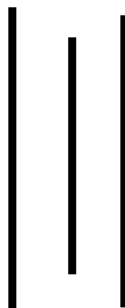
Exploring Hyper-Tolerant Arsenic Resistant *Bacillus* Species from Nepalese Agriculture Soil and their Plant Growth Promoting Efficacy

A

Dissertation

In Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science in Biotechnology

2024



Submitted to

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RECOMMENDATION

This is to certify that the research work entitled "**Exploring Hyper-Tolerant Arsenic Resistant *Bacillus* Species from Nepalese Agriculture Soil and their Plant Growth Promoting Efficacy"** has been carried out by **Mr. Nitesh Kumar Patel** under my supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 653. The result presented here is her original findings. We hereby recommend this thesis for final evaluation.

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CERTIFICATE OF EVALUATION

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ACKNOLEDGEMENT

Firstly, I would like to anknowledge **Prof. Dr. Krishna Das Manandhar**, Head of Department, central department of biotechnology, Tribhuvan University, Nepal for providing me this great opportunity.

I would like to express my sincere gratitude to the **University Grants Commission Nepal** for providing funding for my thesis under **Higher Education Reform Project**, University Grants Commission.

I want to express my deep gratitude to my supervisors **Dr. Pramod Poudel, Associate Professor, Central Department of Biotechnology, Tribhuvan University,** Nepal for his generous support, motivating and guidance during research period.

I express my gratitude to **Prof.Dr. Hari Datta Bhatarai**, **Central Department Of Botany, Tribhuvan University**, for invaluable support and providing of Green house for pot assay and him guidance, encouragement, and provision of the greenhouse facilities have been instrumental in the successful completion of this research project.

I am also obliged to all faculties and staff of central department of biotechnology for supporting me directly and indirectly.

I want to express my heartfelt thanks to my classmate **Mr. Milan Parajuli** for his cooperative help and encouraging me throught the research work. My thanks go next to my classmate at cdbt 11 batch, senior **Puja Batta and Suraj Chaudhary** and junior who helped and supported me during my work.

Finally I would like to thanks all good souls and well-wishers who are missed here to mention.Last but above all, I would like to express my deep gratitude to my father **shree Chanardev Raut, Mother Sudama Devi and Guru Ramasewak Prasad** for their overwhelming support of all sphere.

Nitesh kumar Patel

ABSTRACT

Both surface and ground water is significantly used for irrigation and drinking purpose in which deposited arsenic on top of agriculture soil. Nepal Arsenic contamination in agricultural soils possess a significant threat to crop productivity and human health. The main aim of this study is to isolate and characterize hyper tolerant arsenic-resistant bacteria from contaminated agricultural soil of terai region of Nepal and evaluate their potential plant growth-promoting efficacy. For the isolation of arsenic resistant Bacillus, sixteen soil samples were collected from cultivable agricultural fields of Terai region of Nepal and isolated bacteria were screeened based on tolerance to high levels of arsenic through minimal inhibitory concentration assays. Only two S1 and M1 bacterial isolates with high arsenic tolerant arsenic resistant were selected and were characterized. Both bacterial isolates S1 and M1 showed maximum resistance against arsenic; up to 5,500 ppm of As (V) and 1000 ppm of As (III). In addition, both isolates showed heavy metal resistant against cobalt, copper, and zinc (200 ppm) and lead (400 ppm) which make its hyper tolerance in nature. Both S1 and M1 exhibited plant growth-promoting activities such as auxin and hydrogen cyanide production, phosphate and zinc solubilization, nitrogen fixation, biofilm production, heavy metal resistant, organic acid production, phosphatse production, amylase, HCN, protease, Laccase and lipase producers. Bio control activities were also observed against *F. oxisporum, R. solani, A. solani* etc. Based on 16S rRNA gene sequencing, isolates S1 and M1 showed highest similarity with *Bacillus cereus* (99.77%) and *Bacillus weidmanii* (99.69%) respectively.

Pot experiment in green house condition was performed using isolates S1 and M1 to confirm their effect of on growth of *Brassica juncea* L. Czern, in both presence and absence of arsenic. Both the isolates significantly enhanced the growth of *Brassica juncea* such as increase in shoot length, root lengths, leaf area, water content, chlorophyll a & b, total chlorophyll, soluble protein in leaf were observed as compared to uninoculated control in the presence and absence of arsenic. These findings suggest that isolates S1 and M1 are first hyper tolerant arsenic resistant bacteria ever reported in Nepal and could be best promising candidate as plant growth promoters and biocontrol agent in arsenic contaminated soil.

Keywords: Soil, Arsenic resistant Bacillus, Plant growth promotion, Bioremediation, biofilm, Biocontrol

TABLE OF CONTENTS

RECOMMENDATION ii

CERTIFICATE OF EVALUATION iii

ACKNOLEDGEMENT iv

ABSTRACT v

TABLE OF CONTENTS vi

LIST OF FIGURE xii

LIST OF TABLES xv

LIST OF ABBREVIATION xvi

1. INTODUCTION 1

1.1 Background 1

1.2 Research Hypothesis 4

1.3 Objectives 4

1.4 Rationale of study 5

LITRATURE REVIEW 6

2.1 Arsenic contamination in agriculture soil of Nepal 6

2.2 Determination of arenite 7

2.3 Arsenic resistant bacteria 7

2.4 Arsenic oxidising bacteria 8

2.5 Arsenic uptake from soil 9

2.6 Arsenic translocation within plant 10

2.7 Hyperaccumulator 10

2.8. Factors affecting As speciation, availability, and mobilization into plants 11

2.8.1. Plant genotype 11

2.8.2. Rhizosphere microbiome 11

2.8.3. Soil Physico-chemical properties 12

2.9 Impact of arsenic in plant 13
2.10 The Microbial Detoxification of Arsenic Related to Bioremediation 16
2.11 PGPR mediated enhancement of As phytoremediation in plants 18
2.11.1 Biological nitrogen fixation 18
2.11.2 Phosphate solubilization 19
2.11.2. Organic acid production (OA) 19
2.11.3 Zinc solubilization 20
2.11.4 Resistance to heavy metal 20
2.11.5. Phytohormones production 21
2.11.6 Production of hydrogen cyanide (HCN) 21
2.11.7 Biocontrol 22
2.11.8. Biofilm producing ability of bacteria (a next-generation bio-fertilizer) 22
2.11.9. Nanobiofertilizer 23
2.12 Hydrolytic enzyme production 23
2.12.1 Protease 23
2.12.2 Cellusase 23
2.12.3 Amylase 24
2.12.4 Laccase 24
2.12.5. Lipase 24
2.13. Pot assay 24
CHAPTER III 26
Materials and Methods 26
3.1 Laboratory setting 26
3.3 Sampling site and method 26
3.4 Isolation of Bacillus species 26
3.4 Minimum inhibitory concentration 26
3.5 Investigation of oxidation and reduction of arsenic by the isolates by silver nitrate method 27

3.6. Phenotypic and biochemical characterization 27

3.7. Removal of arsenic from culture broth or effect of arsenic on bacterial growth 27

3.8 Optimization of growth physiochemical properties of bacteria 27

3.9 Determination of antibiotic resistance...28

3.10 Heavy metals tolerance 28

3.11 Pathogenicity of isolates 29

3.12 Development of bacterial consortium 29

3.13 IAA-like compound production ability...29

3.13.1 Determination of Optimum Condition for IAA Production 29

3.14 Ammonia production 30

3.15 Nitrogen fixation assay on NFb medium 30

3.16 Phosphate solubilizing activities..30

3.16.1 PVK agar plate 30

3.16.2 Liquid Mpvk medium 30

3.16.3 Phosphatase activity..31

3.17. Organic acid production in Pikovaskya media...31

3.18. Zinc solubilizing activities 31

3.19 In vitro screening of bacterial isolates for their bio control properties 32

3.19.1 Screen for hydrogen cyanide (HCN) production 32

3.19.2 Biofilm formation 32

3.19.3 Dual plate assay for antagonism of isolates 33

3.20. Screening bacterial isolates for hydrolytic enzyme production 33

3.20.1 Protease production activity 34

3.20.2 Cellulose production activity 34

3.20.2 Production of amylase (starch hydrolysis) 34

3.20.3 Laccase production activity 34

3.20.4 Lipase producing activity 34

3.21. Greenhouse assay..34

3.21.1 Seed sterilization 34
3.21.2 Inoculum preparation from bacteria 35
3.21.3 Seed treatment 35
3.21.4 Pot assay in green house 35
3.21.5 Layout, Design and Treatments 35
3.21.6. Irrigation 36
3.21.7. Seedling treatment 36
3.21.8 Measurements of different growth parameter 37
3.22 Water Content Analysis 37
3.23 Chlorophyll content 38
3.24 Total soluble protein 39
3.25. Molecular Identification of hyper tolerant AR *Bacillus* Species 39
3.26 Statistical analysis 40
CHAPTER IV 41
RESULT 41
4.1 Bacterial Isolation 41
4.2 Minimum inhibitory concentration 41
4.3. Investigation of Oxidation and reduction of arsenic by the isolates by silver nitrate method 42
4.4 Morphological Biochemical Characterization 42
4.4 Bioremediation test or effect of arsenic on bacterial growth 44
4.5. Optimization of growth physiochemical properties 45
4.5.1 Effect of Nacl 45
4.5.2 Effect of temperature 45
4.5.3 Effect of pH 46
4.6. Determination of antibiotic resistance 46
4.7. Estimation heavy metal resistance of bacteria 47
4.8. Biocompatibility of isolated bacteria 47

4.9. Hemolysis test 48
4.10. IAA production Assay 48
4.11. Nitrogen fixation assay 49
4.12 Ammonia Producing Assay 49
4.13 Zinc solubilizing assay 49
4.12 Phosphate solubilizing assay 50
4.12.1 Solid agar plate 50
4.12.2 PVK agar plate 50
4.12.3 Liquid pvk medium 50
4.14 Organic acid production 51
4.14.1. PH of culture medium 51
4.14.2. Estimation of organic acid by HPLC 51
4.15. Phosphatase assay 52
4.16 In vitro screening of bacterial isolates for their bio control properties 53
4.16.1 Screen for hydrogen cyanide (HCN) production 53
4.16.2. Dual plate assay for antagonism of isolates 54
4.16.3 Detection of Biofilm production 54
4.16.3.1Congo red agar assay 54
4.16.3.2 Tube assay 54
4.16.3.3 Microtiter plate assay 54
4.17 Hydrolytic enzymes production 55
4.17.1 Protease production activity 55
4.17.2 Cellulose production activity 55
4.17.3 Production of amylase (starch hydrolysis) 55
4.17.4 Laccase production activity 55
4.17.5 Lipase producing activity 56
4.18 Growth parameter of plant 56
4.18.1 Shoot length (SL) 56

4.18.2 Leaf Area (LA) 57
4.18.3 Root length (RL) 58
4.18.3 Fresh shoot and dry shoot weight 59
4.19. Water content (WC) 61
4.20. Chlorophyll Content 62
4.21. Total protein content 65
4.21 Molecular Characterization 66
4.23 Summary 67
CHAPTER V 69
DISCCUSSION 69
CHAPTER IV 79
6.1 CONCLUSION 79
6.2 RECOMMENDATION 80
REFERENCE 81
Appendices I
Appendix I Media Composition I
Appendix II calculation IV
Appendix III standard curve V
Appendix IV photograph of treatment VIII

LIST OF FIGURE

Figure: 1 Impact of arsenic on human health (Prakash & Verma et al., 2021) 7
Figure: 2 Structure of different arsenic (Bergqvist et al., 2011.) 9
Figure: 3 Transport of arsenic in plants and PGPR mediated arsenic amelioration in plants (Kumar et al., 2022)...11
Figure: 4 Arsenic toxicity in plants (Mitra et al., 2017). 13
Figure: 5 Arsenic toxicity in plants (Patel et al., 2023)...16
Figure: 6 Arsenic detoxification and metabolism pathways in bacteria (G. Yan et al., 2019)...17
Figure: 7 Direct and Indirect Mechanisms of action by PGPR on plant growth and development...18
Figure: 1 Presence of brown color indicates arsenate (a & b), colon morphology(c&d) and gram staining (e &f) for S1 and M1 respectively 42
Figure: 2 Effect of sodium arsenite on M1 (a, b,c) and S1(c,d,e) after 24,48 and 72 h respectively. 44
Figure 10: Removal of aresenic by M1 (a) and S1 (b) after 24, 48 and 72 h respectively. 45
Figure: 11 Effect of salt (a), salt (b) and temp. (c) On bacteria S1 and M1 46
Figure: 3 : HM tolerance (a&b), biocompactibility of S1 and M1(c&d) and hemolysis test for M1 (e) &S1 (f).. 48
Figure: 13 IAA production ability of the isolates S1 & M1 after 24h (a), 48(b), 72(c) and 96(d) h of incubation at 37ºC 49
Figure: 14 Retention time of M1 (a), S1 (b), Control(c) & standard (d) of citric acid and lactic acid 52
Figure: 4 Blue colour indicating nitrogen fixation abilities of Both S1 and M1 strains (a), Brown colour indicates ammonia production of both S1 and M1 strains,phosphate solubilizing (c) and clear zone indicate zinc solubiling on ZnO(d) and ZnCO3(e) by S1 and M1 53
Figure: 16 HCN activity (a), black colonies with a dry crystalline consistency which indicated positive for biofilm formation (b), 35 stained with crystal violet and

shows adherence to the walls and bottom of the test tube indicates positive for Biofilm product..55

