed and centrifuged for 5 min at 13,000 rpm at room temperature. 2 mL of the supernatant was vortexed together with 1 mL of 0.56 moL L-1 HCl. Thereupon, 2 mL of the 2, 4-dinitrophenylhydrazine reagent (0.2% 2, 4-dinitrophenylhydrazine in 2 moL L-1 HCl) was added to the glass tube, and the contents were vortexed and then incubated at 30°C for 30 min. Following the addition and mixing of 2 mL of 2 moL L-1 of NaOH, the absorbance of the mixture was measured by using spectrophotometer at 540 nm. The cell suspension without ACC was used as negative control and with $(NH_4)_2SO_4$ (0.2% w/v) as positive control [85].

3.9.3 Qualitative estimation of phosphate solubilizing

Phosphate solubilizing ability of the isolates was determined qualitatively by streaking strains on PVK agar media. The components of this media were prepared in 500ml of distilled water and after autoclaving the media was poured onto petri dishes. After that the strains were spread on that plate through streaking. The inoculants were cultured at 30°C for 72 hours. Strains that could grow in all insoluble phosphate media were selected as phosphate solubilizing bacteria [86].

Table 3: The composition of PVK agar media

Ingredients	500 ml
Dextrose	5g
$Ca_3(PO_4)_2$	2.25g
$MgSO_4$	0.05g
Nacl	0.2 g
Kcl	0.1g
0.1% $(NH_4)_2SO_4$	0.25g
$MnSO_4$	0.00005g
$FeSO_4$	0.00005g
Yeast extract	0.25g
Agar	12.5g

3.9.4 Qualitative analysis of nitrogen fixation capability of endophytic bacteria

Both nitrogen-free and nitrogen-containing media were used for the isolation of nitrogen-fixing bacteria to check the nitrogen fixing capability of endophytic bacteria.

Table 4: Composition of *Azotobacter* medium required for isolation of nitrogen fixing bacteria [87]

Ingredients	500ml
Sucrose	10 g
Dipotassium phosphate (K_2HPO_4)	0.5 g
Anhydrous magnesium sulphate ($MgSO_4$)	0.25g
Sodium chloride (NaCl)	0.25 g
Calcium carbonate ($CaCO_3$)	1g
Bromothymol blue	0.008g
Agar	12 g

Table 5: Composition of *Rhizobium* Medium (Yeast Extract Mannitol Agar Medium) required for isolation of nitrogen fixing bacteria [82]

Ingredients	500ml
Mannitol	5 g
Dipotassium phosphate (K_2HPO_4)	0.25 g
Anhydrous magnesium sulphate ($MgSO_4$)	0.1g
Sodium chloride (NaCl)	0.05 g
Yeast	0.5g
Congo red	0.03g
Agar	12 g

The components of both media were prepared in 500ml of distilled water and after autoclaving the media was poured onto petri dishes. After that the strains were spread on that plate through streaking. The inoculants were cultured at 30^{o}C for 72 hours.

3.10 Effect of one isolate on eggplant growth

3.10.1 Effect of one isolate on germination percentage, seedling growth of eggplant on petri dishes in the dark

3.10.1.1 Seed sterilization and preparation:

The growth promoting activity test of the isolates was carried out on eggplant. The eggplantseeds were purchased from BRAK nursery. These were allowed to sundry first and then washed with 70% ethanol for 50 seconds. After that the seeds were washed with autoclaved water for 3 times.

3.10.1.2 Seed bacterization

Finally all the seeds were kept in different ependroff tubes where 1ml of bacterial suspension was added and this solution was kept in the dark for 8 hours. After that the seeds were ready for germination.

3.10.1.3 Seed inoculation on to petri dishes

Seeds were inoculated in petri dishes the petri dishes in the dark for 14 days. The petri dishes were arranged in a Completely Randomized Design (CRD) with 3 replications. Germinated seeds were recorded 3 times during 14 days. After 7 days of seed germination 0.25ml individual bacterial suspension was applied in each petri dish.

The following parameters were recorded from this experiment:

Germination percentage- Germination percentage was calculated using the following formula: GP = (Total germinated seed)/ (Total number of seed) [88].

Root length and shoot length- Root length and shoot length were measured using a graduated ruler.

Fresh weight and dry weight- Fresh weight and dry weight were measured in gram by analytical balance.

Vigor index- Vigor index was calculated using the formula by Zhu and Hong: Seedling length× germination percentage.

3.10.2 Pot experiment to ensure the effect of one isolate on seedling growth and weight of eggplant

3.10.2.1 Pot preparation and seed inoculation

Seeds were prepared for inoculation after surface sterilization and bacterization according to previously described method (3.10.1.2-3). Then the seeds were inoculated in plastic pot containing soil without fertilizer and compost.

3.10.2.2 Treatment

After seed germination 2ml of bacterial suspension was spread in each pot in every week. The seedlings were treated 4 times. The root length and shoot length were measured in 14 days, 21 days and 30 days for endophytic bacteria treated plants.

3.10.3 Statistical Analysis

Data derived from the present study were assessed with an emphasis on effects of endophytic bacteria on eggplant. Each data was recorded in 3 replicates. Root length, shoot length, germination percentage, seedling length, vigor index, fresh weight and dry weight were analyzed through one way ANOVA method by 'SPSS' software. All data were expressed as the mean value and standard deviation of total measurements for 3 replicates where significant differences were performed by using Fisher's protected LSD test at $\rho \leq 0.05$. Graphical analysis along with standard error was conducted by Microsoft office excel 2013.

3.11 Pesticide biodegradation in liquid medium

Conical flasks (250mL) containing mineral salt medium (100mL) supplemented with 100mg/L of chlorpyrifos was inoculated with 1ml of bacterial suspensions. After 25days the absorbance of all the samples including control (without bacteria in mineral salt medium) were recorded at 600nm in two ways; before centrifugation and after centrifugation. If the isolates can degrade or mineralize the pesticides, the absorbance will be less than the control after centrifugation. Further confirmation of pesticide bio-degradation was done by 'FTIR' analysis. A Thermo Nicolet Nexus FTIR spectrophotometer was used to obtain the IR measurements

connecting with liquid nitrogen cooled MCT-A detector. A miracle, single reflection horizontal ATR accessory (PIKE instruments) having a diamond ATR crystal fixed at incident angle of 458 was used to collect the spectra. The each fiber samples (approximately 10 mm) were mounted on top of ATR crystal and pressed gently by a pre-mounted sample clamp. The IR spectra of each sample fibers were obtained in the range of 500-4000 cm^{-1} with an average of 4 cm^{-1} resolution. The Omnic software was used to correct the ATR effect and atmospheric contributions from CO_2 and H_2O vapor [89].

3.12 Phylogenetic tree analysis

After sequence similarity analysis the evolutionary tree of the identified strains were formed in order to get a better understanding about their origin. With the help of maximum likelihood method the optimal tree was formed. 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. All positions containing gaps and missing data were eliminated and all sites were used for making comparison. Evolutionary analyses were conducted in 'MEGA X' [90].

3.13 Technical protocol of whole genome sequencing

Table 6: Supported Combination

Sequencing Instrument	**Illumina MiniSeq System**
Setup Option	Local Run Manager
Setup Workflow Generate	FASTQ
Library Preparation Kit: Indexing:	Nextera XT DNA Sample Prep Dual Indexing
Reagent Kits:	MiniSeq Kit
Analysis Workflow:	Generate FASTQ
Analysis Software:	Local Run Manager

Nextera Transposase Adapters

The transposase adapters are used for Nextera tagmentation.

Read 1 5′ TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG

Read 2 5′ GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

PCR Primers Index

1 Read 5′ CAAGCAGAAGACGGCATACGAGATGTCTCGTGGGCTCGG

2 Read 5′ AATGATACGGCGACCACCGAGATCTACACTCGTCGGCAGCGTC

3.13.1 Creating a Run with Local Run Manager

Set Run Parameters

- Log in to Local Run Manager.
- Select **Create Run**, and then select **Generate FASTQ**.
- Enter a run name that identifies the run from sequencing through analysis.
- The run name can contain alphanumeric characters, spaces, and the following special characters: `~!@#$%-_{}.
- [Optional] Enter a run description to further identify the run.
- The run description can contain alphanumeric characters, spaces, and the following special characters: `~!@#$%-_{}.

Specify Run Settings

- Select the library prep kit from the Library Prep Kit drop-down list.
- Select **2** to specify a dual-indexed run.
- Select to specify the read type.
- Enter the number of cycles for the run.
- [Optional] For Custom Primers, specify any custom primer information to be used for the run by selecting the appropriate checkboxes.
- Custom primer options vary based on your instrument or Local Run Manager implementation.

Enter Samples Manually

- Adjust the samples table to an appropriate number of rows.
- In the Rows field, use the up/down arrows or enter a number to specify the number of rows to add to the table. Select to add the rows to the table.
- Select to delete a row.

- Right-click on a row in the table and use the commands in the contextual menu.
- Enter a unique sample ID in the Sample ID field.
- Use alphanumeric characters, dashes, or underscores. Spaces are not allowed in this field.
- [Optional] Enter a sample description in the Description field.
- Use alphanumeric characters, dashes, or underscores. Spaces are not allowed in this field.
- Specify an Index 1 sequence.
- Select **Show Index Sequence**/**Show Index Names** to toggle between showing the name of the index and the index sequence
- Specify an Index 2 sequence.
- Select a manifest file from the Manifest drop-down list.
- [Optional] Enter a project name in the Sample Project field.
- Use alphanumeric characters, dashes, or underscores. Spaces are not allowed in this field.
- [Optional] Select **Export Sample Sheet** to export the sample information in *.csv format. The exported sample sheet can be used as a template, or imported when creating new runs.

3.13.2 Creating Nextera XT DNA Libraries

3.13.2.1 Tagment Genomic DNA

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
gDNA	-25°C to -15°C	Thaw on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly.
ATM	-25°C to -15°C	Thaw on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly.
TD	-25°C to -15°C	Thaw on ice. Invert the thawed tubes 3–5 times, and then centrifuge briefly.
NT	15°C to 30°C	Check for precipitates. If present, vortex until all particulates are resuspended.

2. Save the following tagmentation program on the thermal cycler:

- Choose the preheat lid option
- 55°C for 5 minutes
- Hold at 10°C

Procedure

1. Add the following volumes in the order listed to each well of a new Hard-Shell skirted PCR plate. Pipette to mix.
    - TD (10 µl)
    - Normalized gDNA (0.2 ng/µl per sample) (5 µl)
2. Add 5 µl ATM to each well. Pipette to mix.
3. Centrifuge at 280 × g at 20°C for 1 minute.
4. Place on the preprogrammed thermal cycler and run the tagmentation program. When the sample reaches 10°C, *immediately* proceed to step 5 because the transposome is still active.
5. Add 5 µl NT to each well. Pipette to mix.
6. Centrifuge at 280 × g at 20°C for 1 minute.
7. Incubate at room temperature for 5 minutes.

The PCR plate contains 25 µl tagmented and neutralized gDNA, all of which is used in the next step.

3.13.2.2 Amplify Libraries

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
Index adapters (i5 and i7)	-25°C to -15°C	Only prepare adapters being used. Thaw at room temperature for 20 minutes.
NPM	-25°C to -15°C	Invert each tube to mix. Centrifuge briefly. Thaw on ice for 20 minutes.

2. Save the following program on the thermal cycler:

- Choose the preheat lid option.

- 72°C for 3 minutes
- 95°C for 30 seconds
- 12 cycles of:
  - 95°C for 10 seconds
  - 55°C for 30 seconds
  - 72°C for 30 seconds
- 72°C for 5 minutes
- Hold at 10°C

Procedure

1. [24 libraries] Arrange the index adapters in the TruSeq Index Plate Fixture as follows.
   - Arrange Index 1 (i7) adapters in columns 1–6 of the TruSeq Index Plate Fixture.
   - Arrange Index 2 (i5) adapter in rows A–D of the TruSeq Index Plate Fixture.

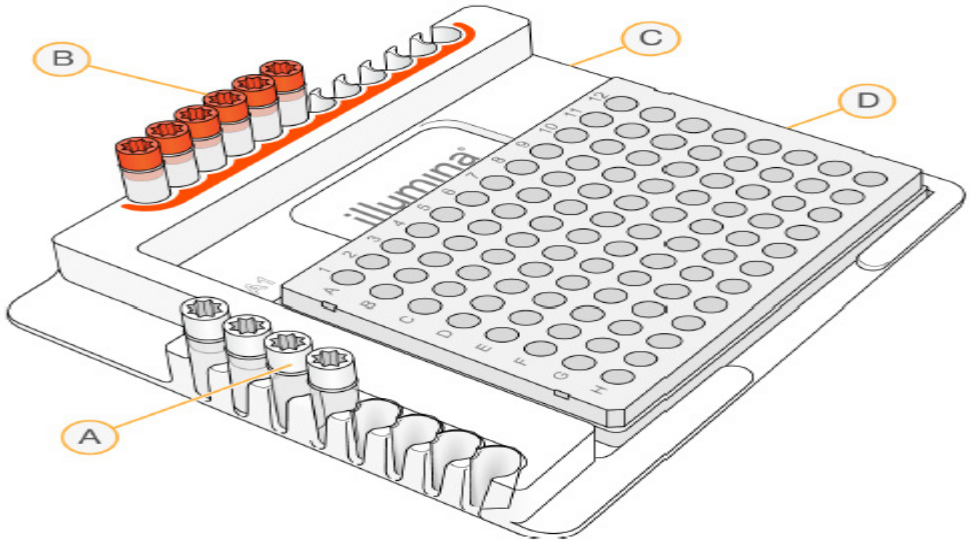


Figure 1: TruSeq Index Plate Fixture Setup for 24 Libraries

A. Rows A–D: Index 2 (i5) adapters (white caps)
B. Columns 1–6: Index 1 (i7) adapters (orange caps)
C. TruSeq Index Plate Fixture

D. Hard-Shell PCR plat

2. [96 libraries] Arrange the index adapters in the TruSeq Index Plate Fixture as follows.
    - Arrange Index 1 (i7) adapters in columns 1–12 of the TruSeq Index Plate Fixture.
    - Arrange Index 2 (i5) adapter in rows A–H of the TruSeq Index Plate Fixture.

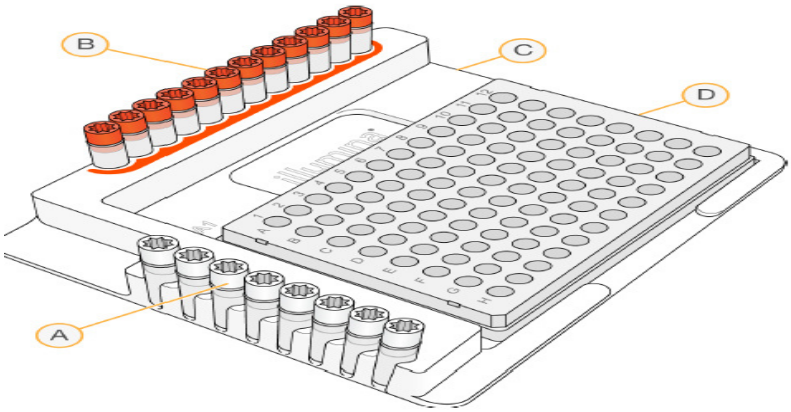


Figure 2: TruSeq Index Plate Fixture Setup for 96 Libraries

A. Rows A–H: Index 2 (i5) adapters (white caps)
B. Columns 1–12: Index 1 (i7) adapters (orange caps)
C. TruSeq Index Plate Fixture
D. Hard-Shell PCR plate

3. Using a multichannel pipette, add 5 µl of each Index 1 (i7) adapter down each column. Replace the cap on each i7 adapter tube with a new orange cap.
4. Using a multichannel pipette, add 5 µl of each Index 2 (i5) adapter across each row. Replace the cap on each i5 adapter tube with a new white cap.
5. Add 15 µl NPM to each well containing index adapters. Pipette to mix.
6. Centrifuge at 280 × g at 20°C for 1 minute.
7. Place on the preprogrammed thermal cycler and run the PCR program. The volume is 50 µl.

3.13.2.3 Clean Up Libraries

Preparation

1. Prepare the following consumables:

Item	Storage	Instructions
RSB	-25°C to -15°C	Thaw at room temperature. RSB can be stored at 2°C to 8°C after the initial thaw.
AMPure XP Beads	2°C to 8°C	Let stand on the benchtop for 30 minutes to bring to room temperature.

2. Prepare fresh 80% ethanol from absolute ethanol.

Procedure

1. Centrifuge at 280 × g at 20°C for 1 minute.
2. Transfer 50 µl PCR product from each well of the PCR plate to corresponding wells of a new midi plate.
3. Add 30 µl AMPure XP beads to each well.

Smaller amplicons in Nextera XT library preps typically yield smaller insert size ranges. To maximize recovery of smaller fragments from the bead cleanup step, use the following conditions.

Input Size (bp)	AMPure XP Recommendation	AMPure XP Volume (µl)
300–500	1.8x AMPure XP	90
> 500	0.6x AMPure XP	30
	(0.5x AMPure XP for ≥ 2 x 250 cycles)*	(25 µl for ≥ 2 x 250 cycles)*
gDNA or other genomic input	0.6x AMPure XP	30

*Applicable only to the MiSeq™ or HiSeq™ 2500 using HiSeq Rapid v2 reagents.

4. Shake at 1800 rpm for 2 minutes.
5. Incubate at room temperature for 5 minutes.

6. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
7. Remove and discard all supernatant from each well.
8. Wash two times as follows.
    a. Add 200 µl fresh 80% EtOH to each well.
    b. Incubate on the magnetic stand for 30 seconds.
    c. Remove and discard all supernatant from each well.
9. Using a 20 µl pipette, remove residual 80% EtOH from each well.
10. Air-dry on the magnetic stand for 15 minutes.
11. Remove from the magnetic stand.
12. Add 52.5 µl RSB to each well.
13. Shake at 1800 rpm for 2 minutes.
14. Incubate at room temperature for 2 minutes.
15. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
16. Transfer 50 µl supernatant from the midi plate to a new Hard-Shell PCR plate.

3.13.2.4 Check Libraries

Run 1 µl of undiluted library on an Agilent Technology 2100 Bioanalyzer using a High Sensitivity DNA chip.

The following figure shows example traces of libraries successfully sequenced on a HiSeq 2500 system. Typical libraries show a broad size distribution of ~250–1000 bp, as shown in the top panel. Various libraries can be sequenced with average fragment sizes as small as 250 bp or as large as 1500 bp.