Figure: 17 Clear zone formed around colony in SMA (a) and CMC (b) indicates positive for protease and cellulose, colourless zone surrounding colonies indicates the production of amylase(d), Development of brown color indicating laccase production(e) and precipitate..56

Figure: 5 Effect of sodium arsenite on shoot height (a&b) and leaf area(c & d) of Brassica juncea with and without S1 and M1 PGP ARB. Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM 57

Figure: 6 Effect of sodium arsenite on Root length of Brassica juncea with and without S1 (b) and M1 (a) PGP ARB. Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM. ..59

Figure: 20 Effect of sodium arsenite on fresh weight and dry weight of Brassica juncea with and without M1 (a&b) & S1(c&d) ARB. Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM 61

Figure: 21 Water content of Brassica juncea with and without M1 (a) and S1 (b) PGP ARB. . Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM. 62

Figure: 7 Effect of sodium arsenite on chlorophyll a, b and total chlorophyll content of Brassica juncea with and without M1 (Fig.a, b &f) and S1(c, d &e PGP ARB. Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean mean ± SEM 63

Figure: 23 Effect of sodium arsenite on total protein content of Brassica juncea with and without M1 (a) and S1 (b) PGP ARB. Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM. 65

Figure: 8 Neighbor-joining tree based on 16S rRNA gene sequences, showing the position of isolates S1 and M1, and their closely related reference strains inclusive of other Bacillus species. Bootstrap values (expressed as percentages of 1000 replications)...67

Figure 25 Standard curve for p estimation V
Figure 26 Standard curve of pNP........ V
Figure 27 Standard curve of IAA........ V
Figure 28 Standard curve of BSA V

LIST OF TABLES

Table 1 Bacterial species able to resist to HM through various mechanism 27
Table 2 Isolation of Bacillus species from various soil sample 50
Table 3 Morphological and biochemical test of isolates 52
Table 4 Inhibition zone of bacteria against antibiotics 56
Table 5 Zinc solubilizing and solubilizing index 59
Table 6 Concentration of phosphate solubilizes for Both S1 and M1 60
Table 7 pH of PSB broth after 7 and 15 days 60
Table 8 Organic acid concentration produced by both isolates 61
Table 9 Concentration of ALP and ACP produced by S1 and M1 62
Table 10 Inhibition zone of fungus by S1 and M1 63
Table 11 Detection of Biofilm by microtiter plate assay 64
Table 12 Efficacy of strains S1 and M1 against shoot lenght and leaf area 68
Table 13 Efficacy of strains S1 and M1 against Root length 69
Table 14 Efficacy of strains S1 and M1 against Fresh weight and dry weight of plant 70
Table 15 Efficacy of water content of Brassica juncea with and without M1 and S1 PGP ARB 72
Table 16 Efficacy of chlorophyll a and b of Brassica juncea with and without M1 and S1 PGP ARB 75
Table 17 Efficacy of total chlorophyll content of Brassica juncea with and without M1 and S1 PGP ARB 75
Table 18 Efficacy of total protein content of Brassica juncea with and without M1 and S1 PGP ARB 76

LIST OF ABBREVIATION

ARB	Arsenic resistant bacteria
ATP	Adenosine triphosphate
BNF	Biological nitrogen fixation
BSA	Bovine serum albumin
Ch.a	Chlorophyll
Ch.a	Chlorophylla
Ch.b	Chlorophylla
CTAB	Hexadecyl trimethyl ammonium bromide
DAP	Diammonium phosphate
DAPG	2,4-diacetylphloroglucinol
DCP	Dicalcium phosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetate
HC	Hydrolysis capacity
HCN	Hydrogen cyanide
HPLC	High performance liquid chromatography
IAA	Indole acetic acid
LA	leaf area
MIC	Minimmum inhibitory concebtration
MR-VP	Methyl red-Voges Proskauer
NB	Nutrient Broth
NPK	Nitrogen phosphorous potassium
NPK	Nitrogen, Phosphorus and potassium
OF	Oxidative fermentative

PCR	Polymerase chain reaction
PDA	Potato Dextrose agar
PGP	Plant growth promotion
PGPR	Plant growth promoting rhizobacteria
PPM	Parts per million
PSB	Phosphate solubilizing bacteria
PSE	Phosphate solubilization efficiency
PSF	Phosphorous solubilizing fungi
PSI	Phosphate Solubilization Index
PSI	Photosystem I
PSII	Photosystem II
PSM	Phosphate solubilizing microorganisms
PSMF	phenylmethylsulfonyl fluoride
PVK	Pikovskaya medium
RL	Root lenght
RNA	Ribonucleic acid
ROS	Reactive oxygen Species
SA	Shoot length
SIM	Sulphur indole motility
TCP	Tricalcium phosphate
TE	Tris EDTA
TSB	Trypticase soy broth
TSI	Triple sugar iron
TSP	Triple superphosphate
Wt	Weight

CHAPTER I

INTODUCTION

1.1 Background

Arsenic (As) is a silver-gray, semimetallic substance that become discoloured in air. Arsenic is originating from both natural and anthropogenic sources (Lombi et al., 2000) . Naturally, Arsenic exists in various compound forms such as sulpher(S) and iron (Fe)as realgar (As_4S_4), orpiment (As_2S_4), arsenolite ($FeAs_2$), arsenopyrite (FeAsS), loellingite ($FeAs_2$), cobalitite (CoAsS), white arsenic (As_2O_3), white cobalt ($CoAs_2$), arsenical iron (As_4Fe_3) and nickel glance (NiAsS) within the Igneous rocks. More than 99 % of the As in the world is found in rocks, mainly in silicate minerals, where As substitutes Al, Fe and Si (Bhumbla & Keefer et al, 1994) .

Anthropogenic activities, like a geothermal, mining operations, excess pumping of shallow ground waters, chemical fertilizer, herbicides, and pesticides are responsible for disperse arsenic in the environment. Phosphate is combined within the detergents that are used in the cloths washing having concentration of arsenic ranging from 70 to 80 parts per billion (ppb). Consequently, the water produced as a byproduct of detergent utilization may contain up to 150 ppb of arsenic, which becomes solubilized within the groundwater. Various insecticides and pesticides composed of Arsenic compounds such as Sodium asenate ($NaAsO_3$), sodium asenite ($NaAsO_2$) and calcium arsenate $[Ca(AsSO_4)^2]$ which become solubilized in the ground water due to their interaction with the soil. Phosphate (PO_4^3) available in the fertilizers converts Fe and Mg Oxide/Hydroxide from arsenic to Arsenate (AsO_4^3) and dissolves into groundwater.

The level of toxicity in the different compounds of Arsenic from higher to lower are arsine gas (Ash_3)> arsenite (inorganic, As^{+3})> arsenical pentavalent> arsenoxide> arsonium compounds> arsenate (As^{+5})> elemental arsenic. Arsenic exists in organic or inorganic forms (Sharma & Sohn et al., 2009). It is impossible to completely eradicate it from the surroundings; however, it has the ability to undergo a transformation in its structure. Inorganic and organic arsenic both exhibit a white color, lack odor, and are devoid of taste. Organic arsenic is typically considered as being non-toxic, or less toxic compared to

inorganic arsenic, and is predominantly present in fish and shellfish (Prakash & Verma et al., 2021).

Arsenic is a noxious metalloid classified as a class 1 carcinogen, which is extensively dispersed within the earth's crust. The contamination of arsenic is widespread globally, encompassing regions such as India, China, Bangladesh, Brazil, France, Taiwan, Pakistan, Malaysia, and the United States (Chen et al., 2017; McClintock et al., 2012). As per the research conducted by Phung et al. (2017), an estimated 35-77 million individuals have reportedly been exposed to this metalloid. Increased levels of As are dangerous to human health since they can cause a number of serious illnesses, such as cancer, respiratory, cardiovascular, and neurological diseases. These harmful effects on health could appear even after extended exposure to low levels of the metalloid (Kumar A. et al., 2021; Phung et al., 2017). Both surface water and ground water such as shallow tube well and deep tube well are significantly used for irrigation which is contaminated with arsenic which is deposited on top of agricultural soil. Soil plays a main role in supporting the agricultural production system. As is hindering to plant growth and development when its concentration within the soil surpasses the limit level (Abbas et al., 2018; Rani & Goel et al., 2009). Increased soil As level influences plant development contrarily by inhibiting cellular metabolism and improving oxidative stress, hence diminishing the grain yield and enhancing As substance in crops (Liu et al., 2007; Mitra et al., 2017) . The accumulation of arsenic in food grains serves as a mechanism for its widespread distribution among the human population (Bhattacharya et al., 2010). A general hierarchy of plant parameters based on sensitivity to various metals, including arsenic, can be outlined as follows: root length > root mass > shoot length > total mass (root plus shoot) > shoot mass > germination (Abedin et al., 2002).

According to (Huq et al., 2006), the contamination of agricultural land with arsenic from soil led to its desolation and had adverse effects on human health by means of the food chain. There are various available technologies that can be used for the removal of arsenic from water and soil such as oxidation, coagulation precipitation, filtration adsorption, ion exchange and membrane. These techniques are expensive and time consuming, so to solve these problems alternative method is required called Bioremediation. Because of its

efficacy, affordability, environmental friendliness, and adaptability in handling different kinds of contamination, bioremediation is frequently chosen.

In order to address arsenic contamination problem, researchers have explored the use of arsenic-resistant bacteria as a potential solution. These bacteria possess the ability to resist and reduce/oxidize arsenic, making them ideal candidates for bioremediation purposes. Arsenic-resistant bacteria could be a valuable tool in mitigating the effects of arsenic contamination in agriculture. The isolation and characterization of ARB from contaminated sites provide promising opportunities for bioremediation and plant growth promotion. In regions where agricultural soil is contaminated, research is conducted on the application of bacteria resistant to arsenic contamination for the goals of bioremediation and plant growth promotion.

For the purpose of bioremediation especially focused on isolation of *Bacillus* species due to the widespread presence of *Bacillus* species in the environment and prolonged exposure to arsenic may result in the development of arsenic-resistant mechanisms including arsenite oxidation and methylation (Mallick et al., 2014). As a result, it is crucial to use *Bacillus* species from such an environment for arsenic bioremediation and also exhibited high resistance to arsenite and arsenate, as well as multiple heavy metals due to their involvement in organic acid production, plant growth-promoting (PGP) features, enzyme formation, arsenic bioremediation, and other areas, *Bacillus* species are currently of great interest (Poudel et al., 2016; Sapkota et al., 2019). It has been discovered that *Bacillus* species possess chromosomal or plasmid-borne *arsC* genes (Jain et al., 2011). These genes have the ability to extract arsenic from contaminated environments (Mohamed & Farag et al., 2015).

Performance of ARB PGPR was show on plant green mustard due to their abilities to phytoremediate heavy toxic metal to some extend. Phytoremediation is defined by (Salt et al., 1998) , the utilization of plant species to eliminate or diminish the harmfulness of toxic substances in the surroundings for example *Brassica napus* and *Cicer arietinum* were also utilized for the phytoremediation of soil contaminated with arsenic (Gupta D. K. et al., 2008; Nie et al., 2002) respectively. Broad Leaf Mustard, also referred to as mustard green, is scientifically known as *Brassica juncea (L.) Czern* (Marfa rayo). Green mustard is predominantly distributed in regions ranging from Central to Eastern Asia and is commonly

identified as 'Rayo' within the Nepalese context Mustard greens, known as "*raayo ko saag*," are a prevalent winter vegetable widely cultivated from November to April.

Brassica species (e.g., *Brassica juncea, Brassica napus*) have ability to tolerant heavy metal because of their inherent ability to produce high above ground biomass and extract high quantities of heavy metals from contaminated soils or water (Salido et al. 2003). MicroRNA-specific microarray analysis of *B. juncea*, identified role of As-specific microRNAs in regulating sulfur metabolism, and metabolism and function of hormones like jasmonates, auxins, and ABA (Srivastava et al., 2013). Indian mustard plant, *Brassica juncea (L.)Czern.*, is known to accumulate Zn, Cu, Pb, Cr, Cd and As (Belimov et al., 2005; Ebbs & Kochian et al., 1997). Despite the potential of *B. juncea* to remove many toxic metals from the soil, phytoremediation is yet to become commercially available technology as contradictory results have been reported regarding the efficiency of this plant in phytoextraction (Belimov et al., 2005; Clemente et al., 2005a; Salido et al., 2003). Thus, application of arsenic resitant plant growth promoting *Bacillus* species can be used to enhance the growth of vegetable plants even in arsenic contaminated soil.

1.2 Research Hypothesis

a) **Null hypothesis Null Hypothesis (H0):** There is no significant difference in the arsenic-resistant bacteria and their plant growth-promoting efficacy in agricultural soil.
b) **Alternative Hypothesis (H1):** There is a significant difference in the arsenic-resistant bacteria and their plant growth-promoting efficacy in agricultural soil.

1.3 Objectives

General objectives

i) To explore hyper-tolerant arsenic resistant *Bacillus* species from Nepalese agriculture soil and study their plant growth promoting efficacy

Specific objectives

i) To isolate the arsenic resistant *Bacillus* species from soil
ii) To screen ARB based on minimal inhibitory concentration of arsenic.
iii) To investigate potent plant growth promoting abilities of isolated *Bacillus* specis

iv) To assess the biological control activities of isolates

v) To perform pot experiments using potent PGPB and ARB on *Brassica juncea* L. Czern.

vi) To evaluate the growth performances of the plants under various treatment conditions

vii) To identify the potent arsenic resistant and plant growth promoting *Bacillus* species using 16S rRNA gene sequencing

1.4 Rationale of study

Generally high concentration of As is present in groundwater compared to surface water. The groundwater is mostly used in the irrigation and drinking purposes in Terai region. It has been reported that the Terai agricultural land irrigated through shallow tube wells and deep tube wells arsenic contamination exceeded above the standard limit. Additionaly, there are major health issues reported in Terai region due to consumption of arsenic contaminated tube well water. Arsenic can be accumulated in crops, leading to food chain contamination.Therefore, addressing arsenic contamination in agricultural soil is essential for ensuring food security and safeguarding public health in Nepal. Exploring the potent high tolerant ARB for bioremediation which offers a sustainable and cost-effective approach to mitigate arsenic contamination in agricultural soils.

ARB are also reported to show plant growth-promoting abilities, such as nitrogen fixation, phosphate solubilization, phytohormones production, Zinc solubilization, heavy metal detoxification hydrolylic enzyme production and biocontrol activities against phytopathogen which enhance soil fertility, improve nutrient availability to plants, and ultimately increase crop yields. This study focuses on the isolation and characterization of hyper tolerant arsenic-resistant *Bacillus* from Nepalease agricultural soil and investigates their potential as plant growth-promoting efficacy. The aim is to explore hyper-tolerant arsenic resistant *Bacillus* species from Nepalese agriculture soil and study their plant growth promoting efficacy. This research can contribute to the development of sustainable and eco-friendly strategies for improving plant growth in arsenic-contaminated soils as bio fertilizers for sustainable agriculture.

CHAPTER II

LITRATURE REVIEW

Arsenic mainly exists in the environment as arsine (III), elemental arsenic (0), arsenite (III) and arsenate(V). Among all these forms only arsenite and arsenate are more abundant in natural environment than the other form. The permissible limit of arsenic in agricultural soil is 20 ppm as per the recommendation of the European Community (Kabir et al., 2016). Soil As above this level is detrimental to plant growth and food quality.

2.1 Arsenic contamination in agriculture soil of Nepal

Soil contamination by arsenic, due to highly contaminated irrigation water, and its carryover effect to plants have been reported in Bangladesh and India (Ali et al., 2003).

Almost 66 % of people used groundwater for drinking and 90% of population using groundwater for domestic purpose including irrigation and drinking (Neku & Tandukar et al., 2003; Pokhrel et al., 2009) . According to FAO, the guideline value of arsenic irrigation drinking water is 0.1 mg L^{-1} (10 ppm) (Dahal et al., 2008; Pokhrel et al., 2009).

Approximately 200,000 tube well could exist in terai region (Shrestha R. et al., 2003) and 800,000 according to (World Bank, 2005). Approximately, 69 % of total tube wells have arsenic concentrations 50 ppb and 2.29 million and 0.37 million of population is expected to be drinking water having arsenic concentration 10 - 50 µg/L and >50 µg/L respectively (Kohnhorst et al., 2005; Shrestha R et al., 2012). Half a million people have been exposed to arsenic levels greater than 50 µg/L in their drinking water and 31% of population (3.5 million) in the region are estimated to have been exposed to arsenic levels between 10 and 50 µg/L (Pokhrel et al., 2009).

According to Shrestha et al. (2003), drinking water with As levels above 0.050 mg/L is reportedly consumed by 0.5 million individuals.

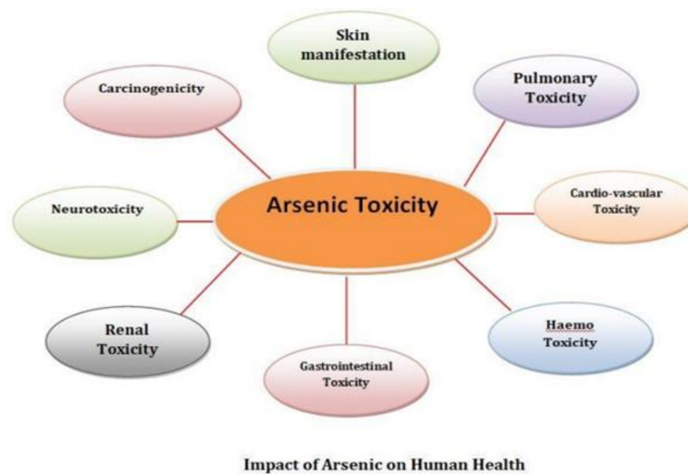


Figure: 9 impact of arsenic on human health (Prakash & Verma et al., 2021)

2.2 Determination of arenite

The arsenic contaminated soil and water may be determined by many methods such as atomic adsorption spectroscopy (AAS), inductively coupled plasma atomic emission spectroscopy (ICP-AES), electrochemistry and spectrophotometry with silver diethyldithiocarbamate. (Tien et al., 2015) presents a study on the arsenic determination from water by spectrophotometric method based on molybdate reagent.

2.3 Arsenic resistant bacteria

Arsenic oxidizing bacteria have a unique kind of enzyme called arsenic oxidase in their protoplasm. This enzyme allows the bacteria to oxidize arsenite to arsenate.Numerous bacteria resistant to high concentrations of arsenic have been found; these microbes may be employed in the bioremediation of arsenic-contaminated ground water.

Isolation of arsenic resistant and plant growth promoting *Bacillus flexus* in Nepalese soil (Pandey et al., 2020). Arsenic contamination in soil and water is a significant environmental problem in Nepal, posing a threat to the ecosystem and public health and to assess the plant growth-promoting activities of arsenic-resistant *Bacillus* species in the Terai region of Nepal (Magar et al., 2022a) . Arsenic-resistant bacteria have been isolated and studied in Nepal. In the Terai region of Nepal, several studies have identified arsenic-resistant *Bacillus* species in soil and water samples (Magar et al., 2022a; Poudel et al., 2019).These Bacillus species, including *B. subtilis subsp. stercosis, B. flexus, B. licheniformis, B. cereus, and B. flexus*, have shown plant growth-promoting activities such as indole acetic acid (IAA) production and phosphate solubilization (Bista & Shakya et al., 2017a). These isolated bacterium from arsenic-enriched water in Rautahat district, exhibited high resistance to

arsenite and arsenate, as well as multiple heavy metals due to their involvement in organic acid generation, plant growth-promoting (PGP) features, enzyme production, arsenic bioremediation, and other areas, *Bacillus* species are currently of great interest (Poudel et al., 2016; Sapkota et al., 2019). Due to the widespread presence of *Bacillus* species in the environment, which includes arsenic and its enriched form, prolonged exposure to arsenic may result in the development of arsenic-resistant mechanisms including arsenite oxidation and methylation (Mallick et al., 2014) . As a result, it is crucial to use *Bacillus* species from such an environment for arsenic bioremediation. It has been discovered that *Bacillus* species possess chromosomal or plasmid-borne *arsC* genes (Jain et al., 2011). These genes have the ability to extract arsenic from contaminated environments (Mohamed & Farag et al., 2015).

2.4 Arsenic oxidising bacteria

Oxidation of Arsenic by Arsenic-resistant bacteria can defend themselves against arsenite and arsenate through the *ArsRBC* efflux mechanism. However, a subset of bacteria resistant to arsenic have generated arsenite oxidation systems that mitigate arsenite's toxicity by changing As(III) into As(V). There are two different bacterial arsenite oxidases, AIO and ARX (Yan G. et al., 2019). The majority of the As(III) oxidizing bacteria possess the AIO arsenite oxidase. The AIO arsenite-oxidizing bacteria are heterotrophs (i.e., bacteria use organic carbon compounds as energy and carbon sources for growth and the As(III) oxidation process is a detoxification mechanism of the bacteria (Santini et al., 2000) . The ARX arsenite oxidase is found in chemolithoautotrophic bacteria that use arsenite as an electron donor to generate energy for growth. They can be aerobic or anaerobic bacteria depending on whether oxygen or nitrate is used as a terminal electron acceptor, respectively (Zargar et al., 2010) .For instance, *Rhizobium* sp. strain NT-26 is an aerobic chemolithoautotrophic As(III) oxidizing bacterium (Vanden Hoven & Santini et al., 2004) and *Alkalilimnicola ehrlichii* MLHE-1 is an anaerobic chemolithoautotrophic As(III) oxidizing bacteriu (Zargar et al. 2010). *Bacillus flexus* strain SSAI1 isolated from agro-industry waste in India displayed high arsenite resistance and efficiently oxidized arsenite to less toxic arsenate (Sun et al., 2023). Additionally, a *Bacillus* strain IIIJ3-1 high arsenic resistance, aerobic arsenite oxidation, and dissimilatory reduction of arsenate, indicating its potential for bioremediation strategies in arsenic-contaminated environments (Herrera et al., 2021).

2.5 Arsenic uptake from soil

The first compartment of the plantpart subjected to a substance via the roots is the apoplast. Localization of As in the apoplast may make a significant contribution to the total amount of As in a plant.

As (V) , As(III) and Methylated arsenic species uptake from soil

Genetic evidence for high affinity phosphate transporters being involved in As(V) uptake by plants (Bienert & Jahn et al., 2010). The major form of As in aerobic soils is AsV , which is structurally a chemical analog of phosphate and enters plant root tissue via phosphate (Pi) transporters (Farooq et al., 2016). Arsenate is mainly absorbed by phosphate transporters from Pht family while arsenite is taken up via aquaporins (Clemente et al., 2005b; Meharg et al., 2005). While As(V) predominates in an oxygen-rich environment (aerobic), As(III) is largely found in anaerobic environments like paddy soil and groundwater (Mondal and Garg et al., 2017). As(III) has been reported sixty times more toxic than As(V) (Ratnaike et al., 2003). As(III) have high affinity for sulfhydryl groups and can bind to reduced cysteines in peptides and proteins (Zhou et al., 2011). Further, As(V) has a higher affinity to iron oxides than As(III), which makes As(V) less mobile in soil (Wang et al., 2019). Structurally, As(V) mimics phosphate, and hence transported inside of the root cells through phosphate transporters (PHTs) (Wang M. et al., 2019; Zhou et al., 2011).

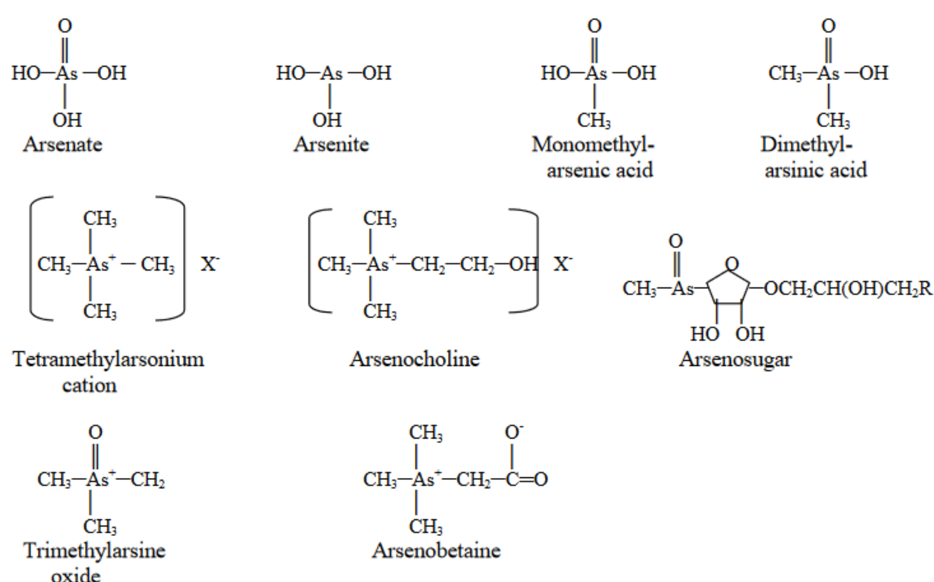


Figure: 10 Structure of different arsenic (Bergqvist et al., 2011.)