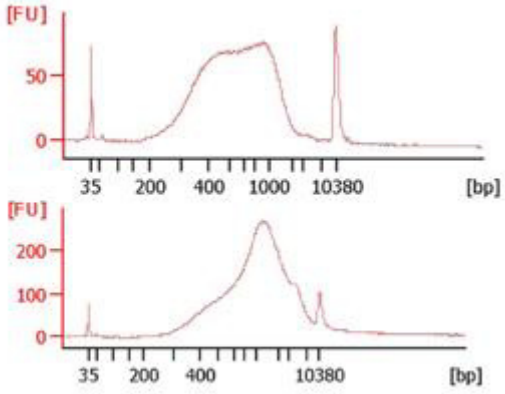


Figure 3: Library Size Distributions of Control gDNA

3.13.2.5 Normalize Libraries

Note

Manually normalize libraries when the final library yield is less than 10–15 nM. Bead-based normalization on low yield libraries can result in overly diluted samples and low sequencing yields. For more information, see Best Practices for Standard and Bead-Based Normalization in Nextera XT DNA Library Preparation Kits (Pub.No. 470-2016-007).

Preparation

Prepare the following consumables:

Item	Storage	Instructions
LNA1	-25°C to-15°C	Prepare under a fume hood. Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNB1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNW 1	2°C to 8°C	Bring to room temperature. Use a 20°C to 25°C water bath as needed.
LNS1	Room temperature	Bring to room temperature.

Procedure

1. Transfer 20 µl supernatant from the Hard-Shell PCR plate to a new midi plate.
2. Add 44 µl LNA1 per sample to a new 15 ml conical tube. Calculate about 5% extra sample to account for sample loss due to pipetting.For example: for 96 samples, add 4.4 ml LNA1 to the tube (100 samples × 44 µl = 4.4 ml).
3. Thoroughly resuspend LNB1. Pipette to mix.
4. Transfer 8 µl LNB1 per sample (including the 5% extra) to the 15 ml conical tube containing LNA1. Invert to mix.For example: for 96 samples, transfer 800 µl LNB1 to the tube of LNA1 (100 samples × 8 µl = 800 µl).
5. Pour the bead mixture into a trough.
6. Add 45 µl combined LNA1 and LNB1 to each well containing libraries.
7. Shake at 1800 rpm for 30 minutes.

8. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
9. Remove and discard all supernatant from each well.
10. Wash two times as follows.
    a. Add 45 µl LNW1 to each well.
    b. Shake at 1800 rpm for 5 minutes.
    c. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
    d. Remove and discard all supernatant from each well.
11. Add 30 µl 0.1 N NaOH to each well.
12. Shake at 1800 rpm for 5 minutes.
13. During the 5 minute elution, label a new 96-well PCR plate SGP for storage plate.
14. Add 30 µl LNS1 to each well of the SGP plate. Set aside.
15. After the 5 minute elution, make sure that all samples in the midi plate are resuspended. If they are not, resuspend as follows.
    a. Pipette to mix or lightly tap the plate on the bench.
    b. Shake at 1800 rpm for 5 minutes.
16. Place on a magnetic stand and wait until the liquid is clear (~2 minutes).
17. Transfer the supernatant from the midi plate to the SGP plate.
18. Centrifuge at 1000 × g for 1 minute.

Note

After denaturation, the libraries are single-stranded DNA, which resolves poorly on an agarose gel or Bioanalyzer chip. For quality control, use the double- stranded DNA saved from step 16 of the cleanup procedure

3.13.2.6 Pool Libraries

Preparation

1. 1.To prepare for the sequencing run, begin thawing reagents according to the instructions for your instrument.
2. If the SGP plate was stored frozen at -25°C to -15°C, thaw at room temperature. Pipette to mix.

Procedure

1. Centrifuge at 1000 × g at 20°C for 1 minute.
2. Label a new Eppendorf tube PAL.

3. Transfer 5 µl of each library from the SGP plate to the PAL tube. Invert to mix.
4. Store unused pooled libraries in the PAL tube and SGP plate at -25°C to -15°C for up to 7 days.

3.13.3 Denaturing and Diluting Libraries and PhiX Control for sequencing

3.13.3.1 Denature and Dilute Libraries Prepare Hybridization Buffer

Prepare Hybridization Buffer

1. Remove the tube of Hybridization Buffer from -25°C to -15°C storage and thaw at room temperature.
2. When thawed, store at 2°C to 8°C until you are ready to dilute denatured libraries.
3. Vortex briefly before use.

Prepare Incubator

1. Preheat the incubator to 98°C.

Dilute Library to Loading Concentration

1. Combine the following volumes of pooled libraries and prechilled Hybridization Buffer in a microcentrifuge tube.

Table 7: Dilute Library to Loading Concentration

Library Pool	Prechilled Hybridization Buffer
2 µl	998 µl
3 µl	997 µl
4 µ	996 µl
l5 µl	995 µl

The total volume is 1 ml.

2. Vortex briefly and then centrifuge at 280 × g for 1 minute.
3. Transfer 250 µl diluted library to a new microcentrifuge tube.

4. Add 250 µl prechilled Hybridization Buffer.
5. Vortex briefly and then centrifuge at 280 × g for 1 minute.

Denature Diluted Library

1. Place the tube on the preheated incubator for 2 minutes.
2. Immediately cool on ice.
3. Leave on ice for 5 minutes.

3.13.3.2 Denature and Dilute PhiX Control

Dilute PhiX to 4 nM

1. Thaw a tube of 10 nM PhiX stock.
2. Combine the following volumes in a microcentrifuge tube.
    a. 10 nM PhiX (10 µl)
    b. RSB (15 µl)

    The total volume is 25 µl at 4 nM.
3. Vortex briefly and then pulse centrifuge.

Note

[Optional] Store the 4 nM PhiX at -25°C to -15°C for up to 3 months.

Denature PhiX

1. Combine the following volumes in a microcentrifuge tube.
    a. 4 nM PhiX (5 µl)
    b. 0.1 N NaOH (5 µl)
2. Vortex briefly and then pulse centrifuge.
3. Incubate at room temperature for 5 minutes.
4. Add 5 µl 200 mM Tris-HCl, pH 7.0.
5. Vortex briefly and then centrifuge at 280 × g for 1 minute.

Dilute Denatured PhiX to Loading Concentration

1. Add 985 µl of prechilled Hybridization Buffer to the tube of denatured PhiX. The total volume is 1 ml at 20 pM.
2. Dilute the denatured 20 pM PhiX to 1.8 pM as follows.

a. Denatured PhiX (45 µl)
b. Prechilled Hybridization Buffer (455 µl)

   The total volume is 500 µl at 1.8 pM.

3. Invert to mix and then centrifuge at 280 × g for 1 minute.

Note

[Optional] Store the denatured 1.8 pM PhiX at -25°C to -15°C for up to 2 weeks. After 2 weeks, cluster numbers tend to decrease.

Combine Library and PhiX Control

1. Combine the following volumes.

PhiX Control and Library	Volume
Denatured and diluted PhiX control	5 µl
Denatured and diluted library	500 µl

2. Set aside on ice until you are ready to load it onto the reagent cartridge.

Note

The library and PhiX mixture provides a PhiX spike-in of 0.5%–2.0%. Actual PhiX percentage varies depending upon the quality and quantity of the library pool.

3.13.4 Preparing Sequencing Consumables

Prepare the Reagent Cartridge

1. Remove the reagent cartridge from -25°C to -15°C storage.
2. Thaw reagents using the following water bath options. Do not submerge the cartridge. When thawed, dry the base before proceeding.

Method	Time to Thaw	Stability Limit
37°C water bath	35 minutes	Up to 2 hours
Room temperature water bath (19°C to 25°C)	90 minutes	Up to 24 hours

If multiple cartridges are thawing in the same water bath, allow additional thawing time. Alternatively, thaw reagents using the following options.

Method	Time to Thaw	Stability Limit
Room temperature air (19°C to 25°C)	5 hours	Up to 24 hours
Chilled at 2°C to 8°C	18 hours	Up to 72 hours

3. Invert the cartridge 5 times to mix reagents.
4. Inspect the large reservoirs from the bottom of the cartridge to make sure that reagents have thawed and the reservoirs are free of ice crystals.
5. Gently tap on the bench to reduce air bubbles.

Prepare the Flow Cell

1. Remove a new flow cell package from 2°C to 8°C storage.
2. Set the unopened flow cell package aside at room temperature for 30 minutes.

Note

Avoid repeated cooling and warming of the flow cell.

3. Remove the flow cell container from the foil package.
4. Put on a new pair of powder-free gloves.
5. Grip the flow cell by the plastic cartridge and remove it from the container.
6. Clean the glass surface of the flow cell with a lint-free alcohol wipe.
7. Dry with a lint-free lens cleaning tissue. Use care around the black flow cell gasket.
8. Inspect the flow cell ports for obstructions. Make sure that the gasket is well-seated.

3.13.5 Performing a Run on the MiniSeq

3.13.5.1 Load Libraries onto the Reagent Cartridge

1. Clean the foil seal covering reservoir **#16** labeled **Load library here** using a low-lint tissue.
2. Pierce the seal with a clean 1 ml pipette tip.
3. Add 500 µl prepared 1.8 pM libraries into reservoir **#16**. Avoid touching the foil seal

as you dispense the libraries.

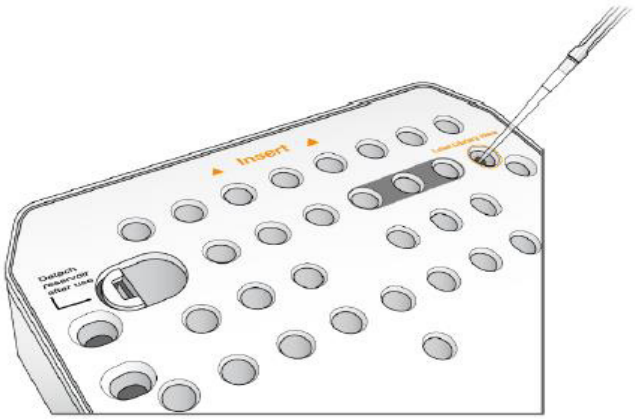


Figure 4: Load Libraries

3.13.5.2 Set Up a Run

1. From the Home screen, select **Sequence**.

The Sequence command releases consumables from a previous run and opens the series of run setup screens.

3.13.5.3 Log in to Local Run Manager

1. Enter your user name and password.
2. Select **Next**.

3.13.5.4 Select Available Run

1. Select a run name from the list of available runs.

   Use the up and down arrows to scroll through the list or enter a run name in the Search field.
2. Select **Next**.

3.13.5.5 Load the Flow Cell

1. Open the flow cell compartment door.
2. Press the release button to the right of the flow cell latch.
3. If present, remove the used flow cell from a previous run.
4. Make sure that the flow cell stage is clean. If debris is present, clean the flow cell stage with an alcohol wipe.
5. Place the flow cell on the flow cell stage over the alignment pins.

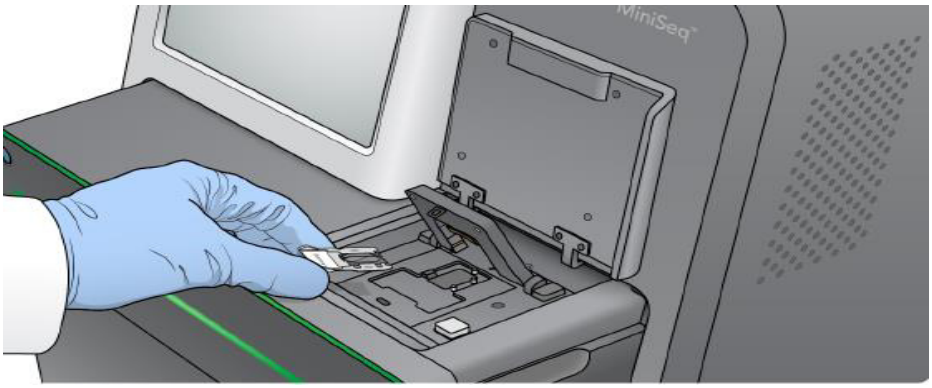


Figure 5: Place Flow Cell on Stage

6. Close the flow cell latch to secure the flow cell.
7. Close the flow cell compartment door.

3.13.5.6 Load the Reagent Cartridge

1. Open the reagent compartment door.
2. If present, remove the used reagent cartridge.

Note

To facilitate safe disposal of unused reagent containing formamide, the reservoir in position #9 is removable.

3. Slide the reagent cartridge into the reagent compartment until the cartridge stops.

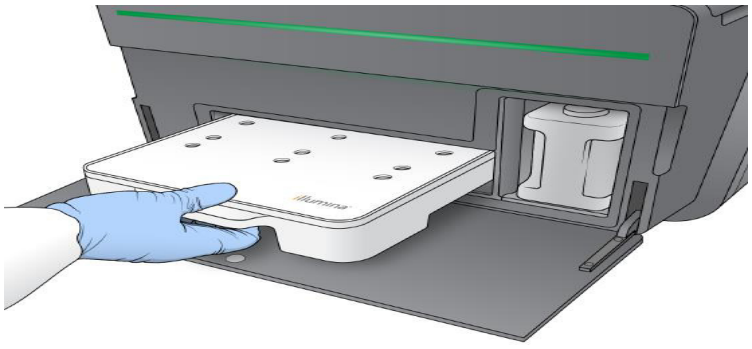


Figure 6: Load Reagent Cartridge

3.13.5.7 Confirm Run Parameters

1. Confirm run parameters.

The control software confirms the number of specified cycles using the following criteria:

- Total cycles do not exceed the maximum cycles allowed based on the reagent cartridge loaded for the run.
- Cycles for Read 1 are greater than the 5 cycles required for template generation.
- Index Read cycles do not exceed Read 1 and Read 2 cycles.

Note

Make sure that you specify the appropriate number of Index Read cycles for the libraries that you are sequencing. For more information, see the library prep documentation.

2. [Optional] Select **Edit** to change run parameters. When finished, select **Save**.
    - **Purge consumables for this run**—Change the setting to purge consumables automatically after the current run.
    - **Run parameters**—Change the read type or number of cycles per read.
    - **Custom primers**—Change the settings for custom primers.
3. Select **Next**.

Note

Do not open the reagent compartment door or the flow cell compartment door during the automated check or during the sequencing run.

4. When the automated check is complete, select **Start**.

3.13.5.8 Review Automated Check

1. Review the automated check results.
    - To stop a check in progress, select **Cancel**.
    - For any items that do not pass, an action is required before you can proceed.
    - To restart the check, select **Retry**. The check resumes at the first incomplete or failed check.
2. To start the run, select from the following options.
    - If the system is not configured to start automatically after a successful

check, select **Start**.

- If the system is configured to start automatically after a successful check, the sequencing run begins automatically. You do not have to be present. However, if any errors occur during the check, the run does not begin automatically.

3.13.5.9 Monitor Run Progress

1 Monitor run progress, intensities, and quality scores as metrics appear on the screen.

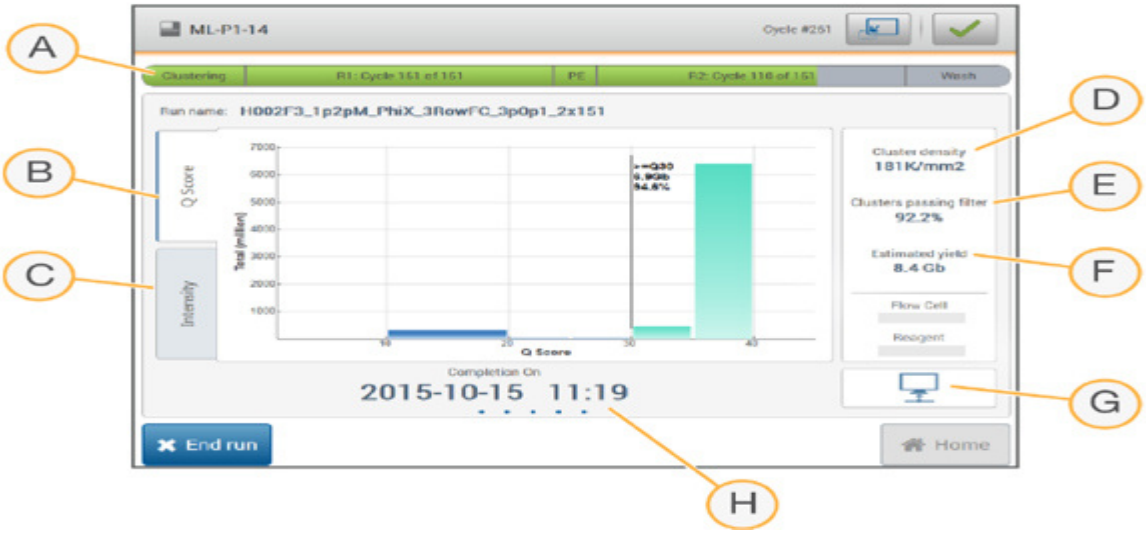


Figure 7: Sequencing Run Progress and Metrics

A. **Run progress**—Shows the current step and number of cycles completed for each read. The progress bar is not proportional to the run rate of each step.

B. **Q-Score**—Shows the distribution of quality scores (Q-scores).

C. **Intensity**—Shows the value of cluster intensities of the 90th percentile for eachtile. Plot colors indicate each base: red is A, green is C, blue is G, and black is T.

D. **Cluster density (K/mm²)**—Shows the number of clusters detected for the run.

E. Clusters passing filter (%)—Shows the percentage of clusters passing filter.

F. Estimated yield (Gb)—Shows the number of bases projected for the run.

G. Data transfer status—Shows the status of data transfer based on analysis configuration.

H. Time to completion—Shows the run completion date and time (yyyy-mm-dd hh:mm).

3.13.6 Viewing Analysis Results in Local Run Manager

3.13.6.1 View Analysis Results

1. From the Local Run Manager dashboard, select the run name.
2. From the Run Overview tab, review the sequencing run metrics.
3. To change the analysis data file location for future requeues of the selected run, select the **Edit** icon, and edit the output run folder file path.The file path leading up to the output run folder is editable. The output run folder name cannot be changed.
4. [Optional] Select the **Copy to Clipboard** icon to copy the output run folder file path.
5. Select the Sequencing Information tab to review run parameters and consumables information.
6. Select the Samples & Results tab to view the analysis report.
    - If analysis was requeued, select the appropriate analysis from the Select Analysis drop-down list.
    - From the left navigation bar, select a sample ID to view the report for another sample.
7. [Optional] Select the **Copy to Clipboard** icon to copy the Analysis Folder file path.

3.13.6.2 Analysis Report

Indexing

Table 8: Indexing Table

Column Heading	Description
Index Number	An assigned ID based on the order that samples are listed in the sample table.
Sample ID	The sample ID provided when the run was created.
Index 1 (i7)	The Index 1 adapter used with the sample.
Index 2 (i5)	The Index 2 adapter used with the sample.

Most Popular Index Sequences

Table 9: Most Popular Index Sequences

Column Heading	Description
Sequence	Shows the sequence of the 100 most popular index sequences.
Reverse Complement	Shows the reverse complement of the original sequences.
Hit Counts	Lists the number of times the sequence was counted.