As(III), monomethylarsonic acid [MMA(V)] and dimethylarsinic acid [DMA(V)] structurally mimics silicic acid and makes its entry via various classes of aquaglyceroporins and more predominantly via nodulin 26-like intrinsic protein (NIP) class of aquaporin channels (Ali et al., 2009; Li et al., 2009; Pajuelo et al., 2019). Lsi1, a NIP class transporter, is located in the root and is a passive aquaporin for both silica and arsenic. Methylated As species like MMA and DMA can be taken up by plant roots but the rates of uptake are less efficient than those of inorganic As species (Li et al., 2009; Jia et al., 2012; Abedin et al., 2002). AsV adsorption was 5 and 2.5 times higher than DMA and MMA, respectively (Raab et al., 2007c).

2.6 Arsenic translocation within plant

Long-distance transportation of arsenic occurs via the xylem and phloem. Generally belived that inorganic arsenic is transported from roots to shoots via the xylem (Zhao et al., 2012). Arsenate have molecular resemblance to phosphate, As(V) disrupts oxidative phosphorylation and the synthesis of ATP once it has entered plant cells (Finnegan and Chen, 2012). However, intracellular As(V) is quickly detoxified to As (III) by arsenate reductase and effluxed out of the plant cell since As(III) efflux pump is present (Duan et al., 2007). The relative distribution of As in plants shows that *Brassica* sp. accumulated As mainly in the roots followed by shoots and flower(Chintakovid et al., 2008). Both species of arsenite and arsenate were found in xylem sap from stems of *Brassica juncea*(Pickering et al., 2000).

However, As(III) interacts with protein thiol groups to cause lipid peroxidation and a decrease in enzyme activity (Tripathi et al., 2007). A portion of As (III) is also uploaded to the xylem and translocated into the shoot through the silicon efflux Lsi2 transporter (Pajuelo et al., 2019) because As(III) resembles polyols chemically, a sugar transporter exports it to the phloem, where it enters the grains (Schneider et al., 2007).

2.7 Hyperaccumulator

According to Rascio and Navari-Izzo (2011), a family of plants known as hyperaccumulators have the ability to tolerate and accumulate extremely high concentrations of hazardous metals.It has been determined that Pteris vittata is an arsenic hyperaccumulator, possessing a remarkable capacity to both accumulate and tolerate arsenic upto 20 mM

arsenate (Ma et al., 2001). Uptake of arsenite by Indian mustard genotypes was higher than that of arsenate and Stunted growth of the plants (Chaturvedi et al., (2006).

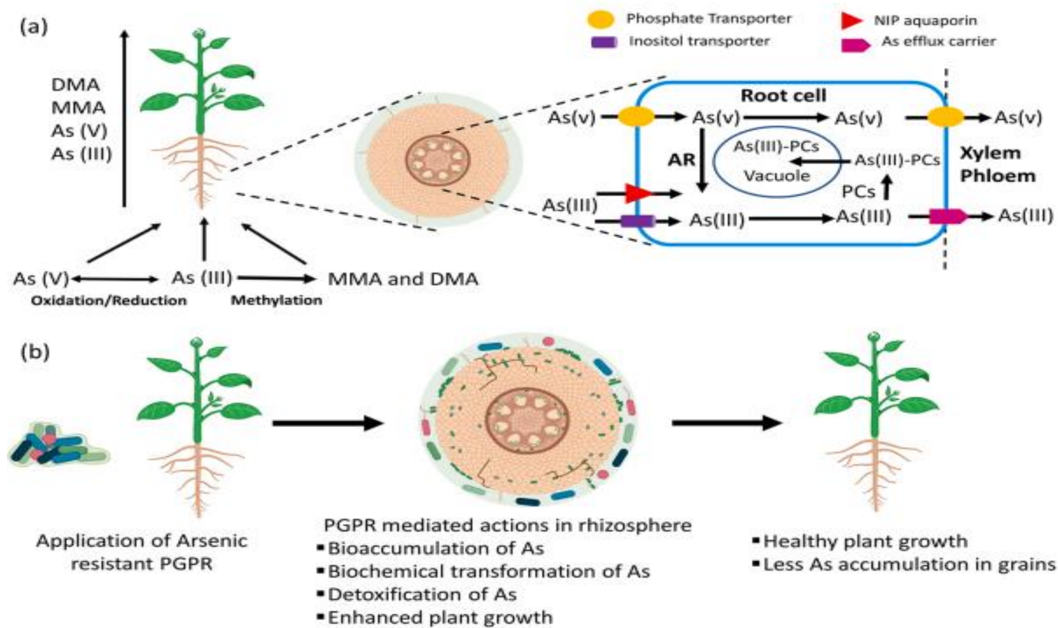


Figure: 11 Transport of arsenic in plants and PGPR mediated arsenic amelioration in plants (Kumar et al., 2022)

2.8. Factors affecting As speciation, availability, and mobilization into plants

2.8.1. Plant genotype

One of the key elements in the As accumulation and mobilization in plants is plant genotype. Under As stress, it has been observed that high-yielding varieties (HYVs) of rice collect more As in the plant and grains(Samal et al., 2021). *Brassica juncea* produces brassinosteroids such as castasterone, typhasterol, teasterone, and 24-Epibrassinolide under As stress, which contribute to the stress protection response (Kanwar et al., 2015) .

2.8.2. Rhizosphere microbiome

Microorganisms mediated changes in As biotransformation processes, such as reduction, oxidation, methylation, and de-methylation affect the As biogeochemical cycling and determine the As bioavailability to the plants (Upadhyay et al., 2020). Anaerobic bacteria possessing respiratory reductase (ArrA) can use As(V) as the terminal electron acceptor, leading to the release of more toxic As(III) (Beigmohammadi et al., 2023). Bacteria in the rhizosphere bio-accumulate As, decreasing its availability to plants (Nabi et al., 2023). The

plant microbiome plays a pivotal role in the release, translocation, and accumulation of As in plants (Anand et al., 2023). Soil microbial activity can affect As adsorption/desorption, solubility, bioavailability, mobility, and soil-plant transfer by altering the chemical speciation of As in soil (Saharan et al., 2023). Bacterial species such as *T. thermophiles, T. Aquaticus, P. arsenitoxidans, C. arsenates, Bacillus , D. auripigmentu, G. barnesi,* and *G. arsenophilus* are capable of synthesizing arsenite oxidase and oxidize As(III) into As(V) (Tsuchiya et al., 2019) . Similarly, microorganisms can also reduce As(V) into As(III) via dissimilatory reduction. The bacteria capable of reducing As(V) include *Bacillus, G. arsenophilus, G.barnesi, C .arsenatis, S. barnesii, S.arsenophilum, Oselenatis, and D. auripigmentu* (Cheng et al., 2022).

2.8.3. Soil Physico-chemical properties

The physico-chemical and biological characteristics of the soil play a major role in determining the speciation of As in the soil, which determines its translocation in plants. Speciation is the existence of an element in distinct mineral phases, oxidation states, and chemical forms(De Mello et al., 2007). Arsenic uptake influence by, soil redox potential (EH), organic matter, soil phosphate, soil pH, silicon, and iron etc.

Soil pH

Both low and high soil pH have been shown to promote arsenic uptake and accumulation in plants (Mitra et al., 2017) . Because arsenic-binding species are more soluble in acidic environments, plant absorption of As is increased (Mawia et al., 2021). In contrast, alkaline soil conditions increase the surface charges that are negatively oriented, which facilitates the As desorption from iron oxides (Mawia et al., 2020).

Soil redox

The redox state of the soil influences As speciation as well. Reduced soil EH in anaerobic soil conditions increases the mobility of soil As(III), particularly in paddy soil that has been flooded with water (Gorny et al., 2015) . Because of the accelerated reduction of As (V) to As(III), lowering the soil EH raises the levels of As(III) in the soil. Elevated levels of organic matter in the soil stimulate microbiological activity and limit the amount of environment that is conducive to Fe-oxyhydroxides dissolving and increasing As mobility (Ma R. et al., 2014) .

Soil phosphate

Due to competitive inhibition, high soil accessible phosphate inhibits arsenate mobilization because plants absorb both through the same transporter. Phosphate, however, also moves arsenate away from iron plaque and soil binding sites, increasing the bioavailability of arsenic (Smith et al., 2002) .

Silicon

Arsenite's chemical counterpart, silicon, is carried by the same transporter in rice (Lsi1 and Lsi 2). Rice requires silicon for proper growth and development, and rice absorbs less arsenite when the soil has a high silica content (Kumar S. et al., 2022).

Irons/Fe plaque formation

As speciation and its movement in soil are also influenced by iron. the oxygen that the rice root releases during anaerobic conditions oxidizes Fe(II), forming iron plaque surrounding the root surface. By scavenging soluble As, this iron plaque lowers the absorption of As (Lee C.H. et al., 2013).

2.9 Impact of arsenic in plant

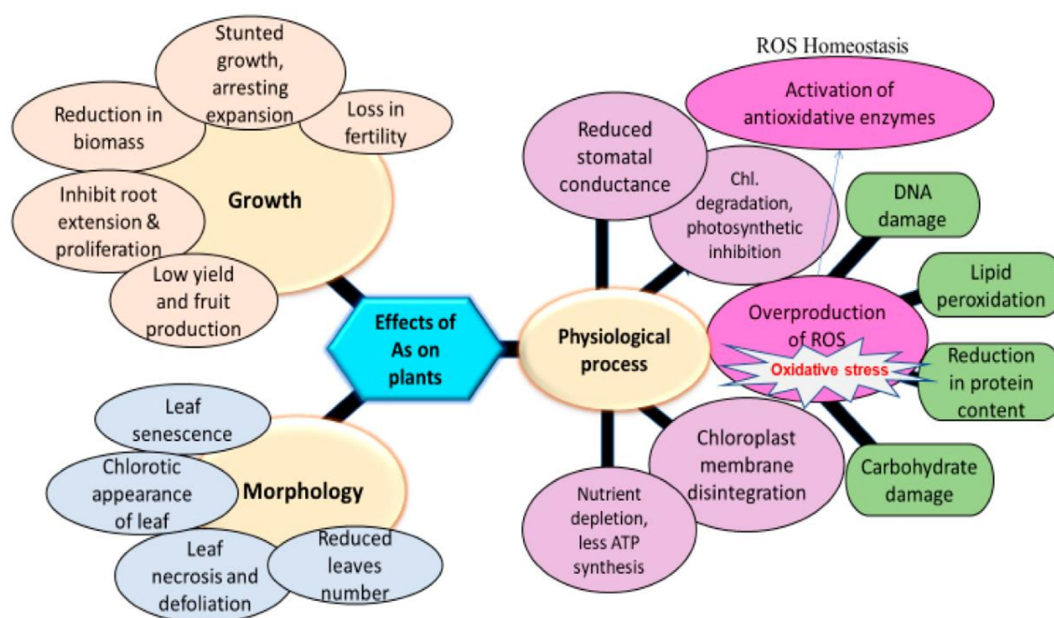


Figure: 12 Arsenic toxicity in plants (Mitra et al., 2017).

A. physiological effect of Arsenic on plant

Effect of arsenic on plant growth

According to (Shahid et al., 2015), arsenic presence in soil might interfere with plants capacity to metabolize nutrients normally, which can result in stunted growth and low crop yield. Exposure to AS stress resulted in noticeable abnormalities to the normal functioning of seedlings such as slowed growth of roots ,shoots and reduces a plant's fresh and dry mass, leaf area, and leaf number of *C. arietium* (Malik et al., 2011) and *O. sativa* (Vromman et al., 2013). The root length is significantly more responsive than shoot height for arsenic (Abedin et al., 2002) . The following chain of events was put by (Abedin & Meharg, et al 2002) due to arsenic: decreased shoot height, decreased leaf area, decreased photosynthesis, and decreased yield.

Impact of arsenic on photosynthesis of plant

Plant photosynthesis is significantly inhibited by arsenic accumulation (Chandrakar et al., 2016; Siddiqui M. H. et al., 2020) . In As-grown plants, the loss of carotenoid pigments occurs concurrently with the concentration-dependent loss of chlorophyll, although the latter process is less sensitive to As than is the synthesis of chlorophyll (Q. Wang et al., 2011). A decrease in chlorophyll biosynthesis in Zea mays (Emamverdian et al., 2015) seedlings was reported due to As-induced stress. Chlorophyll content can decrease due to effects on the light harvesting equipmen (Rahman A. et al., 2007; Porter & Sheridan et al., 1981) and photosystem II activity (Stoeva and Bineva et al, 2003).

Effect of arsenic on ATP synthesis

As(V) is mostly accumulated and metabolized by plants via Pi transport pathways. Chemically, As(V) is quite similar to Pi molecules, which have the ability to interfere with ATP synthesis's at least phosphate-dependent metabolic processes (Singh V.P. et al., 2005). While As(V) and Pi can compete to transport proteins present on the root surface, As(V) does not have the same affinity for Pi molecules as do these transport proteins (Tawfik & Viola et al., 2011). Thus, a decrease in As(V) uptake in plants may result from increased Pi availability in the rhizosphere (Bhattacharya et al., 2012; Tawfik & Viola et al, 2011).

Effect of arsenic toxicity on membrane integrity

Unbalanced nutrient intake and water transport are likely the cause of As detrimental effects on the transpiration process in plants (Singh N. et al., 2006).

B.Biochemical and molecular effect on plant

Arsenic induced Reactive oxygen species (ROS) generation

As(III) is more detrimental to plant growth and generate more ($O^{2\bullet-}$) than As(V), which generate more ($^{\bullet}OH$) and an damage proteins, amino acids, purine nucleotides and nucleic acids and cause peroxidation of membrane lipids (Abbas et al., 2018; Møller et al., 2007).

Impact of Aesenic on carbohydrates Metabolism in plant

The metabolism of important carbohydrates like sugar and starch was negatively impacted by As exposure(Jha & Dubey et al., 2004).

Arsenic effect on lipid metabolism

Several plant species exposed to As stress showed increased cellular electrolyte leakage and cellular membrane damage (Meharg & Hartley-Whitaker et al., 2002; H. P. Singh et al., 2009; Srivastava et al., 2013).

Arsenic effect on protein metabolism

Inorganic As Because of their stronger affinity for sulfhydryl groups in proteins, such forms can effectively impair plant metabolism, resulting in membrane damage and cellular death. Plants that are exposed to arsenic have lower total protein contents (Ismail et al., 2012; . Singh H.P. et al., 2009; Singh N et al., 2006a) .Proteases and peptidases often catalyze the breakdown of proteins into free amino acids. Plant growth and development are suppressed and protease levels are decreased as a result of As exposure(Ismail, 2012). Exposure to As caused decreases in total plant protein in *P. ensiformis* and *P. vittata* (Singh N. et al., 2006), *Trifolium pretense* (Mascher et al., 2002), Oryza sativa (Singh H.P. et al., 2009) .

Arsenic impact on change in DNA structure

According to reports, genotoxic reactions can occur when plants and animals are exposed to harmful amounts of As (Patra et al., 2004) . Numerous investigations have revealed that the early correlation between genotoxicity and ROS generation during its biotransformation (Dalle-Donne et al., 2006). As a result, the generated ROS can lead to oxidative base damage and DNA and protein (Cadet & Wagner et al., 2013) ,a break-up of

chromatid/ chromosome or exchange (Patra et al., 2004), the formation of apyrimidinic/apurinic sites (Faita et al., 2013), DNA protein cross-links (Woźniak & Blasiak et al., 2003) , chromosomal aberrations, sister chromatid exchange, micronuclei formation, and aneuploidy and deletion (Kitchin & Wallace et al., 2008).

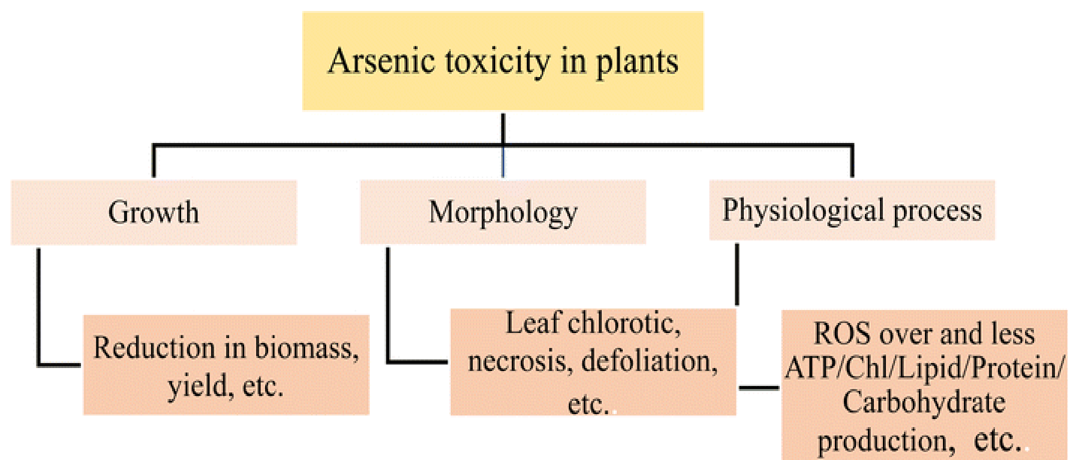


Figure: 13 Arsenic toxicity in plants (Patel et al., 2023)

2.10 The Microbial Detoxification of Arsenic Related to Bioremediation

There are four processes by which microorganism utilizes to metabolically utilize environmental arsenic.

i) Microbial As(III) Oxidation(aio/arx) system;

When microorganisms are faced with As(III), cellular energy is gained from the oxidation of the As(III) by using it as an electron donorin the presence of arsenate oxiade enzyme and conveted in less toxic As(V) (Santini et al., 2000; Vanden Hoven & Santini et al., 2004). An enzyme known as arsenite oxidase and member of the dimethylsulfoxide (DMSO) reductase family, is necessary for bacteria to be able to detoxify arsenic that is present in its surroundings (Anderson et al., 1992). According to (Lett et al., 2012), the geenes *aioA* and *aioB* are linked to arsenite oxidation. ASO-6 aoxB gene and As (III)-oxidase enzyme activity in *Bacillus flexus* have been reported (Das et al., 2016).

ii) Microbial As(V) Reduction (*arr*) system

The cytoplasmic arsenate reduction system *(ArsC)* is one of the reduction mechanisms (Kruger et al., 2013). Arsenate reductase enzyme ArsC (13–15 kDa) mediates the reduction of As(V) to As(III) using ferredoxin or glutathione as an electron donor when As(V) enters the bacterial cell through Pit or Pst (known as phosphate transporters). Genes encoded in

the *arr* operon are found in the respiratory arsenate reductases *ArrA* and *ArrB* are the genes (Macy et al., 2000). The Arr proteins from *Bacillus selenitireducens* (Afkar et al., 2003) and *Chrysiogenes arsenatis* (Anderson et al., 1992) were purified and characterized.

i) Arsenic methylation

A family of As(III) S-adenosylmethionine (SAM) methyltransferase enzymes (ArsM) in microorganisms mediates the detoxifying process of methylation of As (G. Yan et al., 2019). The more toxic As(III) and As(IV) is converted by microorganisms into less toxic methyalated volatile forms like trimethylarsine (TMA) or nonvolatile forms like monomethylarsinate (MMA), dimethylarsinate (DMA), and trimethylarsinate (TMAO) (Bentley & Chasteen et al., 2002; Wu et al., 2002) .

iv)Arsenic resistance (ars) system;

The arsenic resistance (ars) system is the most extensively studied arsenic detoxification pathway in bacteria. The ars operons have been found located either in chromosomes or on plasmids in gram-positive or gram-negative bacteria. The most common are the three-gene operon *arsRBC* as and the extended five gene operon arsRDABC (as on *E. coli* plasmid R773) (Chen et al., 2017).

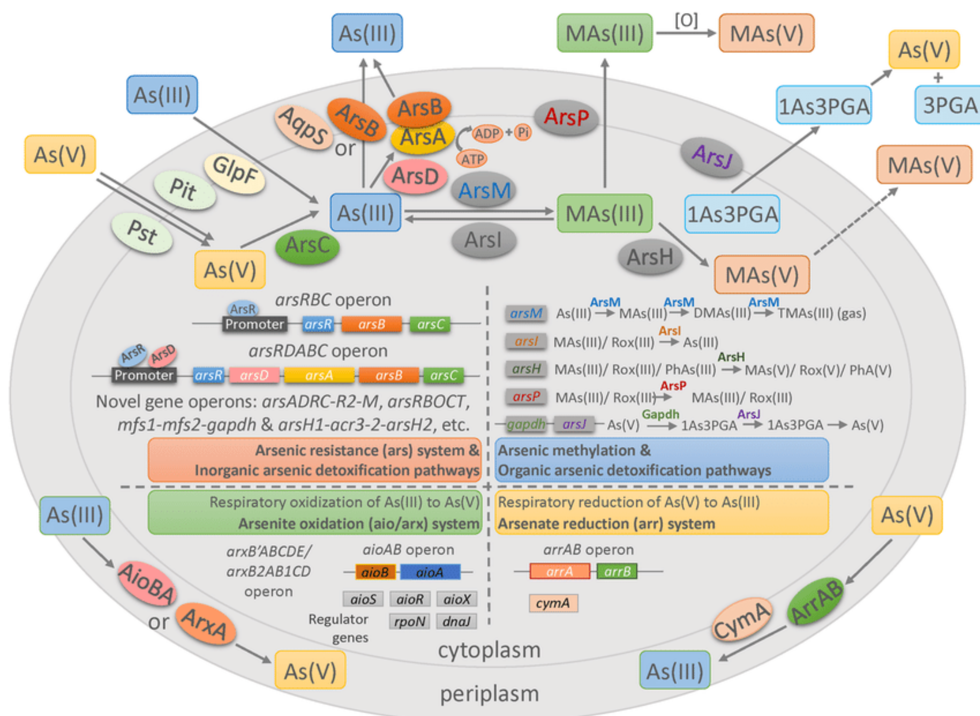


Figure: 14 Arsenic detoxification and metabolism pathways in bacteria (G. Yan et al., 2019)

2.11 PGPR mediated enhancement of As phytoremediation in plants

Brassica juncea produces brassinosteroids such as castasterone, typhasterol, teasterone, and 24-Epibrassinolide under As stress, which contribute to the stress protection response (Kanwar et al., 2015).

Rhizosphere bacteria with plant-growth-promoting ability and As tolerance can be utilized for bioremediation and rhizoremediation (Ramakrishna et al., 2019).

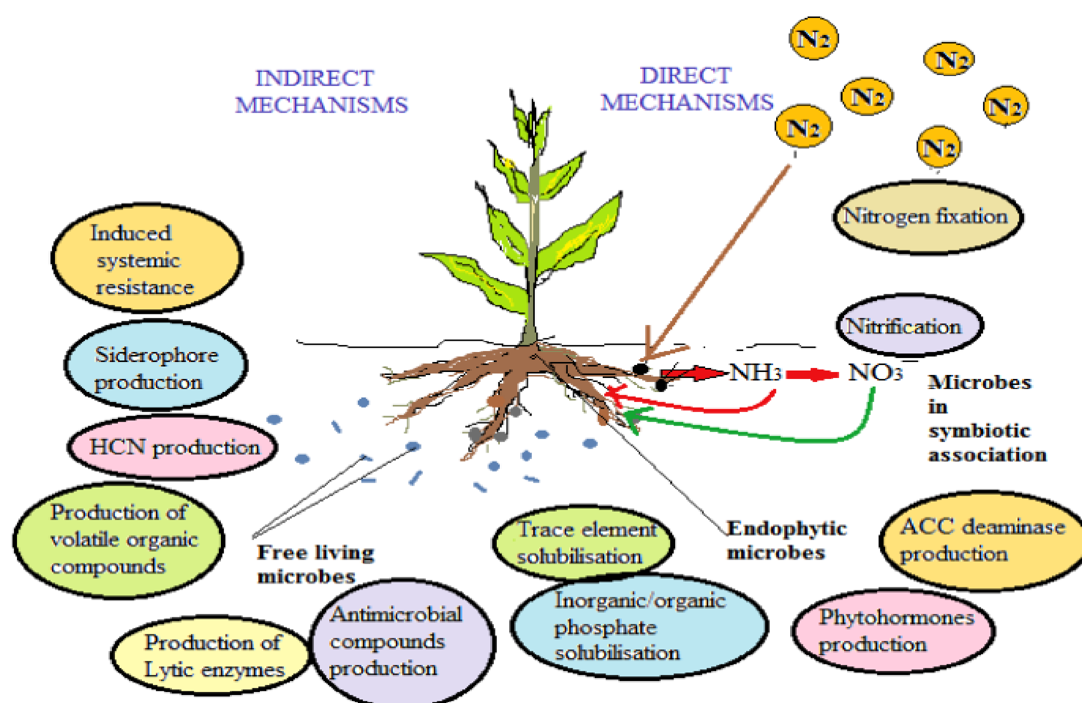


Figure: 15 Direct and Indirect Mechanisms of action by PGPR on plant growth and development

2.11.1 Biological nitrogen fixation

The microbes have the ability to fix nitrogen on their own or in conjunction with plants in symbiotic and associative relationships. Because this metalloid has a detrimental effect on biological nitrogen fixation, only a small percentage of nitrogen-fixing bacteria are able to maintain their nitrogen-fixing potential at high As levels (Mukherjee et al., 2019).