3.13.6.3 Analysis Output Files

The following analysis output files are generated for the Generate FASTQ analysis module.

Table 10: Analysis Output Files

File Name	Description
Demultiplexing (*.demux)	Intermediate files containing demultiplexing results.
FASTQ (*.fastq.gz)	Intermediate files containing quality scored base calls. FASTQ files are the primary input for the alignment step.

3.13.7 Genome analysis using by Basespace Illumina

3.13.7.1 FastQC analysis

Most sequencers will generate a QC report as part of their analysis pipeline, but this is usually only focused on identifying problems which were generated by the sequencer itself. FastQC aims to provide a QC report which can spot problems which originate either in the sequencer or in the starting library material. FastQC can be run in one of two modes. It can either run as a stand alone interactive application for the immediate analysis of small numbers of FastQ files, or it can be run in a non-interactive mode where it would be suitable for integrating into a larger analysis pipeline for the systematic processing of large numbers of files.

It provides the length of the shortest and longest sequence in the set. If all sequences are the same length only one value is reported %GC: The overall %GC of all bases in all sequences. It seems to be optimum range of %GC (40 to 70) [91].

3.13.7.2 ASSEMBLY IN SPAdes (V. 3.9.0)

(1) Stage 1 (assembly graph construction) is addressed by every NGS assembler and is often referred to as de Bruijn graph simplification. We propose a new approach to assembly graph construction that uses the multisized de Bruijn graph, implements new bulge/tip removal algorithms, detects and removes chimeric reads, aggregates biread information into distance histograms, and allows one to backtrack the performed graph operations [92].
(2) Stage 2 (k-bimer adjustment) derives accurate distance estimates between k-mers in the genome (edges in the assembly graph) using joint analysis of distance histograms and paths in the assembly graph.
(3) Stage 3 constructs the paired assembly graph, inspired by the PDBG approach.
(4) Stage 4 (contig construction) was well studied in the context of Sanger sequencing. Since NGS projects typically feature high coverage, NGS assemblers generate rather accurate contigs (although the accuracy deteriorates for SCS) [92]. SPAdes constructs DNA sequences of contigs and the mapping of reads to contigs by backtracking graph simplifications.

a3.13.7.3 Prokka Genome Annotation (V. 1.11.0)

Prokka Genome Annotation BaseSpace App compliments our suite of de novo assembly tools. Prokka expects preassembled genomic DNA sequences in FASTA format [93]. Finished sequences without gaps are the ideal input, but it is expected that the typical input will be a set of scaffold sequences produced by de novo assembly software. This sequence file is the only mandatory parameter to the software. It uses de novo assembly FASTA files as an input that can be generated by the SPAdes Genome Assembler.

Output:

- **.gff** This is the master annotation in GFF3 format, containing both sequences and annotations. It can be viewed directly in Artemis or IGV

- **.gbk** This is a standard Genbank file derived from the master .gff. If the input to prokka was a multi-FASTA, then this will be a multi-Genbank, with one record for each sequence
- **.fna** Nucleotide FASTA file of the input contig sequences
- **.faa** Protein FASTA file of the translated CDS sequences
- **.ffn** Nucleotide FASTA file of all the annotated sequences, not just CDS
- **.sqn** An ASN1 format "Sequin" file for submission to Genbank. It needs to be edited to set the correct taxonomy, authors, related publication etc
- **.fsa** Nucleotide FASTA file of the input contig sequences, used by "tbl2asn" to create the .sqn file. It is mostly the same as the .fna file, but with extra Sequin tags in the sequence description lines
- **.tbl** Feature Table file, used by "tbl2asn" to create the .sqn file
- **.err** Unacceptable annotations - the NCBI discrepancy report
- **.log** Contains all the output that Prokka produced during its run. This is a record of what settings you used, even if the --quiet option was enabled
- **.txt** Statistics relating to the annotated features found

3.13.7.4 GENIUS Metagenomics (V.1.1.0)

The GENIUS® Metagenomics application uses CosmosID's acurated genome database and high performance algorithms to provide rapid, accurate, and actionable bacterial identification at the species, subspecies, and/or strain level.

GENIUS identifies multiple bacteria in a whole genome shotgun metagenomic sample and is able to discriminate pathogens from their 'near neighbors' using statistical and computational methods, with no prior assumptions as to what is present in the sample. GENIUS uses raw, unassembled reads as input and matches the sequence against our curated reference database of known pathogens and microbes.

3.13.7.5 Bacterial Analysis Pipeline (V.1.0.4)

The Bacterial Analysis Pipeline app initially predicts the species of bacterial input genomes using a kmer-based approach. Futher, acquired antimicrobial resistance genes are identified using a BLAST-based approach, where the nucleotide sequence of the input genome is compared to the genes in the ResFinder database. Depending on the identified species,

Multilocus Sequence Typing (MLST) is performed, also using a BLAST-based approach [94,95]. If the input genome is recognised as belonging to Enterobacteriaceae or the gram positive bacteria, BLAST is used to search for plasmid replicons using the PlasmidFinder database. Identified plasmids of the incF, IncH1, IncH2, IncI1, IncN, or IncA/C type are further subtyped by plasmid MLST. Finally, identified *Escherichia coli*, *Enterococcus* spp., *Staphylococcus aureus*, and *Listeria* are compared to the Virulence Finder database containing known virulence genes.

3.13.7.6 Annotating a Genome Using RAST (Rapid Annotation using Subsystem Technology)

The basic steps used to annotate a genome using RAST are described in the subsections below. Input to the process is a prokaryotic genome in the form of a set of contigs in FASTA format. As described below, the actual RAST server will allow a user to specify a set of gene calls, but in the usual case RAST will make its own calls.The service is freely available for the annotation of prokaryotic genomes. The genomes may be "complete" or they may be in hundreds of contigs (which does impact the quality of the derived annotations). A new user must *register* for the service, which involves giving us contact information and acquiring a password. By registering users, we can create a framework in which users have access to only those genomes that they have submitted. It allows us also to contact the user once the automatic annotation has finished or in case user intervention is required. After login the user can monitor his/her submitted job/ jobs on the Job Overview page. After the annotation is complete the user can choose to download the annotated genome in a variety of export formats (e.g. GenBank, FASTA, GFF3, Excel) or browse the genome in the comparative environment of the SEED Viewer without having the data actually installed in the SEED [96]. These options remain for 120 days or until the data are deleted by the user. If desired, the user can request to have the annotated genome added to the SEED. The SEED-Viewer environment presents the user with a variety of options for the immediate analysis of the annotated genome. The Organism Overview page contains basic information on the Genome such as Taxonomy, Size,The number of Contigs, the Number of Coding Sequences and RNAs and counts of non-hypothetical and hypothetical gene annotations. In addition it contains the Number of Subsystems that were automatically determined to be present in the genome [96].

3.13.7.7 Average nucleotide identity (ANI)

Average nucleotide identity (ANI) is a category of computational analysis that can be used to define species boundaries of Archaea and Bacteria. Calculating ANI usually involves the fragmentation of genome sequences, followed by nucleotide sequence search, alignment, and identity calculation. The original algorithm to calculate ANI used the BLAST program as its search engine. An improved ANI algorithm was developed to accommodate the concept of orthology. Here compared four algorithms to compute ANI, namely ANIb (ANI algorithm using BLAST), ANIm (ANI using MUMmer), OrthoANIb (OrthoANI using BLAST). By comparing values to the ANIb that is considered a standard, OrthoANIb and OrthoANIu exhibited good correlation in the whole range of ANI values. ANIm showed poor correlation for ANI of <90% [97].

For average nucleotide identity based on MUMmer (ANIm) calculation, the dnadiff script of the MUMmer tool version 3.0 is used instead of a self-written parser used by the original JSpecies stand-alone implementation.

3.13.17.8 Venn diagram analysis

Venn Diagram is a diagram used in set theory, showing the distribution of the number of elements between 3 sets (4 or more sets can be drawn) It is elements belonging to each set are shown in the circle, and overlapping parts are shown as common elements. It is used for biological applications to illustrate the number of orthologous or unique genes between closely related genomes. In Venn diagram analysis usually used Genebank, fasta file format for genomic comparison. This analysis was done by (http://bioinformatics.psb.ugent.be/webtools/Venn/) web base software [98].

3.13.7.9 Pangenome analysis using by CGView Server

The CGView Server generates graphical maps of circular genomes that show sequence features, base composition plots, analysis results and sequence similarity plots. Sequences can be supplied in raw, FASTA, GenBank or EMBL format. Additional feature or analysis information can be submitted in the form of GFF (General Feature Format) files. The server uses BLAST to compare the primary sequence to up to three comparison genomes or sequence sets. The BLAST results and feature information are converted to a graphical map showing the entire sequence, or an expanded and more detailed view of a region of interest. Several options are included to control which types of features are displayed and how the

features are drawn [99]. The CGView Server can be used to visualize features associated with any bacterial, plasmid, chloroplast or mitochondrial genome, and can aid in the identification of conserved genome segments, instances of horizontal gene transfer, and differences in gene copy number. Because a collection of sequences can be used in place of a comparison genome, maps can also be used to visualize regions of a known genome covered by newly obtained sequence reads. The CGView Server can be accessed at http://stothard.afns.ualberta.ca/cgview_server/ [100]. GC skew was defined as the normalized excess of C over G in a given sequence, (C −□G)/(C + G), which is calculated with sliding windows along the genome. GC skew is defined to be 0 when the amount of C equals that of G. To eliminate the use of window slides, cumulative skew can be calculated as the cumulative sum of the walker graph score at each nucleotide position along the genome, with scores A = 0, T = 0, G = 1, and C = −1 [101]. In this work, however, the cumulative GC skew was calculated by taking the cumulative sum of the GCskew in each of the windows, to normalize the cumulative skew strength without it being affected by the length of the genome. GC skew = (G − C)/(G + C)

Chapter IV
RESULT AND DISCUSSION

This section shows all the results including isolation of endopytic bacteria from tomato *(Lycopersicon esculentum*) root and their biochemical characterization, 16S rRNA gene sequencing, nitrogen fixation, phosphate solubilizing, Indole-3 acetic acid (IAA) production, I-amino-cyclopropane-l-carboxylate (ACC) deaminase activity pesticide mineralizing test and application of these isolates on eggplant to ensure its plant growth promoting activity. After finishing all experiments CS-T.R.3 strain take undergoes for NGS sequencing to ensure plant growth promoting genes The results are presented in this chapter under the following headings.

1.1 Isolation of pesticide degrading endophytic bacteria

One pesticide degrading bacteria was isolated from tomato root. The pesticide degradable endophytic bacteria from tomato root was first grown on PVK agar media and the isolate was transferred from PVK agar media to pesticide containing normal growth media. The colony formation on that pesticide containing plates confirms the biodegradation of pesticides.

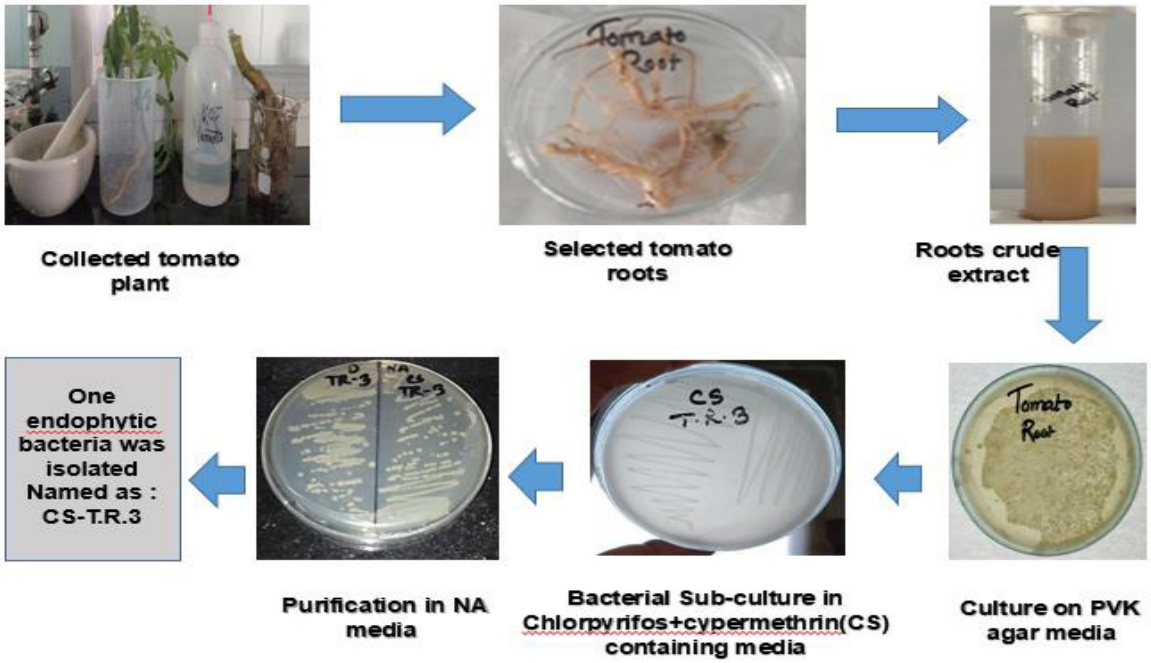


Figure 8: Isolation and purification of pesticide degrading endophyte from tomato root

Figure 9: Pesticide degradation capacity of endophytic bacteria from Tomato root.

Here, CS=Chlorpyrifos + cypermethrin, T.R= Tomato root.

Because of the texture of pesticide in growth media the bacterial colony is quite unclear. The purified colony of these CS-T.R.3 bacteria, these was grown on Nutrient agar plate.

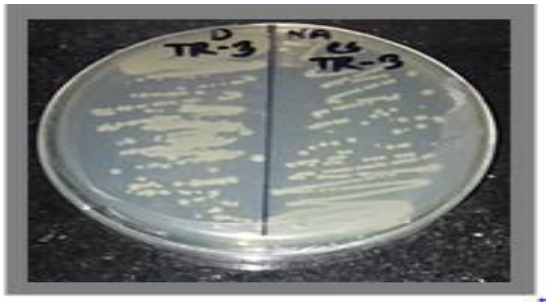


Figure 10: Purification of pesticide degrading isolate on Nutrient Agar (NA) media.

Here, CS=Chlorpyrifos + cypermethrin, T.R= Tomato root.

4.2 KOH string test

A loopful of growth from a bacterial colony was emulsified on the surface of a glass slide in a suspension of 3% KOH. The suspension was stirred continuously for 60 seconds after which the loop was gently pulled from the suspension. The test was considered positive if string occurred within the first 30 seconds after mixing the bacteria in KOH solution [79].

CS-T.R.3 isolate is gram negative bacteria because string formation was occurred in this isolate after stirring these with 3% KOH solution on glass slide.

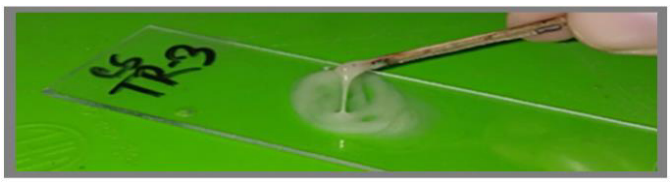


Figure 11: KOH test of endophytic bacteria from tomato root.

4.3 Biochemical Test

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 and the results of the isolates are given below in table:

Table 11. List of performed biochemical test

Performed biochemical tests
• MR-VP test • Catalase test, • Oxidase test, • Triple Sugar Iron (TSI) test, • Citrate utilization test (CIU), • Motility Indole Urease (MIU) Test • Urease test and • Individual sugar fermentation/carbohydrate tests (dextrose, lactose, maltose and sucrose).

4.3.1 Methyl Red-Voges-Proskauer Test/ (MR-VP) Test

MR-VP Test was performed for identifying an organism's metabolic pathway. Methyl Red (MR) test determines whether the microbe performs mixed acids fermentation when supplied glucose. Types and proportion of fermentation products produced by anaerobic fermentation of glucose is one of the key taxonomic characteristics which help to differentiate various genera of enteric bacteria. The development of a stable red color in the surface of the medium indicates sufficient acid production to lower the pH to 4.4 and constitutes a positive test. Because other organisms may produce smaller quantities of acid from the test substrate, an

intermediate orange color between yellow and red may develop. This does not indicate a positive test.

The MR results are shown below:

Figure 12: Methyl Red test of CS-T.R.3 isolate.

The isolate CS-T.R.3 was showed positive result. Some fermenting bacteria undertake the butylene glycol pathway in the fermentation of glucose. These organisms do produce some organic acids but the chief end product of glucose fermentation is 2-3 butylene glycol (2-3 butanediol), a neutral product. However, the VP test detects an intermediate product, the acetyl methyl carbinol (acetoin). Upon the addition of KOH, acetoin is oxidized to diacetyl, which then reacts with the guanidine group of arginine (contained in the peptone) to give rise to a red-coloured product. Following the principle of MR-VP test as stated by [102, 103], VP test result of CS-T.R.3 isolate is negative because of isolate is not capable of producing acetoin.

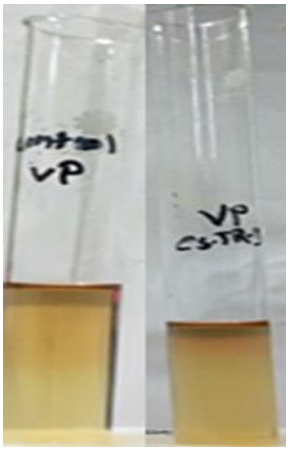


Figure 13: Voges Proskauer (VP) test of CS-T.R.3 isolate

4.3.2 Catalase test

The catalase test differentiates staphylococci (catalase-positive) from streptococci (catalasenegative). The enzyme, catalase, is produced by bacteria that respire using oxygen, and protects them from the toxic by-products of oxygen metabolism. Bacteria which can produce catalase enzyme are strict aerobes as well as facultative anaerobes, although they all have the ability to respire using oxygen as a terminal electron acceptor. Catalase-negative bacteria may be anaerobes, or they may be facultative anaerobes that only ferment and do not respire using oxygen as a terminal electron acceptor [82].

CS-T.R.3 isolated bacteria can produce catalase enzyme that was ensured through formation of bubble after using hydrogen peroxide. The figure is given below;

Figure 14: Catalase enzyme producing capability of CS-T.R.3 isolate.

4.3.3 Oxidase Test

The oxidase test detects the presence of a cytochrome oxidase system that will catalyse 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 [80].

Figure 15: Oxidase enzyme producing capability of CS-T.R.3 isolate

Blue color formation after application of a drop of oxidase reagent (1% tetramethyl-pphenylenediaminedi hydrochloride) is the indication of oxidase positive.But CS-T.R.3 showed no color formation.so,it was oxidase negative.