As stress has been shown to impair the metabolic processes of free-living diazotrophs (Chakraborty et al., 2017). High As levels in free-living nitrogen-fixing bacteria have a deleterious effect on the expression of the nifH gene (Chakraborty et al., 2019) . According to reports, nitrogen fixation capacity was found in the arsenic-resistant PGPR *Bacillus*

aryabhattai MCC3374, and Pantoea dispersa (Govarthanan et al., 2015; Mukherjee et al., 2019).

2.11.2 Phosphate solubilization

Phosphate solubilizing microorganisms are soil microorganisms that have the ability to mineralize and solubilize insoluble soil phosphorus into phosphorus that is accessible to plants (Alori et al., 2017) by producing organic acids and phosphate-solubilizing enzymes (phosphotases, phytases, and C-P lyases).Bacteria such as *Pseudoxanthomonas mexicana* and *Stenotrophomonas maltophilia* have shown resistance to arsenic and the ability to solubilize phosphate (Huda et al., 2022). *Bacillus nealsonii* and *Bacillus tequilensis* have demonstrated phosphate solubilization and plant growth promotion in the presence of arsenic (Pandey et al., 2020).

Alkaline and acid phosphatases

Organic P mineralization is catalyzed by a group of phosphatase enzymes , among which extracellular alkaline and acid phosphatases are considered to play major roles in this process (Nannipieri et al., 2011). ACP is more effective in mineralizing organic phosphorus in acidic soils with pH values less than 7. Alkaline phosphatases (ALPs) mainly catalyze the hydrolysis of phospholipids (i.e., phosphoglucose-6 and ATP) and release inorganic phosphorus in soils with pH values higher than 7 (Luo et al., 2019). *Bacillus amyloliquefaciens* S-1 had an alkaline phosphatase activity of 2594.73 U/L (Bo et al., 2023). Another study isolated a phosphate-solubilizing microorganism identified as *Bacillus* sp. (PSB-29), which produced alkaline phosphatase and solubilized phosphates (Odeniyi & Turaki et al., 2022). Additionally, a proteolytic strain identified as *Bacillu*s sp. Mar64 was found to produce alkaline protease (Kotb et al., 2023) and a novel *Bacillus* species *B. salipaludis* was isolated from soil and found to produce alkaline phosphatase (Xue et al., 2021). Acid phosphatase is primarily derived from plant roots and microorganisms, whereas alkaline phosphatase is mainly produced by microorganisms (Spohn & Kuzyakov et al., 2013).

2.11.2. Organic acid production (OA)

Organic acids are the source of biotical generated H+ ions, which can mineralize bound phosphate present in soil and make it available for the uptake by plants (Bhattacharyya &

Jain et al., 2000). *Bacillus valezensis* produced the most lactic acid, while *Bacillus sp*. and *Bacillus subtilis* produced the most acetic acid (Setiawati et al., 2022). *B. licheniformis* and *B. subtilis* have different production rates of organic acid (Yan Z. et al., 2013). *Bacillus aciditolerans* is a novel species of *Bacillus* that produces acidic phosphatase and was isolated from soil (Bo et al., 2023).

2.11.3 Zinc solubilization

Zinc plays an important role in biosynthesis of many enzymes which are involved in plants metabolic reactions (Saravanan et al., 2007). Zinc can be absorbed by plants as the soluble divalent cation Zn2+, although the majority of zinc in soil is found in insoluble fractions (Alloway et al., 2008). Genus *Bacillus* is one of the most studied genera as they are found to be ubiquitous in nature with multiple growth promoting traits(Ramírez & Kloepper, 2010; Zhao et al., 2015). The Zn solubilizing *Bacillus* strains solubilize unavailable zinc through production of chelating ligands, secretion of organic acids, amino acid, vitamins and phytohormones, and through oxido-reductive systems and proton extrusion (Saravanan et al., 2007a).

2.11.4 Resistance to heavy metal

According to the World Health Organization (WHO), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), lead (Pb), nickel (Ni), mercury (Hg), and zinc (Zn) are most dangerous metals (Pandiyan et al., 2021). As, Cd, and Pb are heavy metals that should be taken very seriously (Zhu et al., 2014). Bacteria can help plants to resist stress and improve plant growth and productivity. Bacterial can transform HMs into less toxic forms and alteration of their availability (Y. Ma et al., 2016; Tirry et al., 2018) through redox reaction and methylation (Fashola et al., 2016).

Table: 1: Bacterial species able to resist to HM through various mechanism

Bacteria	Mechanism	Reference
Bacillus sp. PZ^{-1}	High resistance to Pb^{+2}; it can bioadsorb Pb^{+2} and also resist Cu^{+2}, Zn^{+2}, Cu^{+2}, Ni^{+}	(Ren et al., 2015; Yin et al., 2019)
Bacillus cereus	It resists lead withmetallothioneins production.	(Foysal & Lisa, 2018; Yin et al., 2019)
Bacillus cereus RC^{-1}	It bioadsorbs Cd^{+2} on live and dead cells and bioaccumulates small quantities.	(Y. Ma et al., 2016; Murthy, 2011)
Bacillus cereus XMCr^{-6}	It bioadsorbs Cr+6 and reduces it to Cr^{+3}.	(Cabot et al., 2019; Huang et al., 2013)

2.11.5. Phytohormones production

Auxins, cytokinins, gibberellic acid, abscisic acid, brassinosteroids, jasmonate, and salicylic acid are important plant growth hormones (Q. Wang et al., 2011). According to reports, bacteria that produce phytohormones can help plants become more resilient to stressors brought on by heavy metals (Nazli et al., 2020). Arsenic inhibits the growth of roots in plants with interacting auxin (Ronzan et al., 2018). One of the multipurpose auxins produced by As resistant PGPR is indole acetic acid (IAA), which is essential to the growth of plant roots. Arsenic resistant *Bacillus* such *as B. subtilis subsp. stercosis, B. flexus, B. licheniformis, B. cereus, and B. flexus* were able to produce indole acetic acid (IAA), a plant hormone, and solubilize phosphate (Magar et al., 2022a; N. Pandey et al., 2020). Arsenic resistant *Bacillus* isolated from soil were able to produce IAA,ammonia (Poudel et al., 2019).

2.11.6 Production of hydrogen cyanide (HCN)

HCN is a volatile, secondary metabolite that inhibited the development of microorganisms and negatively affects the growth and development of plants (Z. A. Siddiqui, 2006). HCN is a powerful suppresses of many metal enzymes mainly copper containing cytochrome C oxidases. HCN is formed from glycine through the action of HCN synthetase enzyme, which is associated with the plasma membrane of certain rhizobacteria (Blumer & Haas et al., 2000). To date various bacterial genera have shown to be capable of producing HCN,

including *Alcaligenes, Aeromonas, Bacillus, Pseudomonas, Enterobacter and Rhizobium* species (Kumar A. et al., 2014)

2.11.7 Biocontrol

PGPR can compete with other bacteria to prevent other organism by colonizing quickly and acquiring a larger supply of nutrients (Salomon et al., 2017) and produce antibiotics and their metabolites including bacteriocins, antibacterial proteins, and enzymes are often effective in suppressing pathogen infections including bacteriocins, antibacterial proteins, and enzymes (Raaijmakers et al., 2002). According to studies conducted by (S. Islam et al., 2016), B. subtilis can shield tomato fruits from infection by *Penicillium sp*. and *Rhizopus stolonifer* during the postharvest stage. *Bacillus* species, such as *Bacillus cereus* and *Bacillus velezensis* have been found to inhibit the growth of phytopathogens and promote plant growth (W. I. Ahmed et al., 2023; Sarangi & Ramakrishnan, 2023; Xie et al., 2023). *Bacillus wiedmannii* has been antagonistic effects against root-knot nematodes, such as *Meloidogyne arenaria*, through the production of siderophore, protease, and chitinase (Fallahzadeh-Mamaghani et al., 2023), disease control and plant growth promotion (Singh S. et al., 2022).

2.11.8. Biofilm producing ability of bacteria (a next-generation bio-fertilizer)

Microbial Biofilm is a next-generation bio-fertilizer (Pandit et al., 2020). The microorganisms (planktonic or free-) are frequently organize themselves into a consortium of microbes called biofilm that adheres to a favorable support, interacts and produces extracellular matrix (Monroe et al., 2007). Biofilms provides the protection to the microorganism from altered pH, osmolarity, nutrients scarcity, mechanical and shear forces, antibiotics (Costerton et al., 1995). Formation of biofilms by bacteria during infection due to four reasons (Jefferson, 2004): (i) protection from harmful conditions (defense) (ii) sequestration to a nutrient-rich area (colonization) (iii) utilization of cooperative benefits (community) (iv) biofilms normally grows as biofilms and planktonic cultures are an in vitro artifact. *Bacillus vallismortis* TR01K was found to have an immense biofilm forming potential that ranged approximately 40x times higher than normal standard bacterial biofilm forming potentials (Maitra et al., 2022). Use of biofilm-producing PGPR as biofertilizers shows promise in improving sustainable soil fertility and plant growth (Dewi, 2015).

2.11.9. Nanobiofertilizer

Nanobiofertilizers are a revolutionary class of fertilizers that utilize nanotechnology to improve nutrient use efficiency and promote sustainable agriculture (Ain et al., 2023; Bairwa et al., 2023). These fertilizers are designed to deliver nutrients to plants in a controlled release manner, reducing the excessive use of chemical fertilizers and minimizing environmental impact (Shyamal Mondal, 2022). Nanobiofertilizers, with their unique properties and small particle size, can penetrate deeper into plants and provide nutrients at low concentrations, making them more effective than traditional fertilizers (F. Ahmed et al., 2023; Biswal, 2023). Nanoparticles can be incorporated into controlled-release formulations, enhancing the performance of *Bacillus* biocontrol agents (Pour et al., 2022).

2.12 Hydrolytic enzyme production

PGPR to manage the phytopathogens is a great example of a natural bio-control strategy; the bio-control will lessen the need for chemical fungicides, which is one of the main causes of soil infertility, and may also assist to control the proliferation of the phytopathogens.

2.12.1 Protease

The primary proteins of phytopathogens are broken down by the protease enzyme into peptide chains and/or their constituent amino acids, which eliminates the pathogen's ability to operate on plant cells. *Bacillus* produces a wide variety of extracellular enzymes, including proteases. Several *Bacillus species involved in protease production are e.g. B. cereus, B. sterothermophilus, B. mojavensis, B. megaterium and B. Subtilis* (Shumi et al., 2004).

2.12.2 Cellusase

Cellulolytic bacteria are capable of producing enzyme cellulase which functions to break down cellulose into simple sugar (Padilha et al., 2015a). Cellulase is a group of enzymes consisting of three major components, endoglucanase, exoglucanase, and β-D-glucosidase (Anoop Kumar et al., 2019; P. Gupta et al., 2012) . One of the bacteria that can produce cellulase enzymes is *Bacillus* sp. (Padilha et al., 2015b; Sadhu et al., 2013; Samley Mam et al., 2017).

2.12.3 Amylase

Amylase catalyzes the hydrolysis (splitting) of α-1, 4 glycosidic linkages in polysaccharides (like starch) which breaks the large molecules into smaller molecules of sugar like maltose. Reportedly, *Bacillus* strains have been extensively used industrially to produce α-amylase including *B. amyloliquefaciens, B. subtilis, B. licheniformis, B. stearothermophilus, B.megaterium and B.circulans. & B. subtilis* is aerobic , gram positive , endospore forming bacterium that has ability to produce and secreate the hydrolyzing carbohydrates enzymes , a- amyasle (Panneerselvam & Elavarasi et al., 2015) .

2.12.4 Laccase

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are copper containing enzymes that called blue copper oxidases. Laccases (EC 1.10.3.2, benzenediol oxygen oxydoreductase) are also called as "BLUE ENZYME" because they are multinuclear copper containing enzymes (Ryan et al., 2003) . Recently some laccases have been characterized *from Bacillus species* (Thomas et al., 2023).

2.12.5. Lipase

Lipase-producing bacteria are microorganisms that have the ability to produce lipase enzymes. Lipases are enzymes that can catalyze the hydrolysis of lipids into glycerol and fatty acids and these enzymes have various applications in industries such as food, pharmaceutical, paper, textile, and detergent. *Actinobacteria*, also known as actinomycetes, have shown great potential for lipase production and can be isolated from different types of environments (Sarkar & Chatterji et al., 2018).Lipase was produced by *Bacillus subtilis strain RCPs3 and Bacillus fumarioli strain RCPS4* (Chouhan et al., 2020).

2.13. Pot assay

Arsenic-resistant PGPR strains demonstrate remarkable promotion of plant growth and bioremediation of As under laboratory and pot trial conditions (Basu et al., 2023) . However, only a limited number of strains are capable of fulfilling this function in natural conditions. The effectiveness of PGPR under field conditions relies on various biological and non-biological factors. The ability of PGPR to compete with the existing bacterial community in the rhizosphere is crucial, as failure to do so will diminish the efficacy of the PGPR strains (Ahemad & Kibret et al., 2014).Therefore, an ideal PGPR should possess the ability to thrive in the rhizosphere in order to carry out its intended functions effectively. Within plants, the presence of As can have detrimental effects on growth and productivity

due to an array of morphological, physiological, biochemical and molecular changes. According to Wang et al. (2011), the inoculation of *Rhizobacterium D14* was an increase in plant height, the dry weight of the roots, stems, and leaves, as well as an increase in the amounts of soluble sugar and chlorophyll as well as the activities of catalase and superoxide dismutase. According to (Chintakovid et al., 2008), the relative distribution of As in plants indicates that *Brassica* sp. accumulated As mostly in the roots, followed by shoots and flowers. *Brassica juncea* stem xylem sap contained both kinds of arsenite and arsenate (Pickering et al., 2000).

Pickering et al. (2000) also observed a large accumulation of As by mustard plants, in roots as compared to stems. Srivastava and Singh (2014) reported arsenic-tolerant chickpea growth-promoting bacteria isolated from As-contaminated sites. Dashti etal. Indicated that co-inoculation of soybean with B. japonicum and Serratia species increased grain yield, protein yield, and total plant protein content (Dashti et al., 1997) . PGPR induced increase in leaf protein content of wheat (Afzal & Bano et al., 2008; Islam F. et al., 2014). Cd-tolerant bacteria associated with the roots of the metal accumulating plant *Brassica juncea* L. Czern. grown in heavy metal contaminated soils, and selected PGPR strains which might be increase plant biomass production under unfavourable environmental conditions (Belimov et al., 2005). Accumulation of total As increased with increasing concentration of both As species and exposure time, mainly in roots. Reduction in seed germination, root–shoot length, chlorophyll, and protein content were observed with increasing concentration and exposure time of both As species, being more in As(III)-treated leaves (Ahmad M.& Gupta et al., 2013). Uptake of arsenite by Indian mustard genotypes was higher than that of arsenate and Stunted growth of the plants (Chaturvedi et al., 2006) . The impact of arsenic-resistant Bacillus strains on plant growth parameters has been extensively studied.The various reported *Bacillus* species, such as *B. subtilis, B. amyloliquefaciens, B. pumilus, B. cerus* can significantly influence plant growth under different stress conditions (Domenico Prisa et al., 2021; Lanna-Filho et al., 2017; Maslennikova et al., 2023) and aslo reported to enhance seed germination, to reduce oxidative stress, enhance photosynthetic pigments, limit the accumulation of toxic heavy metals ,increase plant height, improve root and shoot growth, and protect plants against pathogens like *Fusarium oxysporum* (Faisal et al., 2013; Xu et al., 2022).

CHAPTER III

MATERIALS AND METHODS

All the instruments, nutrient media, chemicals and various reagents used during the project and their preparations have been attached in Appendix I, II,III & IV.

3.1 Laboratory setting

This reseach was conducted at the laboratory of Central Department of Biotechnology and partly at green house of Central Department of botany, Kritipur, Tribhuvan University.

3.3 Sampling site and method

The soil sample was collected randomLy from previously reported arsenic contaminated agriculture field (General et al., 2004; Pokhrel et al., 2009) of seven districts of Terai according to their traditional agro-climatic zone percentile composition of Rupandehi, Nawalparasi, Chitwan, Rautahat, Bara Saralahi, and Mahottari. Agricultural top soil (approximately 25 g) was collected at a depth upto 5 cm from each sampling sites and placed into a sterile plastic bag to make soil composite samples. A total of sixteen samples of cultivated top soil were collected and transported to laboratory of central department of biotechnology. All soil samples were preserved at room temperature until use. The pH of soil ranged from 4.5 to 6.5.

3.4 Isolation of Bacillus species

Bacillus sp. were isolated by soil dilution method from collected rhizospere soills. Samples were serially diluted up to 10^{-6} dilution and inoculated following pour plate method into nutrient agar supplemented with sodium arsenite media and incubated at 37° C for 24 h. From all these soil samples of sixty seven distinct colonies were selected based on the colony morphology and they were tested for their arsenic resistant potentiality by culturing in nutrient broth media amended with 200–5500 ppm of sodium arsenate and 200–1000 ppm of sodium arsenite.

3.4 Minimum inhibitory concentration

The levels of As resistance were determined by MIC. MIC is defined as the lowest concentration of arsenite or arsenate added which completely inhibited growth (Muller et

al., 2003). Isolated bacterial colonies were streaked on NB and minimal medium supplemented with varying concentrations of sodium arsenate and sodium arsenite (Mandal et al., 2022).

3.5 Investigation of oxidation and reduction of arsenic by the isolates by silver nitrate method

The ability of the bacterial isolates to reduce and oxidize arsenic (V) and(III) respectively was tested by using silver nitrate solution (Banerjee et al., 2011). Isolates were cultured in the NA plates containing either As(V) or As(III) incubated at 37° C for 72 h. , Then small amount of silver nitrate was added to the media plate. If the media turned brown, it confirms the presence of silver arsenate and if it turns yellow, the presence of silver arsenite was confirmed.

3.6. Phenotypic and biochemical characterization

The isolated bacterial colony was characterized morphologically and physiologically using the accepted techniques of (Logan and De Vos. et al,.2009). The methyle red test, Voges-Proskauer test, nitrate reductibility, citrate, and ability to produce hydrogen sulfide, as well as the utilization of various carbon sources and the utilization of gelatin, starch, and lipid, were among the various biochemical properties of the bacterial isolates that were tested using standard procedures (Logan & de Vos. Et al., 2009).

3.7. Removal of arsenic from culture broth or effect of arsenic on bacterial growth

1 mL of the inoculum was introduced to 50 mL of NB medium supplemented with 200–1400 ppm sodium arsenite from an overnight pure culture and were incubated at 37 ° C for 72 h in an orbital shaker at 120 rpm. Optical density at OD 600 nm was measured using using a spectrophotometer (Chitpirom et al., 2009; Poudel et al., 2019) .

3.8 Optimization of growth physiochemical properties of bacteria

The optimal growth conditions with reference to pH, salt and temperature were determined. The optical density of the growing cultures in NB medium was observed at OD 600 nm using an UV visible spectrophotometer (Iqtedar et al., 2019).

Effect of Nacl

The bacterium was incubated in NB with 2-10 % of NaCl and incubated at 37 ° C for 24h to study the effect of salt on growth.The optical density was measured at 660 nm.

Effect of PH

The influence pH on ARB was studied by growing the bacteria in a NB with pH ranging 4.0-10. The optical density was measured at 660 nm.

Effect of Temperature

The bacterium was grown in NB at temperature ranging from 20-70° C to study the effect of temperature on growth of ARB. The optical density was measured at 660 nm.

3.9 Determination of antibiotic resistance

Several standard antibiotic discs, including ciprofloxacin ,ceftazidime CAZ ,gentamicin GEN ,bacitracin,cefaclor,amikacin,ertapenem,colistin,cepalpthin,linezolid,Tetracycline, chloramphenicol , erythromycin , kanamycin, neomycin , nalidixic acid , rifampicin ,and streptomycin were used to examine the antibiotic sensitivity of the isolated bacteria.

Using the disc diffusion method, the antibiotic sensitivity of the ARB was measured. Antibiotic impregnated discs were placed on Muller Hinton Agar (MHA) plates spread with bacterial culture and incubated at 37 ° C for 24 h. After a 24 h incubation period, an inhibition zone was observed, and resistance was registered as positive. Measurements were made of the widths of the inhibition zones surrounding the discs (Magar et al., 2022b; Weinstein & Lewis et al., 2020) .

3.10 Heavy metals tolerance

The tolerance of bacterial isolates to copper, zinc, and lead were determined by Minimum Inhibitory Concentration (MIC). One loop of bacterial culture was streaked on agar plates containing above 50 ppm concentration of heavy metal like copper, lead, cadmium, zinc,chromium,cobalt ,mercury, zinc until the isolate enabled to give colonies. The MICs value was determined after 48 h of incubation at 37 ^{o} C (Kyaing et al., 2019). The lowest concentration of each metal at which no growth occurred when compared to the control plates was considered as the MIC (Mandal et al., 2022).

3.11 Pathogenicity of isolates

In order to determine the pathogenicity of the isolates bearing ARB, their "hemolytic activity" was tested. The isolates were spot-inoculated onto blood agar plates then incubated at 37±1 °C for 48–72 h. According to (Bhatt & Maheshwari et al., 2020; Lad et al., 2022; Yasmin et al., 2020), the plates were examined to see if clear (ß), greenish-brown (α), or no zones (γ) appeared. These colors correspond to total, partial, and no hemolytic activity, respectively.

3.12 Development of bacterial consortium

The both isolates were tested for their compatibility with each other in all possible combination by cross streak assay on nutrient agar medium (Raja et al., 2006; M. Y. Khan et al., 2022a). One bacterial strain was streaked on the solidified nutrient agar plate and incubated at 28 ± 1°C for 24 h for growth. The counter bacterial strain was streaked vertically to the growth of the already streaked bacterial strain. The plates were incubated at 28 ± 1°C for 48 h and bacterial growth was observed. The growth suppression of counter bacterial strain by already streaked bacterial strain was considered as un-compatible and mixing of bacterial growth of both strains was considered as compatible which considered as appropriate for the development of consortium.

3.13 IAA-like compound production ability

Quantitative analysis of IAA was performed using the method of (Emami et al., 2019) at different concentrations of tryptophan (0, 1, 3, 3, 4 and 5 mg/ mL). Isolates were grown for 48 h on their respective media at 37 ° C. Fully-grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 mL) was mixed with 4 mL of the Salkowski reagent (50 mL, 35% of per chloric acid, 1 mL 0.5 M $FeCl_3$ solution). Development of pink color indicated IAA production.

3.13.1 Determination of Optimum Condition for IAA Production

L-tryptophan as a precursor in IAA production was examined at concentrations of 0, 1, 2, 3, 4 and 5 (mg mL^{-1}). The effect of incubation time for IAA production by isolates was grown in NB medium supplemented with 1 mg mL^{-1} L-tryptophan at pH 7.2 and incubated at 30 °C in a shaker at 120 rpm for 5 days. Production of IAA by isolates was monitored at every 24-h interval (Emami et al., 2019).

3.14 Ammonia production

For the ammonia production, freshly grown cultures were inoculated in 10 mL peptone water in each tube and incubated for 48–72 h at 28° C. Nasser's reagent (0.5 mL) was added in each tube. Development of brown to yellow colour indicated a positive test for ammonia production (Chakra et al., 2019).

3.15 Nitrogen fixation assay on NFb medium

Isolated bacteria were screened on NFb solid medium for nitrogen fixation activity. Isolates showed blue colour zone around the colony considered as positive for nitrogen fixation (Chakra et al., 2019; Swamy et al., 2016).

3.16 Phosphate solubilizing activities

3.16.1 PVK agar plate

The qualitative test of phosphate solubilizing bacteria was evaluated on solid Pikovskaya medium. The clear zone, or area of P solubilization, surrounding each bacterial colony was measured to determine the degree of phosphate solubilization (Ibarra-Galeana et al., 2017).The phosphate solubilization index was calculated using the formula:

(Colony diameter + halo zone diameter)/colony diameter.

3.16.2 Liquid Mpvk medium

The quantification of phosphate solubilization was done in liquid Mpvk medium after 7 days of incubation at 37 ^{o}C. Calcium tri phosphate was used as standard for quantification by spectrophotometer. Estimation of free phosphate released in the medium as a result of phosphate solubilization by the bacterial isolates was estimated as per the method of (Ibarra-Galeana et al., 2017; Shrivastava et al., 2015). Reagent A: 10% Ascorbic acid (stored at 4 ^{o} C) Reagent B: 0. 42% Ammonium molybdate in 1 NH_2SO_4 (stored at room temperature), Reagent C: 1 part of reagent A + 6 part of reagent B. 1.5 mL of 3 days old grown cultures was harvested by centrifugation at 8000 rpm for 2 min. 3.5 mL of reagent C was added to the culture supernatant of each bacterial sample and after mixing, incubated at room temperature for 1h. Optical density was measured at 660 nm against blank. Simultaneously, standard with various concentrations of K_2 HPO_4 was prepared separately.