4.3.4 Triple Sugar Iron (TSI) test

This test is used to identify the capability of bacteria to ferment 3 sugars including lactose, sucrose, glucose and iron. If lactose (or sucrose) is fermented, a large amount of acid is produced, which turns the phenol red indicator yellow both in 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 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 production of ammonia from the oxidative deamination of amino acids (remember peoptone is a major constituent of TSI agar) [83]. According to the principle, CS-T.R.3 isolate bacteria can ferment glucose and produce gas.

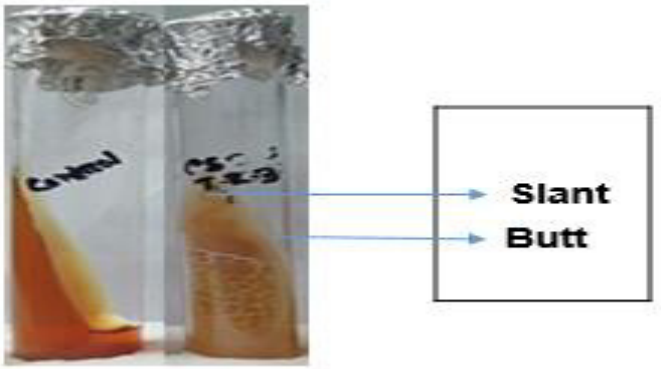


Figure 16: Triple Sugar Iron (TSI) test result of CS-T.R.3 isolate

4.3.5 Motility Indole Urease (MIU) Test

Motility Indole Urea (MIU) medium base is used for this test to differentiate the Enterobacteriaceae. This medium is based on motility, urease and indole production. This medium contains peptones that provide carbon and nitrogen required for growth of bacteria. Urea provides a source of nitrogen for those microorganisms producing urease. This is indicated by a color change of the pH indicator, Phenol Red, from yellow – orange, (pH 7.0) to red – pink, (pH 8.1). The low agar concentration allows the demonstration of motility. The

indole Cap is impregnated with indole reagent for the detection of indole production, shown by the appearance of a pink – mauve colour in the bottom of the cap [80]. Motility Indole Urea (MIU) test result of CS-T.R.3 isolate is given below;

Figure 17: Motility Indole Urea (MIU) test result of CS-T.R.3 isolate.

According to the principles CS-T.R.3 isolate was shown motility and urease positive result that was further confirmed by urease test that is described below in 4.3.6 no. point.

4.3.6 Urease test

The urease test was done by using urea agar base. Urea provides a source of nitrogen for those microorganisms producing urease enzyme. Organisms containing urease hydrolyses urea into ammonia and CO_2. With formation of ammonia 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 result due to the presence of phenol red indicator in the urea agar media [104]. This is indicated by a color change of the pH indicator, Phenol Red, from yellow- orange, (pH 7, 0) to red– pink, (pH 8.1).The urease test result is given below;

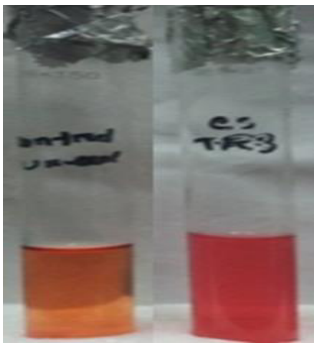


Figure 18: Urease test result

CS-T.R.3 bacteria showed positive result as there was confirmed through color changing from yellow –orange to red.

4.3.7: Citrate utilization test (CIU)

The citrate test is utilized to identify the ability of the bacterial isolate to utilize citrate as a carbon and energy source. This test is basically necessitates in the identification of gram negative bacteria based on their metabolic product since gram negative isolates contain citrate-permease that uses citrate present in the medium as a carbon source of energy. This test is also used to distinguish between members of the Enterobacteriaceae family. A positive CIU test will rely on the generation of alkaline by-products of citrate metabolism which will subsequently increase the pH of the medium demonstrated by the color change of a pH indicator. Citrate enters into the cell and is cleaved by citrate lyase releasing oxaloacetate and acetate after which 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. This test is used to determine the ability of bacteria to utilize sodium citrate as its only carbon source and inorganic ammonium di-hydrogen phosphate ($NH_4H_2PO_4$) is the sole fixed nitrogen source [105].

The CIU test result of CS-T.R.3 isolate is given below in following figure:

Figure 19: Citrate utilization (CIU) test

According to the principles CS-T.R.3 isolate was positive that was confirmed through color changing from green to blue which was compared with a control.

Biochemical characterization of CS-T.R.3 isolate is given below in following table:

Table 12: Biochemical characterization of CS-T.R.3 isolate

Sample name	MR	VP	Catalase	Oxidase	TSI	CIU	MIU	Urease
CS-T.R.3	+	-	+	-	+	+	+	+

4.3.8 Carbohydrate fermentation test (lactose, Sucrose, dextrose and Maltose)

The carbohydrate fermentation test is used to determine whether or not bacteria can ferment a specific carbohydrate. Carbohydrate fermentation patterns are useful in differentiating among bacterial groups or species. It tests for the presence of acid and/or gas produced from carbohydrate fermentation. Four different sugars were used in this test. These were lactose, Sucrose, dextrose and Maltose.

The test result of CS-T.R.3 isolate in these 4 types of sugar fermentation is given in following figure:

(a) (b) (c) (d)

Figure 20: Carbohydrate fermentation test. (a) lactose, (b) dextrose, (c) maltose and (d) sucrose.

The utilization of lactose, dextrose, maltose and sucrose were studied by using fermentation broth in test tubes.CS-T.R.3 can ferment 4 types of sugar and the results are presented in table 13 and figure 20.

Table 13: Carbohydrate utilization test of CS-T.R.3 isolate

Sample name	Lactose	Dextrose	Maltose	Sucrose
CS.T.R.3	+	+	+	+

4.4 Characterization of endophytic bacteria isolate for Plant Growth Promotional (PGP) traits

4.4.1 Quantitative analysis of Indole-3 acetic acid (IAA) production

Organic substances capable of regulating plant growth produced either cndogeneously or applied exogeneously are called plant growth regulators. They regulate growth by affecting physiological and morphological processes at very low concentrations [65]. Sevcral microorganisms arc capable of producing auxins, cytokinins, gibberilins, ethylene or abscisic acid. Bacteria synthesize indole acetic acid (IAA) predominantly by an alternate tryptophan-dependant pathway, through indole pyruvic acid. However, the role of bacterial IAA in plant growth remains undetermined. Promotion of root growth is one of the major function of IAA. The isolates were tested for quantitative IAA production. Isolates producing reddish pink colour reaction with Salkowaski‟s reagent indicated their ability to produce IAA. The result of IAA production test of CS-T.R.3 isolate is given below in following figure:

Figure 21: Quantitative IAA production test of endophytic bacteria from tomato root.

In figure 14, CS-T.R.3 showed strong reddish pink color comparing with control at the left side indicating higher production of IAA. According to the result, the maximum IAA production was observed in CS- T.R.3 (6.563 mg/ml).

4.4.2 Quantitative analysis of I-amino-cyclopropane-l-carboxylate (ACC) deaminase activity test

A number of PGPB contain the enzyme I-amino-cyclopropane-l-carboxylate (ACC) deaminase plays a vital role in plant growth promotion and this enzyme catalyzes the degradation of ACC, the immediate precursor of the plant hormone ethylene into α-ketobutyrate and ammonia in the biosynthetic pathway for ethylene in plants. ACC deaminase activity can decrease ethylene production in the roots of host plants which results in root lengthening. For most of the plants, ethylene is very essential to break seed dormancy but after germination, high-level ethylene would inhibit root elongation. This enzyme also plays significant role in sustaining plant growth and development under biotic and abiotic stress conditions by reducing stress induced ethylene production in plants [69, 70]. CS-T.R.3 bacterial isolate was tested for quantitative ACC deaminase activity test. The result of ACC deaminase activity test of CS-T.R.3 isolate is given below in following figure:

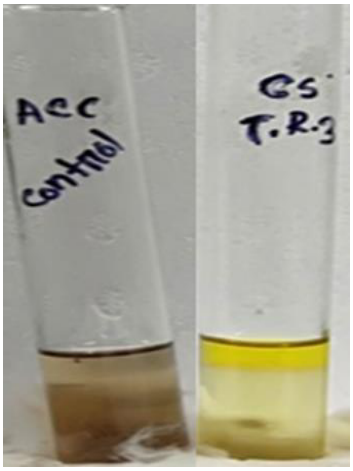


Figure 22: Quantitative estimation of ACC deaminase activity test of endophytic bacteria.

The absorbance of the isolate including control at 600nm for spectrophotometric analysis is given below in following table;

Table 14: Quantitative estimation of ACC-deaminase production

Sample name	Negative control	Sample absorbance with ACC	Positive control -$(NH_4)_2SO_4$	Sample absorbance with $(NH_4)_2SO_4$
CS.T.R.3	0.067	0.076	0.109	0.076

According to result, ACC deaminase activity was observed in CS-T.R.3 (Absorbance 0.076 where the absorbance of negative control is 0.067 and positive control is 0.109). This indicated the ability of the isolates to use ACC as a sole nitrogen source for their metabolic activities due to the presence of ACC deaminase activity.

4.4.3 Qualitative analysis of phosphate solubilizing test

Phosphorus is so important element that is required for plant growth which does not exist as elemental form in the soil. Phosphorus in the soil solution exists as insoluble inorganic phosphorus and insoluble organic phosphorus. Phosphate solubilizing microorganisms (PSMs) are group of beneficial microorganisms capable of hydrolyzing organic and inorganic phosphorus compounds from insoluble compounds. Soil is a natural basal media for microbial growth. Mostly, one gram of fertile soil contains 10^1 to 10^{10} bacteria, and their live weight may exceed 2,000 kg ha^{-1}. Among the whole microbial population in soil phosphorus, solubilizing bacteria comprise 1–50% [106].

Bacteria isolate was grown in PVK (Pikovskaya agar) media which relies on the use of calcium phosphate and numerous inorganic components for the screening of phosphate solubilizing bacteria.

The result of phosphate solubilizing capability of the CS-T.R.3 isolate is given below in following figure:

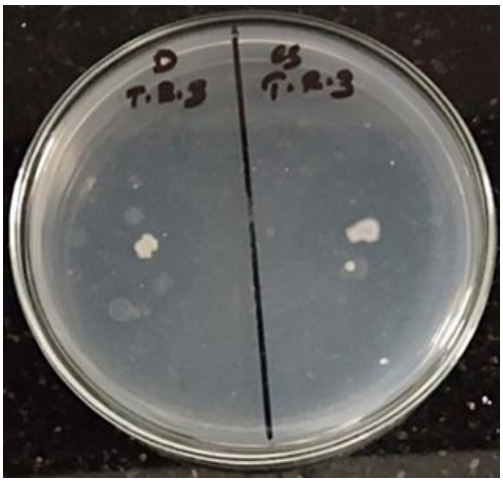


Figure 23: Qualitative analysis of phosphate solubilizing test

The CS-T.R.3 isolate could utilize calcium phosphate and was grown in that media. It is capable of solubilizing phosphate.

4.4.4 Qualitative analysis of nitrogen fixation capability of endophytic bacteria

Nitrogen is a major component of chlorophyll, the most important pigment needed for photosynthesis, as well as amino acids, the key building blocks of proteins which is a critical limiting element for plant growth and production and it is also found in other important biomolecules, such as ATP and nucleic acids. Even though it is one of the most abundant elements (predominately in the form of nitrogen gas (N_2) in the Earth's atmosphere), plants can only utilize reduced forms of this element [107].

Biological nitrogen fixation (BNF) is the conversion of atmospheric N_2 to NH_3, a form that can be used by plants. However, the process is restricted to bacteria and archaea and does not occur in eukaryotes. Symbiotic nitrogen fixation is part of a mutualistic relationship in which plants provide a niche and fixed carbon to bacteria in exchange for fixed nitrogen. The endophytic bacteria fix atmospheric dinitrogen (N_2) in plants through colonization into the host plant root system and cause the roots to form nodules to house the bacteria that acts as the factories of ammonia production. The bacteria then begin to fix the nitrogen required by the plant. Access to the fixed nitrogen allows the plant to produce leaves fortified with nitrogen that can be recycled throughout the plant. This allows the plant to increase photosynthetic capacity, which in turn yields nitrogen-rich seed [26].

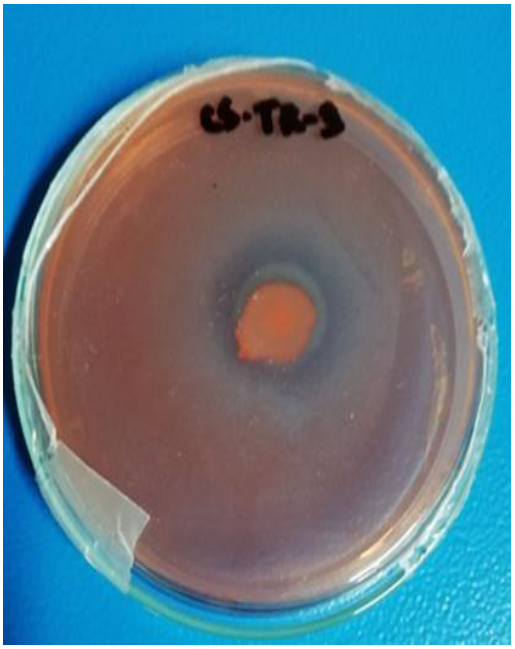


Figure 24: Qualitative analysis of nitrogen fixation capability of endophytic bacteria in *Rhizobium* Medium (Yeast Extract Mannitol Agar Medium)

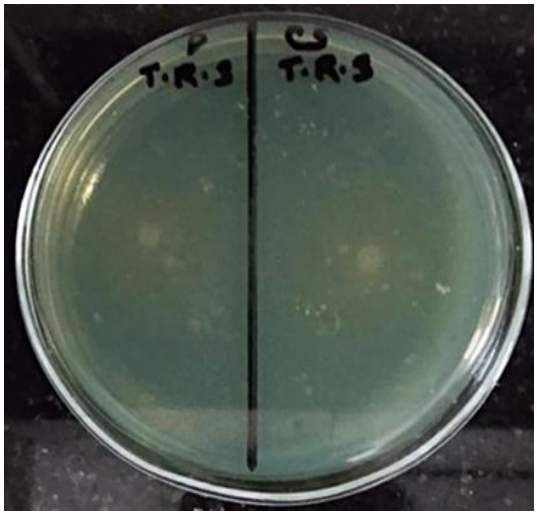


Figure 25: Qualitative analysis of nitrogen fixation capability of endophytic bacteria in *Azotobacter* medium

Isolation of nitrogen-fixing bacteria was carried out on Yeast Extract Mannitol Agar selective media and *Azotobacter* medium. One of the main concerns about this investigation is to isolte and characterize the nitrogen-fixing bacteria from soil sample, tomato and cauliflower root. The growth of bacteria on Yeast Extract Mannitol Agar and *Azotobacter* medium indicate their ability to fix atmospheric nitrogen without association or symbiotically to the plants. The use of Yeast Extract Mannitol Agar and *Azotobacter* medium for the isolation of nitrogen-fixing bacteria has earlier been reported by some researchers [82, 87].

The isolate CS-T.R.3 was showed growth rate and zone formation of nitrogen fixing bacteria in Rhizobium Medium (Yeas Extract Mannitol Agar Medium) are positive by measuring zone of area which showed diameter of halozone (8cm)

4.5 Effect of the CS-T.R.3 bactrial isolate on seedling growth of eggplant on petri dishes in the dark

Seeds were treated with bacterial suspension for 6 hours before inoculating on petri dish. After 7 days of seed inoculation in the petri dish 0.25ml of bacterial suspension was spread on germinating seeds. After 13 days of germination, the effect of the CS-T.R.3 isolate on seedling growth of eggplant was recorded.

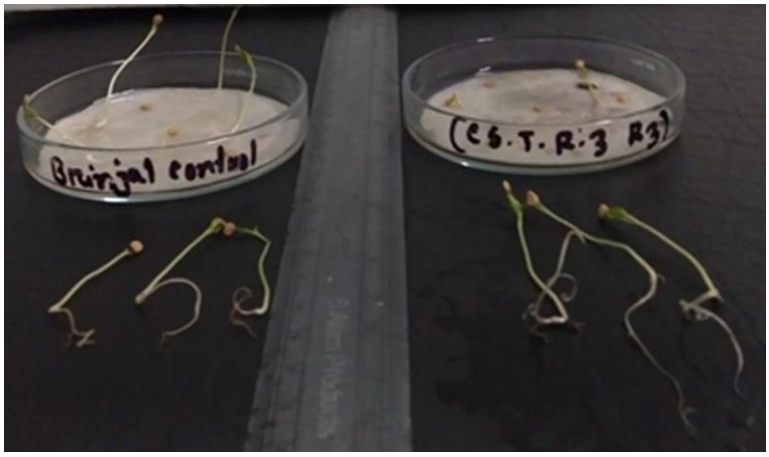


Figure 26(a): Effect of pesticides (Chlorpyrifos and cypermathrine) degrading endophytic bacteria on eggplant growth comparing with control in the left side of this image.

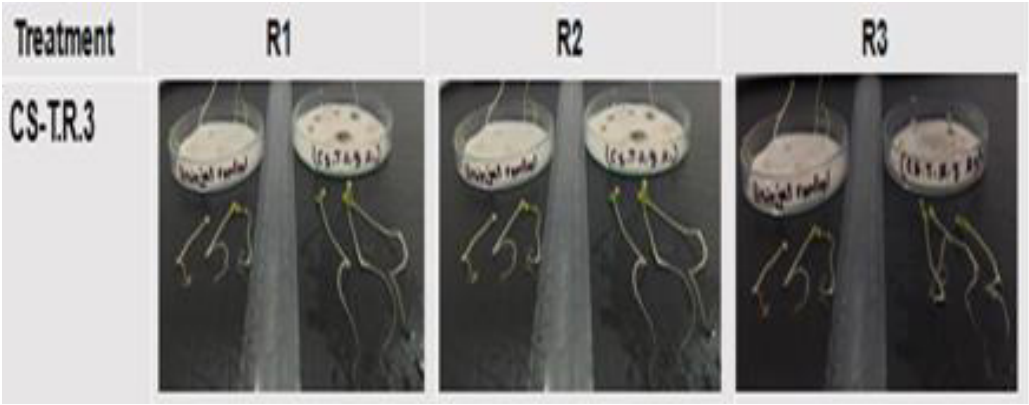


Figure 26(b): Effect of pesticides (Chlorpyrifos and cypermathrine) degrading endophytic bacteria on eggplant growth comparing with control in the left side of this image.

Table 15: Root and shoot length, germination rate and vigor index of eggplant comparing with control after treated with CS-T.R.3 isolate after 13 days of seed germination

Treatment	Germination percentage	Root Length	Shoot Length	Seedling Length	Vigor Index
Control	76.92 ± 7.70^{bcd}	3.48 ± 0.61^{g}	3.09 ± 0.07^{c}	6.57 ± 0.62^{f}	508.30 ± 98.20^{d}
CS-T.R.3	82.05 ± 11.75^{abcd}	4.84 ± 0.47^{f}	3.27 ± 0.21^{c}	8.88 ± 0.69^{e}	734.40 ± 156.11^{abc}

4.6 Pot experiment to ensure growth promoting activity of the isolate

After 7 days of seed germination in the pot 1ml of bacterial suspension were inoculated in each pots and 4th time these seedlings were treated with bacterial suspensions. After 21 days of seedling stage the plant were treated with bacterial stains. Then after the 4th time 10 days later these all plants were treated with bacterial strains. After 40 days of germination the result of plants is given below in following figure:

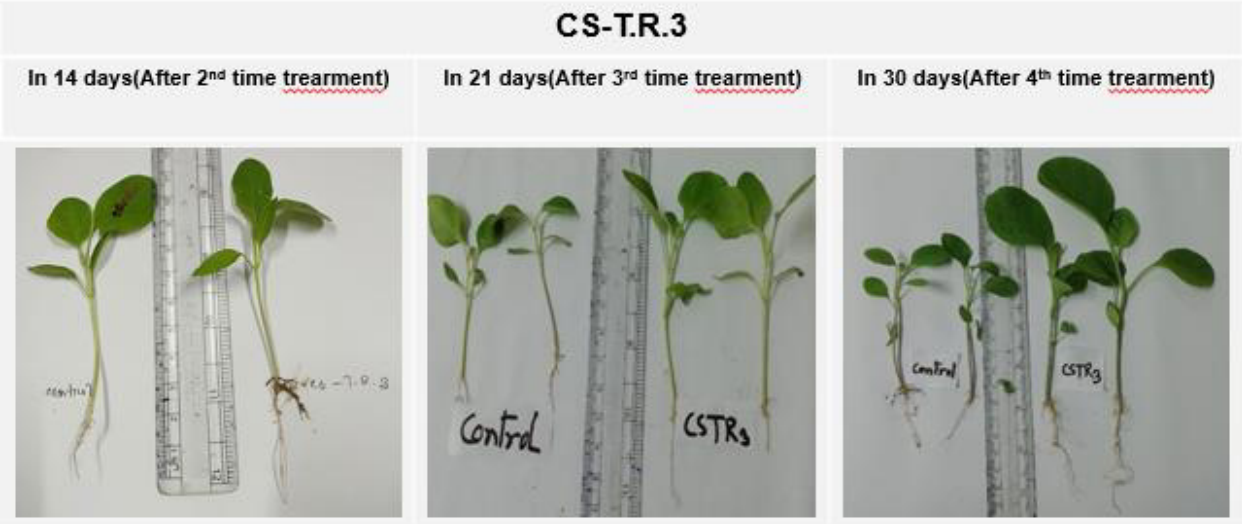


Figure 27(a): Effect of pesticides (Chlorpyrifos and cypermathrine) degrading endophytic bacteria on eggplant growth comparing with control in the left side in each image.

Figure 27(b): Effect of pesticides degrading endophytic bacteria on eggplant growth comparing with control in the left side.

Table 16: Root and shoot length and weight of eggplant comparing with control after treated with endophytic bacteria after 40 days of seed germination

Treatment	Root length	Shoot length	Seedling length	Fresh weight	Dry weight
Control	3.83 ± 0.35^{f}	6.86 ± 0.41^{e}	10.70 ± 0.26^{c}	0.26 ± 0.01^{f}	0.03 ± 0.01^{d}
CS-T.R.3	8.16 ± 1.04^{bcd}	8.83 ± 0.76^{cd}	17.00 ± 1.80^{ab}	0.93 ± 0.12^{cd}	0.19 ± 0.07^{c}

4.7 Pesticide biodegradation in liquid medium

The complete degradation of organophosphates pesticides like chlorpyrifos is not possible due to its structural complexity. The microbial degradation of organic pollutant can be either completely degraded into harmless compounds in mineralization or partial as intermediate metabolites in cometabolism [108]. The biodegradation of organophosphates by bacteria were reported by several researchers as follows: *Pseudomonas aeruginosa, P. putida. Bacillus pumilus, Bacillus cereus*, *Serratia marcescens, Klebsiella* sp., *Alcaligenes* sp., *Alcaligenes faecalis*, Flavobacterium sp., and *Enterobacter* strain B- 14 [109-111]. The bacterial strains were able to utilize chlorpyrifos as the sole source of carbon. These pesticide degradation by bacterial strains was exhibited as a decrease in pesticide concentration that was proportional to increase in bacterial growth [108].

Table 17: Spectrophotometric analysis of chlorpyrifos bio- degradation

Sample name	Absorbance of chlorpyrifos containing bacterial solution (600nm) Absorbance after centrifugation
Chlorpyrifos control	2.365
CS-T.R.3	1.934

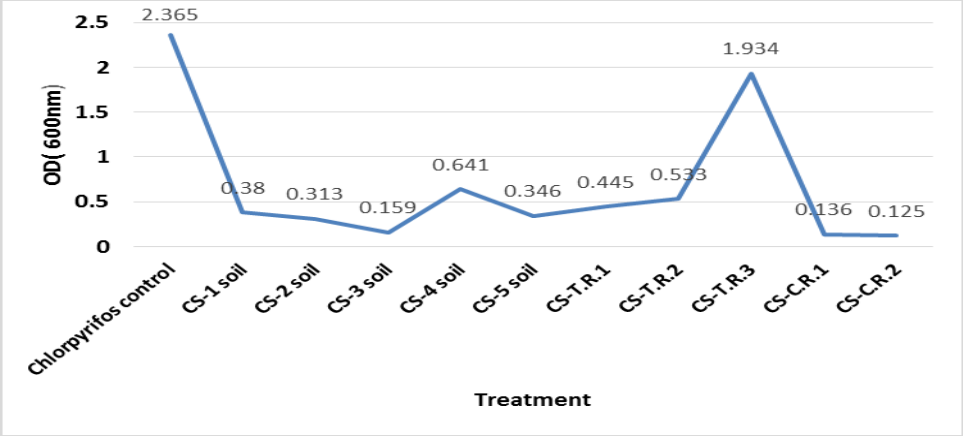


Figure 28: Absorbance of chlorpyrifos containing bacterial solution (600nm) after centrifugation

Figure 29: Chlorpyrifos control (without bacteria in the left) and chlorpyrifos with bacteria in MSM (minimal salt medium)

4.7.1 FTIR analysis

4.7.1.1 FTIR analysis of Chlorpyrifos biodegradation in MSM

During the biodegradation of Chlorpyrifos, The IR spectrum for pure Chloropyrifos showed C-Cl within 600–880 cm^{-1}, C-H stretch at 2991 cm^{-1}, 1, 4- benzene substitution at 889.78 cm^{-1}, the benzene special vibration at 1400-1700 cm^{-1}.[114] In this experiment the IR peaks of chlorpyrifos control (without bacteria in MSM media) were at 673.4 indicating C-Cl bond and the benzene special vibration at 1410 and 1640 cm^{-1} wavelength with intensity 48.96, 87.32 and 76.36 respectively.

In Figure 30, chlorpyrifos control was compared with CS-T.R.3 treated chlorpyrifos. In CS-T.R.3, C-Cl stretching vibration and benzene special vibration were appeared at 648 and 1631cm^{-1} respectively with peak intensity 43.5 and 71 respectively for this two bonds which

are lower than chlorpyrifos control (48.96 and 76.36). In CS-T.R.3 treated chlorpyrifos peak the benzene stretch at 1410cm^{-1} was totally disappeared.

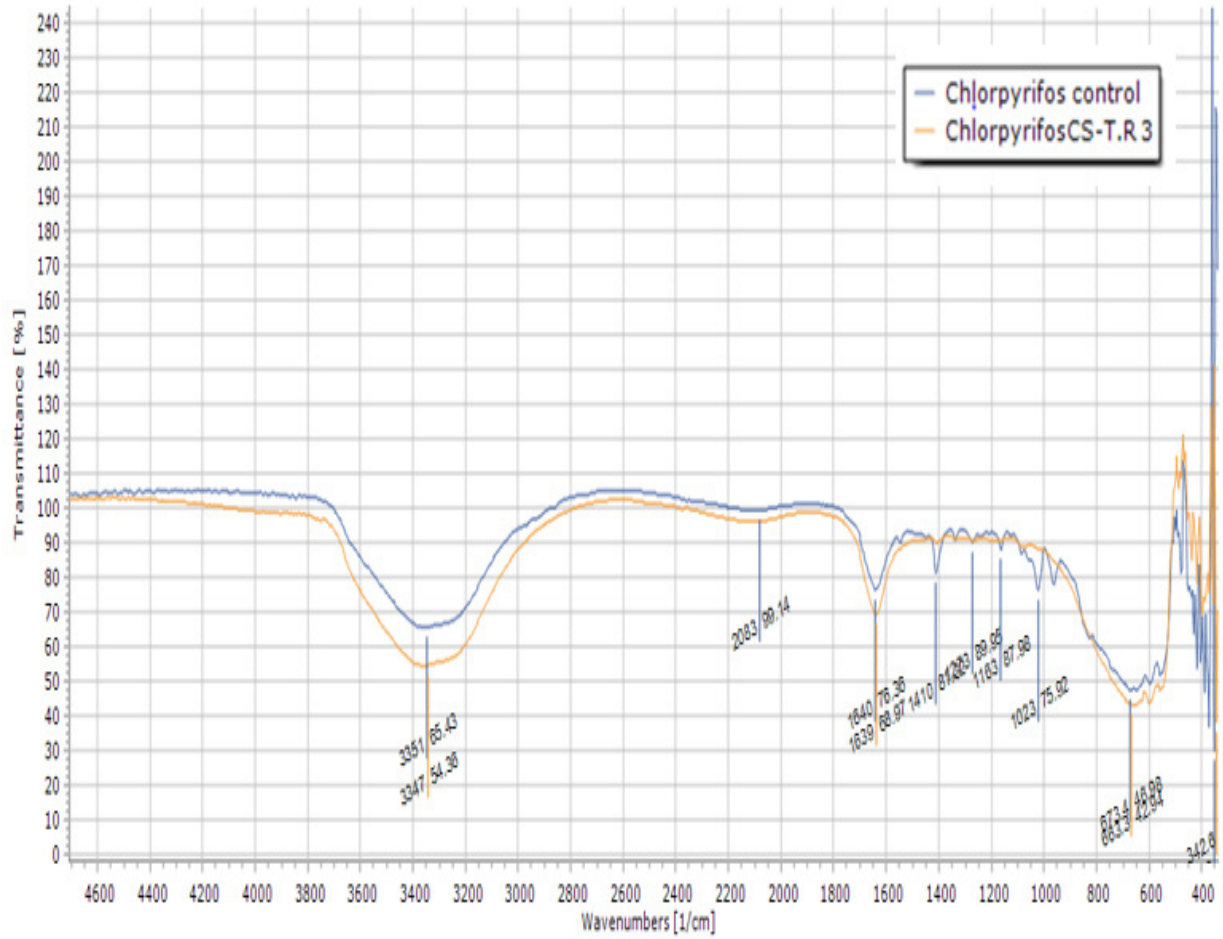


Figure 30: FTIR spectra of chlorpyrifos after in MSM (minimal salt medium) along with the isolates after 21 days of incubation

4.8 NGS analysis using by list of some bioinformatics tools

4.8.1 FastQC analysis

FastQC was analyzed using Basespace (Illumina) web base server. After completing WGS, 2 fastq file have been gotten which name R1 and R2 file. Those file was using check their quality by fastQC analysis, quality depend on GC%. Previous report says the optimum range of GC% (40-70). So HSTU-Sny5 strain was showed 57.8% that indicate sequence quality is good [91].

Table 18: FastQC analysis using Illumina V.1.9

Filename	5_S1_L001_R2_001.fastq.gz
File type	Conventional base calls
Encoding	Sanger / Illumina 1.9
Total Sequences	928055
Filtered Sequences	0
Sequence length	35-151
%GC	57.8

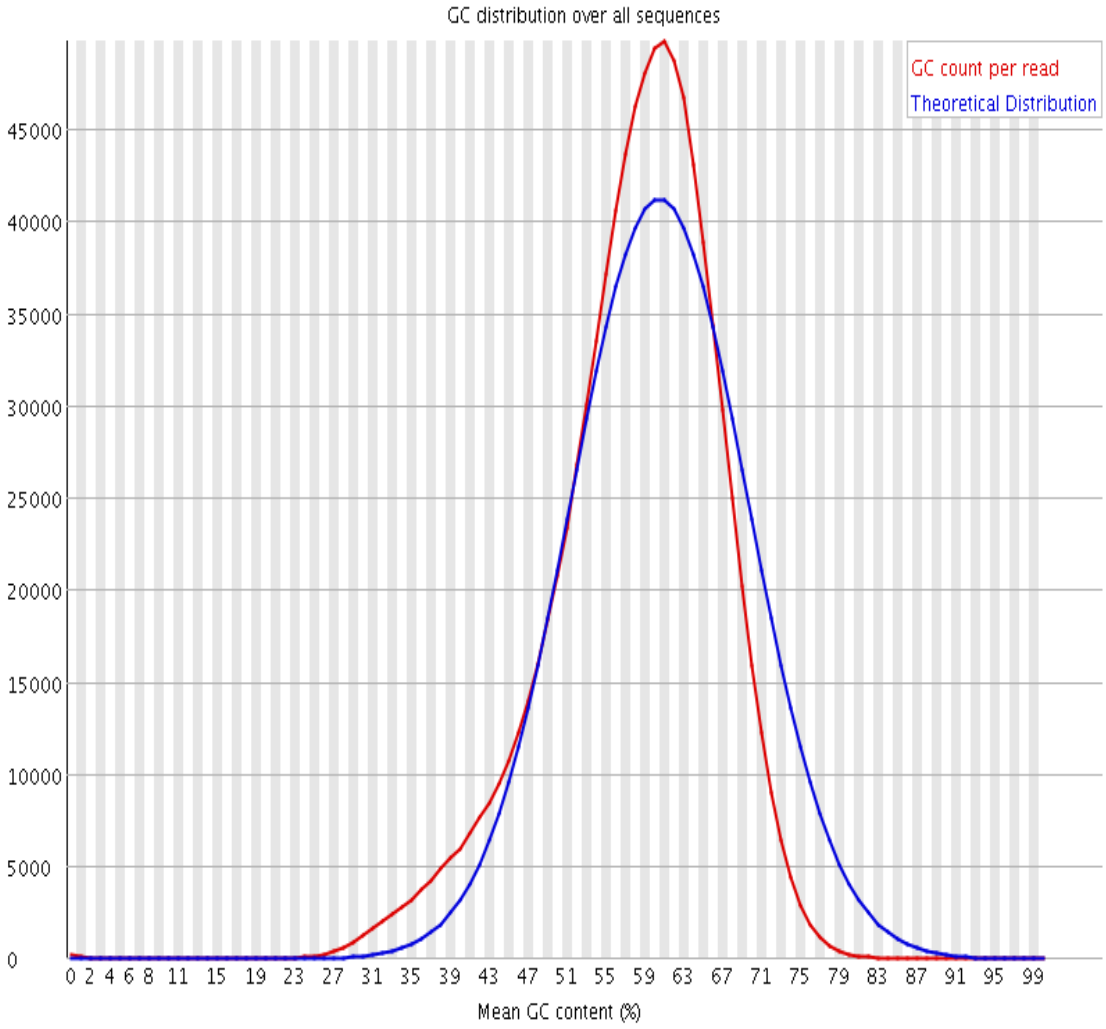


Figure 31: FastQC analysis

4.8.2 Assembly in SPAdes

NGS projects typically feature high coverage, NGS assemblers generate rather accurate contigs (although the accuracy deteriorates for SCS). SPAdes constructs DNA sequences of contigs and the mapping of reads to contigs by backtracking graph simplifications [92]. Using SPAdes V.3.9.0 algorithm HSTU-Sny5 strain have been generated for construct mapping and contigs sequence which showed 206 contigs sequence and number of read sequence 5394379 bp.

Table 19: List in details assembly report

Assembly	contigs	scaffolds
# contigs (>= 0 bp)	206	201
# contigs (>= 1000 bp)	40	36
Total length (>= 0 bp)	5432112	5431423
Total length (>= 1000 bp)	5391587	5391393
# contigs	45	41
Largest contig	500316	599206
Total length	5394573	5394379
GC (%)	57.82	57.82
N50	309326	309326
N75	141812	189855
L50	7	7
L75	15	12
# N's per 100 kbp	0	0

4.8.3 GENIUS Metagenomics analysis

The GENIUS Metagenomics application have been used to acurated genome database and high performance algorithms to provide rapid, accurate, and actionable bacterial identification at the species, subspecies, and/or strain level [95]. The result of HSTU-Sny5 strain (Figure 32) was showed 94% genus conformation.

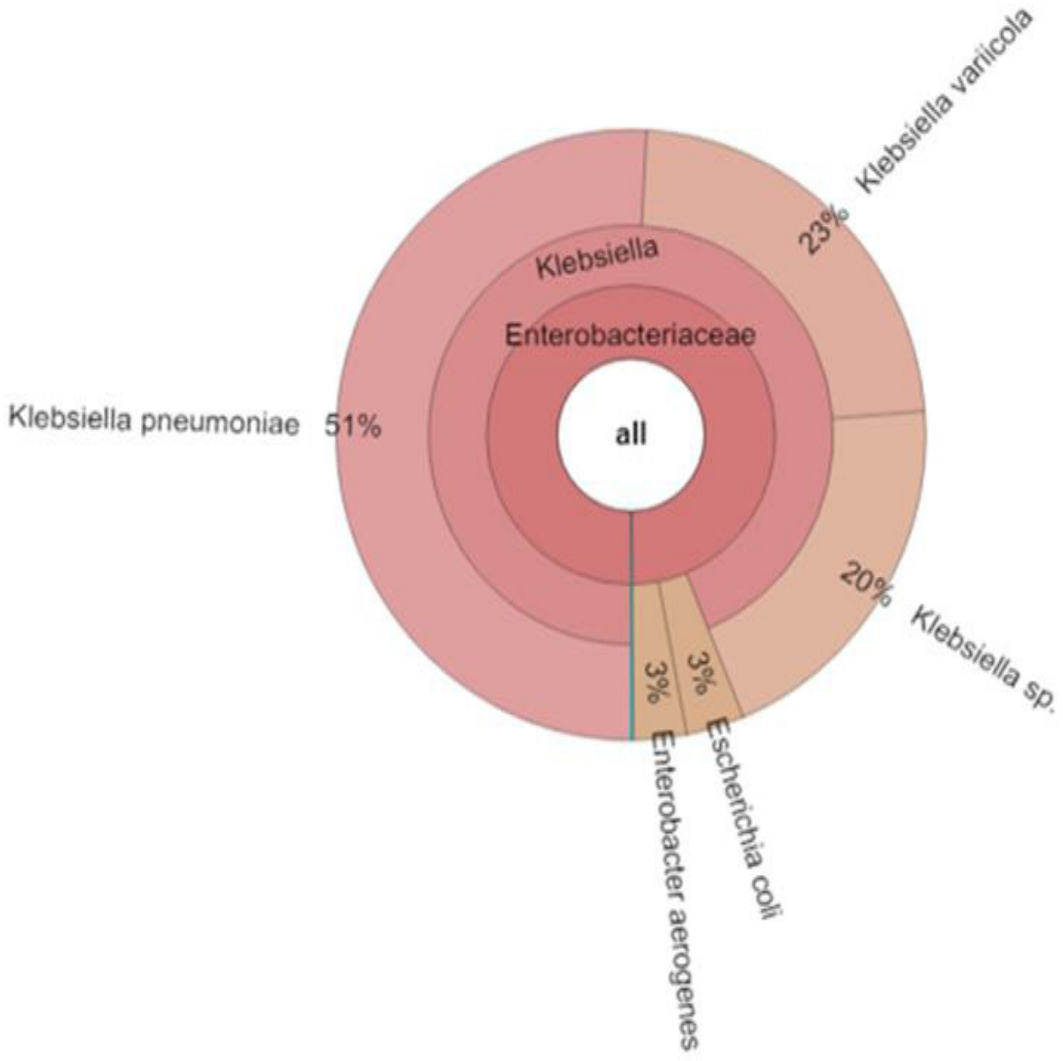


Figure 32: Metagenomics analysis of *klebsiella* sp. HSTU-Sny5 strain

4.8.4 ANI value calculation

Average nucleotide identity (ANI) is a category of computational analysis that can be used to define species boundaries of Archaea and Bacteria. Calculating ANI usually involves the fragmentation of genome sequences, followed by nucleotide sequence search, alignment, and identity calculation [97]. *Klebsiella* sp. HSTU-Sny5 genome have been compared with 10 same genus where as highest result showed 99.09 % nucleotide sequence identity. ANI calculation have been done by MUMmer tool version 3.0 web base sarver. All result shows below Table no 20;

Table 20: ANI calculating result

	***K* sp. HSTU-Sny5**	***K. quasipneumoniae* subsp. A708**	***K. quasipneumoniae* subsp. strain G747**	***K. quasipneumoniae* strain G4584**	***K. variicola* strain WCHKP19**	***K. pneumoniae* strain AR 0079**	***K. aerogenes* strain G7**	***K. grimontii* strain SS141**	***K. oxytoca* strain NCTC11683**	***K. pneumoniae* strain MNCRE69**	***K. pneumoniae* strain NCTC13635**
***Klebsiella* sp. HSTU-Sny5**	*	**99.09**	**96.71**	**96.71**	93.60	93.84	87.03	86.15	86.12	93.84	93.67
***Klebsiella quasipneumoniae* subsp. A708**	**99.09**	*	**96.69**	**96.69**	93.72	93.90	87.18	86.33	86.25	93.88	93.70
***Klebsiella quasipneumoniae* subsp. strain G747**	**96.71**	**96.69**	*	**99.99**	93.69	94.06	87.27	86.02	85.99	94.08	93.89
***Klebsiella quasipneumoniae* strain G4584**	**96.71**	**96.69**	**99.99**	*	93.70	94.07	87.29	86.03	85.98	94.10	93.90
***Klebsiella variicola* strain WCHKP19**	93.60	93.72	93.69	93.70	*	94.69	87.57	86.07	85.93	94.68	94.54
***Klebsiella pneumoniae* strain AR 0079**	93.84	93.89	94.06	94.07	94.70	*	87.78	86.01	85.92	**99.76**	**99.05**
***Klebsiella aerogenes* strain G7**	87.03	87.18	87.27	87.29	87.57	87.78	*	85.84	85.83	87.99	87.52
***Klebsiella grimontii* strain SS141**	86.15	86.33	86.02	86.03	86.07	86.01	85.85	*	**99.49**	86.05	85.92
***Klebsiella oxytoca* strain NCTC11683**	86.12	86.25	86.00	85.99	85.93	85.92	85.83	**99.49**	*	85.93	85.82
***Klebsiella pneumoniae* strain MNCRE69**	93.84	93.88	94.08	94.10	94.68	**99.76**	87.99	86.04	85.92	*	**99.04**
***Klebsiella pneumoniae* strain NCTC13635**	93.68	93.71	93.89	93.90	94.54	**99.05**	87.51	85.92	85.82	**99.04**	*

4.8.5 Phylogenetic tree analysis

The evolutionary history was inferred by using the Maximum Likelihood method based on the REALPHY online tool (fig.33). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. *Klebesiella* sp. HSTU-Sny5 strain was showed 0.004 distances from nearest species and made cluster form.

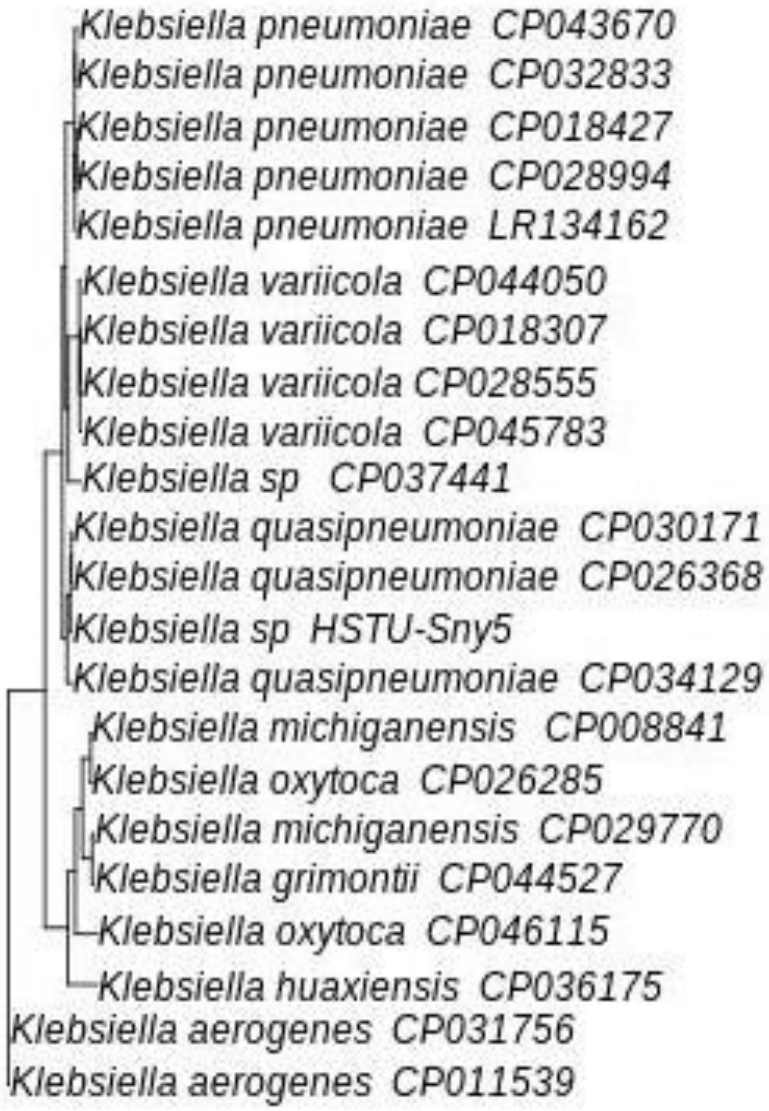


Figure 33: Phylogenetic analysis of HSTU-Sny5

4.8.6 Venn diagram analysis

Venn diagram was showing the *Klebsiella* sp. HSTU-Sny5 predicted essential amino acid sequence in different studies. HSTU-Sny5 strain was showed total 151619 numbers of amino acid sequence present in this genome in which 117102 amino acid sequence were showed unique and 22044 numbers of amino acid sequence were common among each other (fig 34). In addition to maximum 20795 amino acid sequence were matched with reference genome (CP030171)

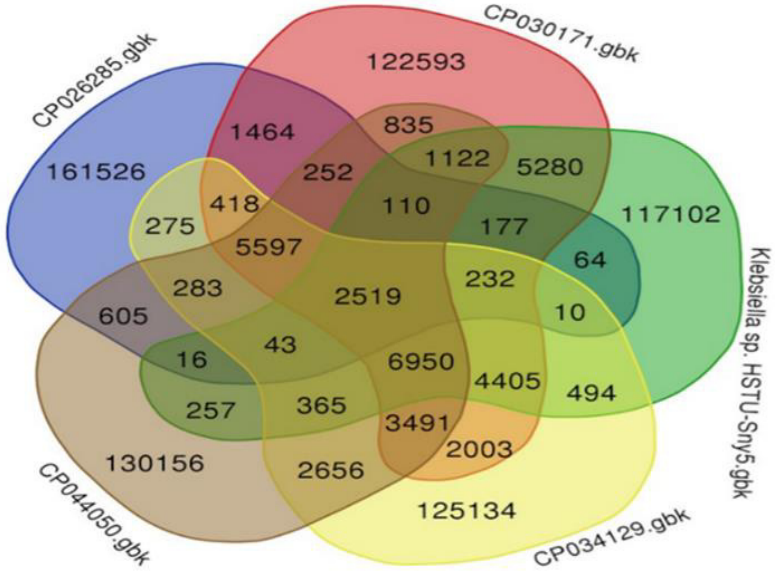


Figure 34: Venn diagram of *klebsiella* sp. HSTU-Sny5 predicted essential amino acid sequence

4.8.7 Pan-genome analysis

CGview shows all the proteins in a bacterium in a proper way, it's called genomic map. We hereby transcribed the *Klebsiella* sp. HSTU-Sny5 bacterial protein into a genomic map where 4,999 CDS, 77 tRNAs were shown in 5.4mbp length sequences. GC skew is observed in local genomic regions primarily introduced by RNA synthesis, but the overall genomic polarity due to replication is present regardless of these local effects, and the GC skew is thus observed in intergenic regions as well as in the third nucleotide positions in codons. Because only a few *origin* and *terminus* positions had been identified by experimental means, analysis of GC skew was first utilized for the computational prediction of *ori* and *ter* positions by genome sequences. Here GC-skew positive indicates that the protein coding sequence (CDS) is present in

downstream and GC-skew negative indicates that the protein coding sequence (CDS) is present in upstream (fig 35).

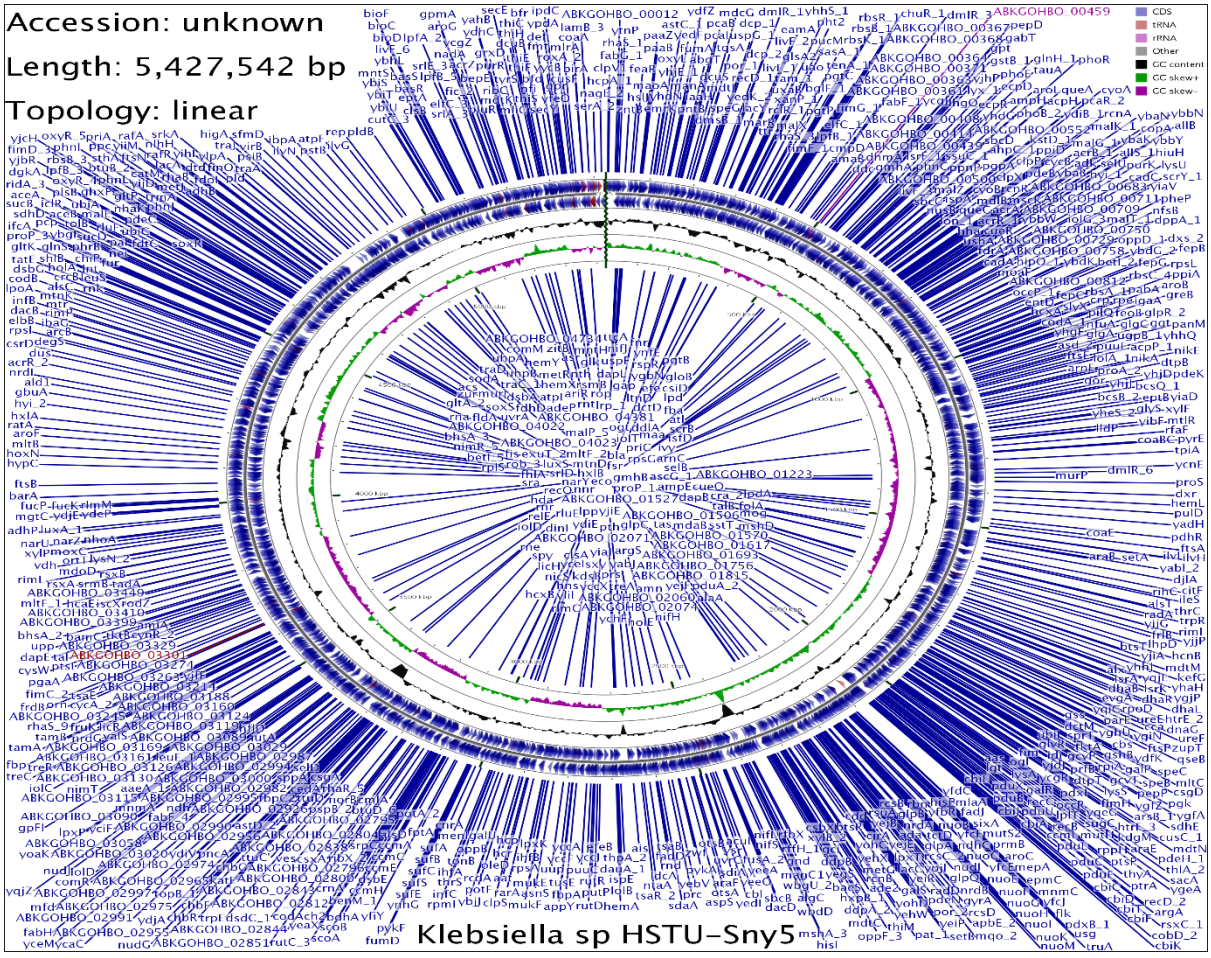


Figure 35: Pan genome of *klebsiella* sp HSTU-Sny5

4.8.8 Annotating a Genome Using RAST

The basic steps was used to annotate a genome using RAST are described in the subsections below. Input to the process is a prokaryotic genome in the form of a set of contigs in FASTA format. After annotating contig fasta file showed in seed viewer result below table and also showed genomic feature in subsystem about 59% genome coverage .In subsystem –total 3038 proteins are involved where the strain HSTU-Sny5 is predicted to encode for about 801 proteins involved in carbohydrate metabolism and 564 proteins involved in amino acid metabolism (fig 36). Isolated protein show below table and figure;

Table 21: Annotating feature of HSTU-Sny5

Genome	*klebseilla* sp. sny5
Domain	Bacteria
Taxonomy	Bacteria; *klebseilla* sp. sny5
Size	5,432,112
GC Content	57.8
N50	309326
L50	7
Number of Contigs (with PEGs)	206
Number of Subsystems	588
Number of Coding Sequences (CDS)	5120
Number of RNAs	111

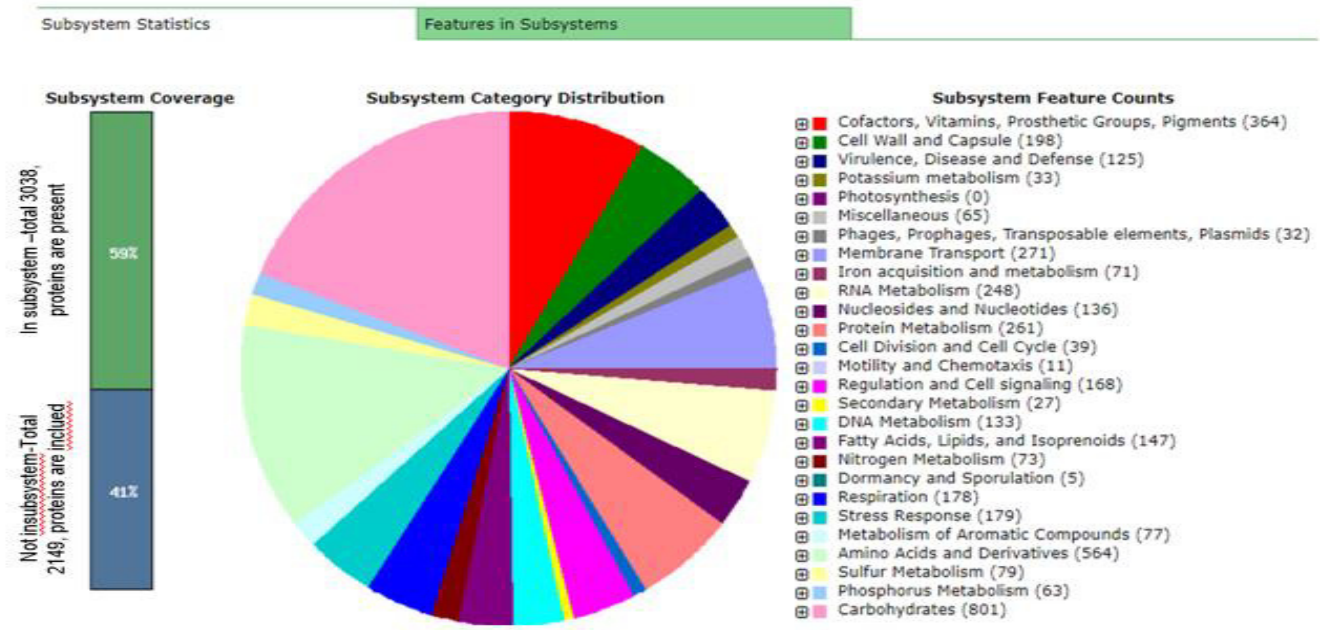


Figure 36: Genes connected to subsystems and their distribution in different categories.

4.9 Housekeeping genes

Housekeeping genes are typically constitutive genes that are required for the maintenance of basic cellular function, and are expressed in all cells of an organism under normal and patho-physiological conditions[112-114]. Although some housekeeping genes are expressed at

relatively constant rates in most non-pathological situations, the expression of other housekeeping genes may vary depending on experimental conditions [115].

The origin of the term "housekeeping gene" remains obscure. Literature from 1976 used the term to describe specifically tRNA and rRNA. For experimental purposes, the expression of one or multiple housekeeping genes is used as a reference point for the analysis of expression levels of other genes. The key criterion for the use of a housekeeping gene in this manner is that the chosen housekeeping gene is uniformly expressed with low variance under both control and experimental conditions. Validation of housekeeping genes should be performed before their use in gene expression experiments such as RT-PCR. So we found some housekeeping genes from *Klebsiella* sp. HSTU-Sny5 such as (*recA, tusA, tusB, tusC, tusD, rpoD, gyrA, gyrB, 16S, arcA, ffh, cinA, rho, yaeO, tsx, malE)* which has capable to protect physiological condition and cellular function. Those genes were identified from RAST server annotation, genes were present in different NODE with different position which shows table 1 and also expressed those genes in phylogenetic analysis.

Table 22 : Housekeeping genes and their position

Activity	**Gene Designation**	**Locus tag : Gene position**
Housekeeping genes of HSTU-Sny5	*recA*	NODE_15 : (135904-136962)
	tusB	NODE_3 : (3756-3529)
	tusC	NODE_3 : (4183-3824)
	tusD	NODE_3 : (4569-4183)
	tusA	NODE_3 : (119539-119363)
	rpoD	NODE_5:(72599-70758)
	gyrA	NODE_22:(40305-37891)
		NODE_6 : (128295-130928)
		NODE_7 : (32311-32784)
	16S	NODE_3 : (114810-115406)
	arcA	NODE_4 : (254976-255692)
	ffh	NODE_16 : (19011-17647)
	cinA	NODE_15 : (135316-135813)
	rho	NODE_23 : (19430-20689)
	yaeO	NODE_4 : (9780-10040)
	tsX	NODE_2 : (151722-150838)

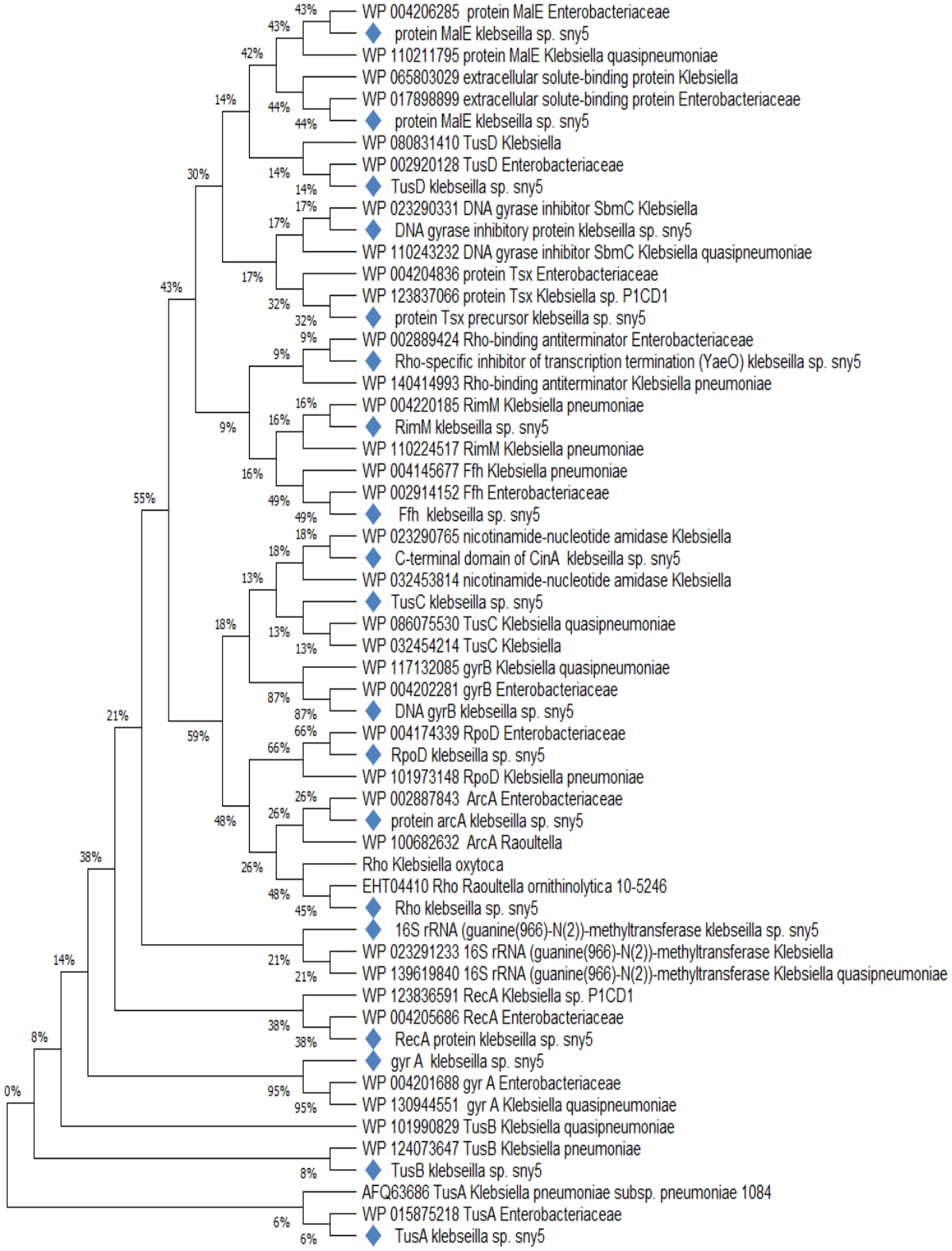


Figure 37: Phylogenetic tree according to housekeeping genes of *Klebsiella* sp. HSTU-Sny5

4.10 List of some identified plant growth promoting genes traits from RAST analysis

4.10.1 Effective genes of phosphate metabolism

Phosphate is an important macronutrient in a plant. Making up about 0.2% of a plants dry weight. High-energy phosphate, of ADP and ATP, is the source of high energy that is play important role in chemical reactions of the plant. When ADP and ATP transfer the high-energy phosphate to other molecules (by phosphorylation), is important process in many plant chemical reaction. Inorganic phosphates are important for plant growth and thus microbes can assist plants by mobilizing complex phosphates into more solubilized forms [116]. Several bacteria such as Gluconobacter oxydans, Pseudomonas fluorescens, Azospirillum spp. and Mesorhizobium mediterraneum have shown phosphatesolubilizing abilities [117]. The pst operon of E. coli and B. subtilis is composed of pstS, pstC, pstA, and pstB as well as a two-component signal transduction system consisting of phoP/phoR for phosphate uptake [118]. In the present study, genomic analysis of HSTU-Sny5 revealed that it carries the pst operon [*pstA, pstB, pstC, and pstS* genes; Locus tag:Gene position-Node_22:(60972-60082), Node_2:(84201-83383), Node_22:(61883-60972) and Node_22:(63099-62059) respectively], as well as *phoB* {Locus tag:Gene position- Node_2:(126460-127149)} and *phoR* {Locus tag:Gene position- Node_2:(127171-128466)} genes for phosphate transport.

Table 23 : Phosphate metabolism genes, their position and product

Phosphate metabolic genes	Locus tag:Gene position	Product
pstC	Node_22:(61883-60972)	Component of phosphate ABC transporter
pstA	Node_22:(60972-60082)	Phosphate ABC transporter permease
phoU	Node_22:(59211-58486)	PhoU phosphate transport system protein
phoR(SphS)	Node_2:(127171-128466)	Two-component sensor kinase
phoB(SphR)	Node_2:(126460-127149)	PhoB transcriptional dual regulator
pstB	Node_2:(84201-83383)	Component of phosphate ABC transporter
pstS	Node_22:(63099-62059)	Component of phosphate ABC transporter
phoH	Node_18:(67115-66069) Node_8:(1273-485)	ATP-binding protein
phoQ	Node_11:(80785-79319)	
pntA	Node_1:(153379-154908)	Component of pyridine nucleotide transhydrogenase
pntB	Node_1:(154919-156307)	Component of pyridine nucleotide transhydrogenase

This table shows the genes potentially involved in phosphate metabolism with the locus tag and gene position in the HSTU-Sny5 genome;

4.10.2 Effective genes of IAA production

Indole acetic acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L- tryptophan metabolism produced by several microorganisms including Plant Growth-Promoting Rhizobacteria (PGPR) [119]. The common traits include production of plant growth regulators (like auxin, gibberellin, and ethylene), siderophores, HCN and antibiotics [120]. Bacteria synthesize auxins in order to perturb host physiological processes for their own benefit [121]. We were isolated *Klebsiella* sp. HSTU-Sny5 from tomato plant root have an ability to produce Indole acetic acid as secondary metabolites due to rich supply of substrates. Indole acetic acid helps in the production of longer roots with increased number of root hairs and root laterals which are involved in nutrient uptake [122]. IAA stimulates cell elongation by modifying certain conditions like, increase in osmotic contents of the cell, increase in permeability of water into cell, decrease in wall pressure, an increase in cell wall synthesis and inducing specific RXA and protein synthesis. We were ensured to applied in field experiment which helps to enhance plant growth promoting with root and shoot length. We were also confirm by whole genome analysis of Klebsiella sp HSTU-Sny5 which represent tryptophan producing genes was involved in this genome.

In this study,We found that HSTU-Sny5 contains most of the genes. they are given below in the table:

Table 24: IAA producing genes in various locus

Genes name	Locus tag : Gene position
trpB	NODE_10 : (87330-88523)
trpA	NODE_10 : (88523-89332)
trpD	NODE_10 : (84363-85958)
trpE	NODE_10 : (82801-84363)
trpC	NODE_10 : (85962-87320)

4.10.3 Effective genes of trehalose metabolism

Salinity of agricultural soils is one of the main problems for farmers, since growth and plant production can be adversely affected. Soluble salts decrease the fertility of soils, producing osmotic stress, affecting water balance, and ion homeostasis. This alters the plant's hormonal status, disturbing transpiration, nutrient acquisition and photosynthesis, among others [123]. Plants contain various mechanisms to cope with salt stress, including a microbiome associated

with the rhizosphere, phyllosphere, and endosphere [124]. A group of organisms that stand out in this plant microbiome are the beneficial bacteria known as plant growth-promoting bacteria (PGPB). These bacteria, such as *Azospirillum, Arthrobacter, Azotobacter, Bacillus, Burkholderia, Enterobacter*, and *Pseudomonas*, have been shown to improve salt tolerance in several plant crops [125].

Among the arsenal of mechanisms utilized by PGPB to reduce stress and promote growth in plants in the presence of both drought and high levels of salt is the production of trehalose [126]. Trehalose is a non-reducing disaccharide in which the two glucose units are linked via an α,α-(1,1)-glycosidic bond. This disaccharide has been isolated from all domains of life including plants, animals, fungi, yeast, archaea, and bacteria [127]. Trehalose is also industrially produced as it is used in the food, cosmetics, and pharmaceutical industries [128]. In addition, this disaccharide can play important and different roles in biological cells, such as, as a signaling molecule, reserve carbohydrate, and protectant against various stresses (e.g., drought, cold, and salt stress; [129]. Interestingly, the trehalose content has been strongly associated with the ability of some microorganisms to survive in harsh environments [130]. In the present study, genomic analyses of HSTU-Sny5 revealed that it carries some trehalose metabolic genes. The genes that carried by HSTU-Sny5 are *treS, treR* and these genes are located NODE_14: (128563-130188) and NODE_12:(41338-42285) respectively.

Table 25: Trehalose metabolism genes and their position

Trehalose metabolic genes	Locus tag : Gene position
treS	NODE_14 : (128563-130188)
treR	NODE_12 : (41338-42285)
treC	NODE_12 : (43886-45541)
lamb	NODE_14 : (71354-69750)
treB	NODE_12 : (42416-43834)
OtsB	NODE_7 : (105080-105868)
OtsA	NODE_7 : (105843-107267)

This table shows the genes potentially involved in trehalose metabolism with the locus tag and gene position in the HSTU-Sny5 genome.

4.10.4 Effective genes of chitinase

Table 26: Chitinase gene and their position

Plant growth promotion traits	**Genes with potential for PGP traits**	Locus tag:Gene position
Chitinase	Chitinase	NODE_10:(144978-146231)

4.10.5 Effective genes of H_2S production & sulfur assimilation

Sulfur is a macronutrient which is essention for growth and development of plant. Cystine and methionine amino acids contain sulphur which involve in protein and enzyme synthesis and therefore functionally important for plant growth and development [131]. Sulphur promotes root growth and seed production. It's also helps in nodule formation of legume plants and make the plants in winter hardiness [132]. Formation of chlorophyll, the green substance in leaves that permits photosynthesis. Plants produce starch, sugars, oils, fats, vitamins and other vital compounds through photosynthesis [133]. Sulphur increase the crops yield and healthy production as well as increase the market price. In sulphur deficiency firstly the younger leaves become pale green in color then the whole plant become light yellow appearence.

In this study, we found some H_2S production and sulphate assimilation genes which given below in the table with location of gene;

Table 27: H_2S production and sulfur assimilation genes and their location

Genes name	**Locus tag:Gene position**
cysB	NODE_10:(72614-71640)
cysW	NODE_13:(16937-16062)
cysP	NODE_13:(18786-17770)
cysT	NODE_13:(17770-16937)
cysA	NODE_13:(16072-14978)
cysZ	NODE_13:(6000-6761)

4.10.6 Effective genes of superoxide dismutase

Environmental stresses are the major limiting factor in plant productivity. Much of the injury to plants due to various environmental stresses is associated with oxidative damage.under a range of environmental stresses, the formation of reactive oxygen species may be in excess of the amount present under physiological conditions, thus creating oxidative stress. Antioxidative enzymes are critical components in preventing such oxidative stress in plants [134]. Plant antioxidants include enzymes such as catalase, superoxide dismutase (SOD), and glutathione reductase. Superoxide dismutase (SOD)catalyses the dismutation of the superoxide anion (O_2-) intohydrogen peroxide and molecular oxygen and is consideredto be a key enzyme in the defence against oxidative damage [135]. Superoxide dismutase (SOD) levels may increase in response to increased oxidative stress depending on the species of plant, stage of development and degree of the stress condition [136].

In this study, we have found some superoxide dismutase gene. HSTU-Sny5 genome contains the superoxide dismutase genes such *sodA, sodB, sodC*. The location of these genes with locus tag shows table 28;

Table 28: Respective genes of superoxide dismutase in the HSTU-Sny5 genome

Activity	Gene Designation	Enzyme Name	Locus tag : Gene location
Superoxide dismutase	*sodA*	Superoxide dismutase [Mn]	NODE_10 : (100753-101379)
	sodB	Superoxide dismutase [Fe]	NODE_3 : (353607-353029)
	sodC	Superoxide dismutase [Cu-Zn]	NODE_3 : (365763-366284)

4.10.7 Effective genes of chemotaxis

Motility is an important characteristic for plant-associated bacteria and endophytes, enabling bacteria to move and colonize plants and also to systematically spread within the plant [137].We identified 3 genes involved in chemotaxis and biosynthesis. The most widespread bacterial chemotaxis signaling pathway centers on a fixed core of signaling genes, consisting

in the *cheA/cheY*, methyl-accepting chemoreceptor proteins (MCP) and an adaptor protein CheW (Capra and Laub, 2012). Consistently with its chemotactic nature, we found that HSTU-Sny5 is able to synthetize the *cheY*, which genes present in locus no; NODE_10: (5855-4641)). We also found chemotaxis genes coding (*rbsB* and *malE)* present in locus NODE_19:(87240-86299) and NODE_2:(216128-217360).

Table 29: Chemotaxis genes and their location

Plant growth promotion traits	Genes with potential for PGP traits	Locus tag:Gene position
Chemotaxis	*rbsB*	NODE_19:(87240-86299) NODE_1:(377625-376672) NODE_22:(88323-89213) NODE_2:(436981-436040) NODE_7:(265075-265989)
	malE	NODE_19:(29227-28037) NODE_2:(216128-217360)
	cheY	NODE_10:(5855-4641)

4.10.8 Effective genes of heavy metal resistance

The analysis of genome data from *Klebsiella sp.*HSTU-Sny5 exposed that there was a gene of heavy metal resistance gene. The annotated genomes *Klebsiella sp.*HSTU-Sny5 revealed the presence of various putative proteins related to multiple heavy metals resistance, including resistance proteins, transporters, and metal reductases. A total of seven putative proteins conferring- arsenic resistance, cadmium resistance, copper resistance, Zinc resistance-associated protein, homeosta- sis, or transport were identified in the genome, including copper resistance protein CopC and CopD, Cu(I)-responsive transcriptional regulator CueR, Blue copper oxidase CueO precursor, Cobalt-zinc-cadmium resistance protein CzcA; Zinc resistance-associated protein, Cobalt-zinc-cadmium resistance-ZitB, Cobalt/zinc/cadmium efflux RND transporter, membrane fusion protein, CzcB family Cobalt-zinc-cadmium resistance, Cation efflux system protein CusA. Meanwhile, A CzcCBA operon encoding

cobalt–zinc–cadmium resistance (czc) determinants, and a mercury resistance operon consists of one detoxifying enzyme genes (*merR*) were also found in the genome [141,143]. Strain HSTU-Sny5 carries three operons responsible for arsenic resistance and metabolism in the genome. The first operon contains two genes, including *arsR*, encoding the arsenate reductase; and *arsH*, encoding the organoarsenical oxidase. Previous research has shown that the *ars* operons are quite diverse in *Pseudomonads* such as *P. aeruginosa*and *P. putida* [138-141].Sequence analysis indicated that two *ars* operons in *Klebsiella* sp.HSTU-Sny5,were differentiated from previously described *ars* operons in other *Pseudomonas* species. This may imply a new strategy for arsenic metabolism in this strain. In addition, the genome sequence analyses revealed the presence of Integral membrane protein TerC, chromate transporter ChrA, resistance to chromium compounds, magnesium and cobalt efflux protein CorC, Mg/Co/Ni transporter MgtE, Molybdenum cofactor guanine dinucleotide biosynthesis protein MobA, molybdenum ABC transporter ATP-binding protein. The presence of these genes in the genome suggest that *Klebsiella* sp. HSTU-Sny5 is adapted to thrive in environments with metal(loid) contamination. Meanwhile, the unique mechanism of heavy metal resistance in this bacterium suggested its great bioremediation potentials in heavy metal- contaminated environment.

Table 30: Heavy metal resistance genes and their location

Heavy metals	Gene Name	Locus Number	Start	Stop
Arsenic resistance	*arsH*	NODE_21	57745	58443
Arsenic resistance and Transcriptional regulator	*arsR*	NODE_11	92689	91919
Copper resistance	*copC*	NODE_7	146589	146963
Copper resistance	*copD*	NODE_7	146967	147836
Cu(I)-responsive transcriptional regulator	*cueR*	NODE_2	260479	260889
Copper homeostasis	*cueO*	NODE_4	93636	92035
Cobalt-zinc-cadmium resistance protein	*czcA*	NODE_26	27987	24922
Cobalt-zinc-cadmium resistance	*zitB*	NODE_26	3486	2542
Cobalt-zinc-cadmium resistance	*czcB*	NODE_31	5778	7040
Cobalt-zinc-cadmium resistance	*cusA*	NODE_31	7052	10201
Cation efflux system protein				
Cobalt-zinc-cadmium resistance	*merR*	NODE_29	16034	16459
Transcriptional regulator, MerR family	*merR*	NODE_31	260426	257871
Integral membrane protein	*terC*	NODE_5	17999	16959
Chromium resistance	*chrA*	NODE_9	165337	166704
Magnesium and cobalt efflux protein	*corC*	NODE_18	65503	64574
Magnesium and cobalt efflux protein	*corC*	NODE_7	173305	174864
Magnesium transport	*mgtE*	NODE_3	154490	155503
Magnesium transport	*mgtE*	NODE_6	143968	145404
Molybdenum cofactor guanine dinucleotide biosynthesis protein	*mobA*	NODE_20	94395	94976

4.10.9 Effective genes of nitrogen fixation

Nitrogen-fixing bacteria**,** microorganisms capable of transforming atmospheric nitrogen into fixed nitrogen (inorganic compounds usable by plants). More than 90 percent of all nitrogen fixation is effected by these organisms, which thus play an important role in the nitrogen cycle. The symbiotic nitrogen-fixing bacteria invade the root hairs of host plants, where they multiply and stimulate formation of root nodules, enlargements of plant cells and bacteria in intimate association [144]. We were isolated nitrogen fixing bacteria from tomato root which showed nitrogen fixing activity in Rhizobium medium. After that those bacteria was applied in field experiment on plants, they showed highly significant with control which ensured that nitrogen fixing genes are present in this bacteria. A complete set of genes encoding enzymes involved in nitrogen fixation was found in the genomic analysis of Klebsiella sp. HSTU-Sny5 (Table1). The main genes involved in this process are nif genes, of which *nifQBA* genes present in locus (NODE_7:3441-2926),NODE_7:(4847-3441)and NODE_7:(6586-5012)) annotated as nitrogenase molybdenum-iron proteins and *nifH* gene NODE_7:(22535-21654) encoding dinitrogenase reductase protein have been identified. In the this genome, we have found that *nifEN* genes (NODE_7:(17075-15702) and NODE_7:(15692-14307)) involved in synthesis of the molybdenum-iron cofactor of nitrogenase are clustered into a single operon together with *nifX* (NODE_7:(14320-13850)). Furthermore, the genome of Klebsiella sp. HSTU-Sny5 has *nifUSVW* genes (NODE_7:(13655-12816), NODE_7:(12800-11598), NODE_7:(11582-10440) and NODE_7:(10437-10180)), which are separated from the structural *nifENX* operon[145,146]. Organization of the nitrogen fixation gene cluster in Klebsiella sp. HSTU-Sny5 is presented in Fig 38.

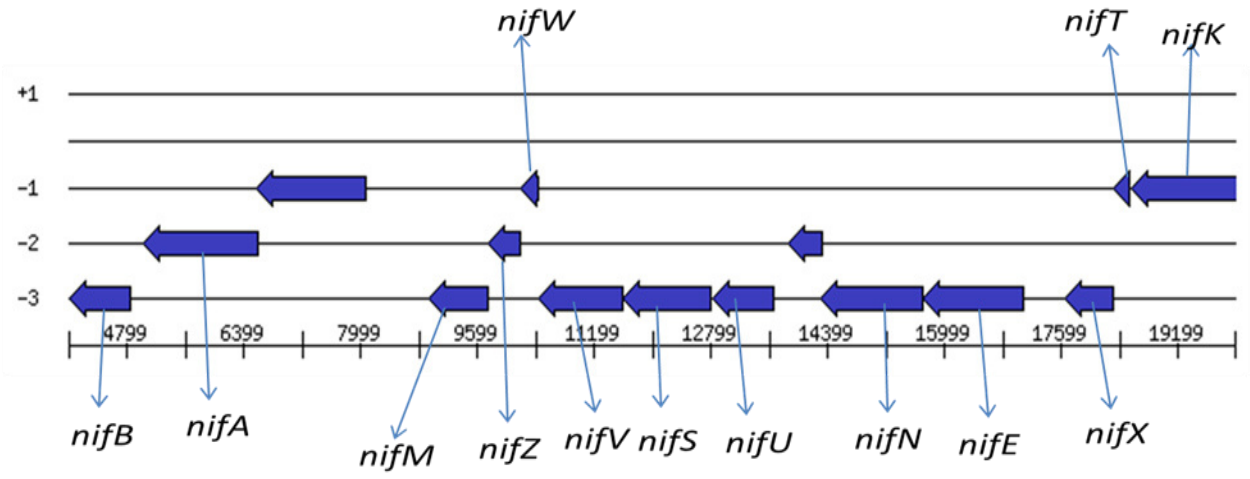


Figure 38: Nitrogen fixing gene present in locus NODE_7 upstream

Table 31: Nitrogen fixation genes, their position and products

Nitrogen fixation gene	Locus tag: position	Products
nifQ	NODE_7:(3441-2926)	Nitrogenase molybdenum-cofactor biosynthesis molybdenum delivery protein
nifE	NODE_7:(17075-15702)	Nitrogenase molybdenum-cofactor biosynthesis protein
nifH	NODE_7:(22535-21654)	Nitrogenase iron protein
nifA	NODE_7:(6586-5012)	Nif-specific transcriptional activator
nifB	NODE_7:(4847-3441)	Nitrogenase FeMo cofactor biosynthesis protein
nifM	NODE_7:(9740-8940)	
nifZ	NODE_7:(10183-9740)	Nitrogenase P-cluster assembly
NifS	NODE_7:(12800-11598)	Nitrogenase metalloclusters biosynthesis protein
NifU	NODE_7:(13655-12816)	Nitrogen fixation protein
NifN	NODE_7:(15692-14307)	Nitrogenase molybdenum-cofactor biosynthesis protein
nifT	NODE_7:(18531-18313)	
nifX	NODE_7:(14320-13850)	Nitrogenase molybdenum-iron protein
nifK	NODE_7:(20133-18571)	Nitrogenase molybdenum-iron protein subunit beta
nifW	NODE_7:(10437-10180)	Nitrogenase-stabilizing/protective protein
nifV	NODE_7:(10582-10440)	Homocitrate synthase

4.10.10 Effective genes of siderophore production

Iron is one of the most vital microelements for virtually all living cells, is usually abundant in the environment, particularly in soils. Despite being most abundant element in earth's crust, the availability of iron is limited due to very low solubility of the dominant ferric iron (Fe3+) in soil and become unavailable to plants as a micronutrient [147]. Some bacteria have the capability to produce low molecular weight (500-1000 dt) metal chelating compound including iron, called as Siderophore. Siderophore chelate iron from mineral phases by production of soluble Fe3+ complexes that can be taken up energy dependent membrane transport mechanism and create it available to plants or bacterial cells [148]. Siderophore forms complex with free iron and transport it into the cell by membrane receptor molecules.In this study, the present investigation has been undertaken to identify the potential siderophore producing gene such as (*fhuB, tonB, fepG, fhuD, fhuC, fhuA, entS,exbB, exbD, fecD, entS, lutA, entB*) from Whole genome sequencing of HSTU-Sny5 bacteria. There are three main genres of siderophore known as hydroxamate, catacholate and carboxalate. Most of the bacterial siderophores are catecholates (i.e. enterobactin), and some are carboxylates (i.e. rhizobactin) and hydroxamates (i.e. ferrioxamine B) [149]. Hydroxamatesiderophore are produced by bacteria and fungi. Siderophore-mediated iron uptake in microorganisms is both a receptor- and an energy-dependent process. Siderophores are part of a multi-component process for transporting ferric iron into a cell. Other components include a specific outer membrane receptor protein Fec A, Fep A and TonB-ExbB-ExbD protein complex in the inner membrane, a periplasmic binding protein. Under iron insufficiency, bacteria synthesize siderophore and increase number of receptor molecules once the siderophore excreted outside of cell thorough membrane receptor it bind with iron complex and transport the iron in to the cell via Fec A and Fep A outer membrane receptor(OM), after it transported to Fec C,D,E and Fep C,D,E so called ABC-Transporter systems (from ATP-binding cassette) [150,151] assembled of two proteins, one to span the membrane acting as a permease and a second one which can hydrolyse ATP to provide the energy for transport. Later siderophore iron complex release in cytoplasm with the favour of membrane protein Ton B. In the cell cytoplasm, the iron released from the complex by a mechanism which is still in doubt: it may involve hydrolytic destruction of the siderophore molecule or the reduction of Fe3+ by a NAD(P)Hlinked siderophore reductase or Ent A,B,C,D protein. The resulting Fe2+ does not have a high affinity for siderophore and therefore dissociated from the complex.When plants under conditions of Fe deficiency. These plants secrete Fe(III)-chelating compounds called phytosiderophores. The phytosiderophore is

delivered to the rhizosphere, it chelates Fe from the soil by creating Fe(III)–phytosiderophore complexes that can be subsequently transported across the root plasma membrane[152].

Table 32: Siderophore producing genes and their location

Activity	Gene Designation	Enzyme Name	Location
Siderophore production of HSTU-Sny5	*fhuA*	Ferric hydroxamate outer membrane receptor	NODE_4:(55083-52954)
	fhuB	Ferric hydroxamate ABC transporter, permease component	NODE_4:(52111-51221)
	fhuC	Ferric hydroxamate ABC transporter, ATP-binding protein	NODE_2:(343114-342317)
	fhuD	Ferric hydroxamate ABC transporter, periplasmic substrate binding protein	NODE_4:(51224-49242)
	tonB	Ferric siderophore transport system, periplasmic binding protein	NODE_2:(440053-437825)
	fepB	Ferric enterobactin-binding periplasmic protein	NODE_2:(450954-449995)
	fepG	Ferric enterobactin transport system permease protein	NODE_2:(447492-446500)
	entS	Enterobactinexporte	NODE_2:(448609-449850)
	exbB	Biopolymer transport protein	NODE_5:(122269-123000)
	exbD	Biopolymer transport protein	NODE_5:(123007-123432)
	fecD	Iron-dicitrate transporter subunit	NODE_2:(344193-343111)
	lutA	Aerobactinsiderophore receptor	NODE_11:(56111-53922)
	entB	Enterobactin biosynthesis bifunctional protein	NODE_22:(453949-454800)

4.10.11 Heat shock proteins

Stresses condition like droughts, intense rains and flooding extrem impacting productivity in crops.

Endophytic bacteria could play a significant role in Stresses, if we can exposs their unique properties of tolerance to extremities, genetic diversity, their interaction with crop plants and develop methods for their successful deployment in crop production.

Endophytic bacteria have formed a number of mechanisms for coping with stress and adapting to changing environmental conditions.

Heat shock proteins are involved in the folding of newly synthesized proteins and also play a crucial role in protecting plant cells from the damaging effects of heat stress [153]

Heat shock proteins consist of chaperons such as DnaK, DnaJ, GroES, ClpB, ClpA, ClpX, small heat shock proteins. Chaperons are involved in the proper folding of denatured proteins and proteases are required for the degradation of irreversibly damaged protiens.[154]

In this study, we have found some chaperons like heat shock proteins.HSTU-Syn5 genome contains the heat shock protein genes *dnak, dnaj, rpoH, smpB, grpE.* The location of these genes with locus tag shows in the table 33;

Table 33: Respective genes of heat shock protein in the HSTU-Syn5 genome

Gene Name	Node location	Start	Stop
dnaK	NODE_4	240732	238816
DnaJ	NODE_4	238728	237595
RpoH	NODE_3	111025	110252
SmpB	NODE_16	26156	26638
grpE	NODE_16	21980	21390

The activity of such chaperones is essential for cell survival during heat shock and for subsequent recovery. Similarlycold tolerant bacteria respond to a decrease in temperature by induction of cryoprotective protein.

4.10.12 Cold shock proteins

Cold shock proteins (Csps) comprise afamily of small proteins that are structurally highlyconserved and bind to single-stranded nucleic acidsvia their nucleic acid binding motifs RNP1 and RNP2.Bacterial Csps are mainly induced after a rapidtemperature downshift to regulate the adaptation to cold stress, but are also present under normal conditions to regulate other biological functions.[155]

The cold shock protein family comprises small, structurally related, and highly conserved nucleic acid-binding proteins that appear to contribute significantly to the management of numerous microbial physiological processes.[156]

The HSTU-Syn5 genome contains the cold shock protein genes *cspD, cspA, cspC, cspE*. These genes have been linked to the modulation of cold adaptive functions. The location of these genes with locus tag shows table 34.

Table 34: Respective genes of cold shock protein in the HSTU-Sny5 genome

Gene Name	NODE location	Start	Stop
cspA	NODE_3	244969	245181
cspC	NODE_7	165654	165863
cspD	NODE_8	167023	167244
cspE	NODE_18	36811	37020

The expression of bacterial cold shock protien (Csp A and Csp B) was shown to improve tolerance of transgenic rice, maize and arabidopsis plants to a number of abiotic stresses including cold, heat and water deficit resulting in improved yields under field conditions.

4.10.13 Flagellar protein

In many bacterial species, the flagellum is an acknowledged virulence factor, and non-flagellated strains have in several cases been observed to be less virulent. The flagellum can act directly as an adhesion, but can also affect virulence by other means. Motility towards a host cell is a prerequisite for adhesion and invasion, and flagella can play an essential role in colonization by facilitating bacterial motility even if the flagella do not directly participate in

the adhesion or invasion. Flagella can also contribute to virulence by regulating the expression of other virulence factors which allows the bacterium to move through the soil matrix and inside the plant. We were confirmed by the presence of a large number of genes involved in *Klebsiella* sp. HSTU-Sny5 in where some flagella genes such as *fliP, fliY, murJ* with genes coding for the flagellar motor proteins MotA and MotB were present in locus respectively (Table 35).

Table 35: Flagella genes and their position

Plant growth promotion traits	Genes with potential for PGP traits	Locus tag:Gene position
Flagellar protein	*flip*	NODE_11:(25350-26885)
	motB	NODE_18:(130877-131401)
	motA	NODE_18: (126878-127570)
		NODE_5: (122269-123000)
	fliY	NODE_7:(81114-81914)
	murJ	NODE_11:(25350-26885)

4.11 Effective genes of pesticides bio-degradation activity

4.11.1 Organophosphate pesticides degrading gene

Organo-phosphorus pesticides account for ~38% of the world's total pesticide usage, which includes more than 100 OPPs that are in use, such as chlorpyrifos, parathion-methyl, triazophos, mala-thion and phosphamidon (2). Several genomes of organophosphate pesticides degrading strains have been published to date (3–5), including *Pseudomonas* spp., *Burkholderia* spp., *Burkholderia zhejiangensis*, etc. Strain HSTU-Sny5 efficiently degrades several pesticides such as chlorpyrifos, parathion-methyl, and diazinon. The analysis of the genome data from *Klebsiella* sp. HSTU-Sny5 revealed that there was a gene of organophosphorus hydrolase (*opdE*) in NODE_1: (319513-320757) that contributed to the degradation of most organophosphate pesticides. Carboxylesterase and phosphotriesterase enzymes are directly involved in the degradation of organophosphate pesticides. The first isolated phosphotriesterase belongs to the *Pseudomonas diminuta* MG species, which shows a highly catalytic activity towards organophosphate pesticides. The phosphotriesterases gene

called *opd* (organophosphate-degrading). *Flavobacterium* ATCC 27551 presents the *opd* gene encoding to a phosphotriesterase [157]. In esterases A, the organophosphates interact with the functional group -SH forming a bond between P=S, which is easily hydrolyzed by H_2O. In the esterase B, organophosphates interaction with the SER-OH forming a P=O bond that is not hydrolyzed by H_2O. Organophosphates that bind to the esterase B stoichiometrically inhibit its enzymatic activity. In this study, the strain *Klebsiella* sp. HSTU-Sny5 showed total seventy three xenobiotics genes in RAST analysis. Among them 20 genes are related with esterase family. In particular, *opdE*, carboxylesterase and phosphotriesterase are available in the genome of *Klebsiella* sp. HSTU-Sny5 that indicates its involvement in organophosphates pesticide degradation. Besides esterase, the two main enzyme families implicated in degradation of pesticides glutathione S-transferases (GSTs) and cytochrome P450 [158]. Importantly, the GSTs and cytochrome proteins are found in the NODE_14, 19, 27 and NODE_11, 12, 13, 14, 17, 18, 2, 9 of *Klebsiella* sp. HSTU-Sny5 genome, respectively. These results suggested that the strain Sny-5 is enriched with pesticides degrading enzymes. As a matter of fact, the concentration of chlorpyrifos was declined when it was grown in presence of those organophosphate pesticides as their sole carbon sources. As seen in Table 36, the pesticides mineralizing others genes such as *pepA, AmpD, PhnO, PhnJ, PhnI* etc genes are commonly found in the genome of Sny5 strain. Therefore, it is supposed that the strains are responsible for mineralizing other pesticides along with organophosphates.

Table 36: Pesticides degrading genes and their position

Genes name	Locus tag : position
opdE	NODE_1 : (319513-320757)
pepA	NODE_12 : (25840-27351)
ampD	NODE_4 : (118452-117889)
PhnO	
PhnO	NODE_19 : (21061- 20606)
PhnJ	NODE_19 : (97562- 96714)
PhnI	NODE_19 : (98619- 97555)

Chapter VI

CONCLUSION

This research was carried out to exploit the chlorpyrifos, mineralizing endophytic bacteria associated with plant growth promotion traits to promote sustainable development. The endophytic bacteria was isolated from tomato root, strain named as CS-T.R.3. Bacteria was isolated in pesticide containing Normal growth media in which pesticides were used as the sole source of carbon whereas organophosphate pesticides used named as Chlorpyrifos with cypermethrin in minimal concentration. Eleven biochemical tests were done to characterize these bacteria. On the basis of biochemical characterization of endophytic bacterial isolates, for Methyl Red test, Vogus Proskauer (VP) test, catalase test, oxidase test , TSI, CIU, Sucrose, Dextrose and MIU test, lactose and Urease and maltose were positive along with all the isolates have extracellular enzyme (Cellulase, xylanase, protease and amylase) producing capability and Congo red (CR), Bromothymol blue (BTB), Toluidine blue (TDB), Avitera blue (ATB) and Trypan blue (TPB) dyes degradation capacity.

These isolates was applied in eggplant for further confirmation of having growth promoting traits and it was noticed that the individual bacterial treatment showed significant result on root length, shoot length, total seedling length, germination rate and weight comparing with control gave higher result in seedling length than compost and fertilizer treated eggplant.

The pesticides bio-degradation was carried out in liquid medium where pesticides (chlorpyrifos + cypermethrin 500 μL/150 ml) were treated with the individual isolates in minimal salt medium for 25 days and then the degradation was confirmed by FT-IR analysis.

After initial endophytic assessment, the NGS whole genome analysis and careful bioinformatics analysis have ensured the presence of specific genes responsible for plant growth promoting endophytic activity. So This work reflects the increasing demand of agro-economic development of a country. An endophyhtic growth promoting strain has been found by evaluating, it's growth promoting activity via different analysis. This valuable data generated will greatly contribute to the already existing huge database that we already have for Bioinformatics and is a benchmark for more complex and productive research in future.

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