3.16.3 Phosphatase activity

The modified test was used to measure the bacterial acid and alkaline phosphatase activity (Juma & Tabatabai et al., 1988). 400 µL of modified universal buffer (MUB) pH 5 and 100 µL of 25 mM p-nitrophenyl phosphate were incubated at 37° C with 100 µL of culture supernatant after centrifuging the bacterial cultures (2300 g, 15 min). The reaction was stopped after one hour by adding 400 µL of $CaCO_3$ (0.5 M) and 100 µL of $CaCl_2$ (0.5 M) then the absorbance was measured at 410 nm. Using a p-nitrophenol (p-NP) calibration curve (µmol p-NP mL^{-1}), the amount of product obtained in the reactions was calculated.

Alkaline Phosphatase (ALP) Activity: - MUB stock solution was adjusted to pH 11

Acid Phosphatase (ACP) Activity: - MUB stock solution was adjusted to pH 6.5

3.17. Organic acid production in Pikovaskya media

Inoculation of PSB medium with ARB strains and incubated at 37 °C in rotatory incubator for 7 day and pH of medium noted along with un-inoculated control

Quantitation of organic acid production by isolates

Overnight culture of ARB isolates were inoculated on Pikovskaya broth medium and incubated for seven days at 37 °C on a 120 rpm shaker. At the end of incubation, the culture was centrifuged at 10,000 rpm at 5 min. The filtrate obtained was used to determine the levels of organic acids, namely: citric, oxalic, formic, succinic, butyric, acetic, and propionic acids. Determination was carried out according to standards using High-Performance Liquid Chromatography (HPLC) Aminex HPLC columns, with a flow rate of 0.5 $mL.minute^{-1}$ and an injection volume of 5µl (Behera et al., 2017).

3.18. Zinc solubilizing activities

Zinc solubilizing capacity of isolated bacteria with two insoluble sources of zinc, zinc oxide (ZnO) and zinc carbonate ($ZnCO_3$). The selected bacterial isolates were inoculated on Bunt and Rovira medium containing insoluble Zn source to determine Zn solubilization qualitatively (Bunt and Rovira, 1955). Overnight bacterial culture were then spotted on media to check clear halo zone formation and incubated at 37 ° C for 7 days. The colony and halo zone diameter were measured by measuring scale.

Zinc solubilization efficiency (SE) was calculated as the ratio of total diameter i.e., clearance zone including bacterial growth and the colony diameter as described by Sharma et al. (2014).

SE = (Diameter of solubilization halozone $/Colony\ diameter$)*100%

Zn solubilizing index (ZSI) was calculated as the ratio of (halo+colony)/colony diameters (Saravanan et al., 2007b).

3.19 In vitro screening of bacterial isolates for their bio control properties

3.19.1 Screen for hydrogen cyanide (HCN) production

The isolates were inoculated on the nutrient media plates containing 4.4 g glycine per liter. The top of the plate was covered with Whatman filter paper no. 1 soaked in 2 % sodium carbonate in 0.5 % picric acid solution and sealed with parafilm. The plates were incubated at 30 ° C for 4 days and observed for a color change of the filter paper from deep yellow to reddish-brown indicated production of HCN (Zerihun et al., 2019).

3.19.2 Biofilm formation

Congo red agar method

The ARB were streaked on the Muller Hinton agar supplemented with 0.8 g/L of Congo red dye and incubated for 48 h at 37 ° C. Biofilm creation was indicated by the development of black colonies with a dry crystalline quality, while strains that do not produce biofilm produce red colonies (Mathur et al., 2006).

Tube assay method

The tube staining technique was used to determine the qualitative evaluation of biofilm formation (Christensen et al., 1982). ARB were inoculated in 10 mL TSB with 5 % sucrose and incubated for 24 h at 37 ° C at static and rotary condition. The tubes were decanted and washed with phosphate buffer saline (PBS pH 7.3), dried and Stained with 0.1 % crystal violet. Excess stain was removed by washing the tubes with deionized water. The appearance of a visible coating on the tube's bottom and wall verified the formation of biofilm. Nevertheless, biofilm growth was not indicated by the liquid interface (Mathur et al., 2006).

Microtiter plate assay

The quantitative estimation of the biofilm formation was done by Microtiter plate assay (Mathur et al., 2006). The ARB were grown in TSB with 5 % sucrose at 37 ° C for 24h at static condition. The cultures after 24 h were diluted 100 times with the same medium and 200 µl of the culture were inoculated in the 96 well plate. The 96 well plate was incubated for 24 h in static condition at 37 ° C at static condition. After respective incubation period content of each well was gently removed by slightly tapping the plates. The wells were then washed with phosphate buffer saline (PBS pH 7.3) to remove free-floating planktonic bacteria. The plates were then stained with 0.1% (w/v) crystal violet solution. Excess stain was washed off thoroughly with 95 % ethanol and plates were kept for drying. Optical density (OD) was measured using micro ELISA auto reader at wavelength of 570 nm. These OD values were considered as an index of attachment to surface.

3.19.3 Dual plate assay for antagonism of isolates

The antagonistic effect of a bacterial suspension culture of ARB Bacillus examined by a dual-culture assay (Tariang et al., 2018). Fungus were cultured on PDA at 28 ° C for 7 days. A mycelia disks (5 mm diameter) of fungus were placed in the centre of PDA plates. At the same time, four drops of bacterial suspension (10^7 CFU) were spotted around the fungal inoculums at a distance of 3 cm. PDA inoculated with the pathogen alone was used as control. Plates were incubated at 28 ° C for 7 days and then the antagonistic effect of test strain on fungus was recorded. Three pathogenic fungus (*F. oxysporum*, *R.solani and A. solani*) already identified were collected from culture storage of central department of biotechnology and were used for antagonism assay.

% of growth inhibition= $[(C-T)/C] \times 100$,

Where, C is the radial growth of the test pathogen in the control plates (cm), and T is the radial growth of the test pathogen in the test plates (cm)

3.20. Screening bacterial isolates for hydrolytic enzyme production

Bacterial isolates were screened for their hydrolytic enzyme production like protease, cellulose, and amylase.

3.20.1 Protease production activity

According to (Chang et al., 2009), bacterial isolates were chosen based on their capacity to produce proteolytic enzymes on skim milk agar or SMA (3% v/v) medium. The ARB was inoculated on SMA and incubated at 30 ^{o}C for 48 h.The formation of clear zone around colonies indicates positive for protease.

3.20.2 Cellulose production activity

The ARB isolates were performed for cellulose production activity by spot inoculation on the CMC (Zerihun et al., 2019). ARB isolates showing clear halo zone on cellulose medium was indicated a positive result for cellulose synthesis after addition of Congo red solution.

3.20.2 Production of amylase (starch hydrolysis)

The bacterial isolates were spot inoculated on starch agar medium plates and incubated at 30 ° C for 48 h. At the end of the incubation period, the plates were flooded with iodine solution kept for a minute and then poured off. Iodine reacts with starch to form a blue color compound. This blue color fades rapidly. Hence the colorless zone surrounding colonies indicates the production of amylase (Zerihun et al., 2019).

3.20.3 Laccase production activity

ARBs were screened for laccase production by plating on Nutrient agar supplemented with Guaiacol (0.01 %). Development of brown color zone, surrounding the bacterial growth, was indicating the production of Laccase (Sonali B. Darade et al., 2017).

3.20.4 Lipase producing activity

Lipase activity was performed by (Ilesanmi et al., 2020). The ARB isolates were streaked onto Tween 80 agar medium and incubated at 37 ^{o}C for 48 h (10 mL/L of Tween80 was added to the cooled media). A white precipitate around colony indicates lipase activity.

3.21. Greenhouse assay

3.21.1 Seed sterilization

To observe the effect of ARB on Broad leaf green mustard (*Brassica juncea* L. Czern), Broad leaf green mustard seeds from Budhathoki agro and Seed trades were sterilized. Seeds were put in falcon tube with 70 % of ethanol for 2 min and properly suspended then

ethanol was discarded and rinsed with autoclaved distilled water by 3 times. Then 4 % bleach with 2 drop of tween20 in falcon tube and suspended with the help of vortex and leaved the seed to settled in the tube then removed Naocl with pipette finally seed were rinsed with sterilized distilled water by 8 times.

3.21.2 Inoculum preparation from bacteria

For the preparation of bacterial inoculum, isolates were first streaked on Nutrient Agar (NA) plates and incubated at 30 ^{o}C for 24h. Single colonies on NA plates were picked with an inoculating loop then transferred to 100 mL NB and grown aerobically in a rotary shaker at 160 rpm at 37 ^{o}C for 48h. The bacterial suspension was diluted with sterilized distilled water to obtain a minimum final cell concentration of 10^8 - 10^9 CFU/mL at (OD = 0.5 - 1 at 550 nm).

3.21.3 Seed treatment

Surface-sterilized broad leafed mustard seeds were suspended for 30 min in suspensions (10^8 CFU/mL) of ARB Bacillus. The sterilized seeds were soaked in prepared bacterial inoculum for 1h with shaking at 120 rpm and untreated seeds was used as control.

3.21.4 Pot assay in green house

A pot experiment was carried out in a green house under ambient air and temperature conditions at the central department of Botany (TU), kritipur. Each pot was filled with 3 kg air-dried and ground (<2mm) soil with three replicate (Samayoa et al., 2020). Both ARB isolated S1 and M1 were selected for evaluation in the soil pot trial with broad leaved mustard. Experiments were conducted in pots (upper diameter 8.5 cm, lower diameter 6.1 cm, and height 15.1 cm) containing 3 kg air-dried and sieved (2 mm mesh) soil. Soil (pH=7.4) was collected from farm land of Tribhuvan University behind the central department of biotechnology new building.

3.21.5 Layout, Design and Treatments

In these experiments, two ARB-PGPR bacteria were applied along with the total ten treatment were laid out in a complete Randomized Block Design (CRBD) with three replications.The treatments included the following:

Normal soil + individual strain

Arsenic soil +individual strain

Half-dose fertilizer (1/2F) +individual strain + arsenic soil

Half-dose fertilizer (1/2F) +individual strain +Normal soil

Arsenic soil control

Normal soil control

Half-dose fertilizer control (1/2F) +Normal soil

Half-dose fertilizer control (1/2F) + Arsenic soil

Full-dose fertilizer control (1F) + Normal soil

Full-dose fertilizer control (1F) + Arsenic soil

Full dose of fertilizer (F) is 120:80:60 kg ha^{-1} and half dose (1/2) is 50 % of full dose.

Three replicates for each strain were used (Lai et al., 2008). Fertilization followed the pots of each treatment were arranged randomly and NPK at the rate of 120:80:60 kg ha^{-1} were used (Bp et al., 2018). N, P, and K were applied as urea, DAP, and potassium sulphate, respectively (Appendix II)

3.21.6. Irrigation

Twenty-four inoculated seeds were planted in each sterilized pot filled with air dried soil. Seeds without bacterial inoculation were planted as the control in both normal soil and arsenic soil. Throughout the experiment, plants were irrigated three times a week with 100 mL of sterilized distilled water in both arsenic amended soil and normal soil. Control pots received sterilized distilled water without additional arsenic solution and with addition of arsenic solution. 100 mL of full dose of NPK and half dose of fertilizer were used as another control to pots containing normal soil and arsenic amended soil immediately after sowing. The concentration of arsenic was maintained 20 ppm with the addition arsenic solution prepared in sterilized distilled water in arsenic amended soil pot (Yahaghi et al., 2019).

3.21.7. Seedling treatment

As Booster Dose

After 10 days, 100 mL aliquots of the bacterial suspensions were added to the pots having normal soil and arsenic soil. Normal sterilized distilled water and autoclaved arsenic

solution was added to the control having normal soil and arsenic amended soil respectively. A predetermined volume of arsenic solution was added to the pot to maintain the metal concentration at 20 ppm. 100 mL of NPK was added to NPK pots having normal soil and arsenic amended soil. Half dose of NPK and individual isolates were added in normal soil and arsenic amended soil.

3.21.8 Measurements of different growth parameter

Growth parameters examined were plant height (cm), leaf area (cm^2), and number of leaves (NOL) (Y. M. Khan et al., 2022b). Efficacy test was applied to determine the effectiveness of each isolate from each variety on each growth parameter by the following formula: efficacy = treated − control/treated × 100 %.

Plant or shoot height (HOP):-plant height was measured using meter rular from the base of the plant to the tip of the leaf.

Number of leaves (NOL):-Number of leaves was obtained by visual counting of the leaves.

Leaf area (AOL):-Leaf area was measured as the product of the length and the width of the leaves.

Formula of AOL cm^2 = length of leaf (cm) × width of leaf (cm).

Root length (RL), fresh shoot weight

Plants were harvested after 24 dyas. The shoots and roots were rinsed thoroughly in deionized water and the roots rinsed in 10 mM Na_2EDTA at pH 6.0. The lengths of roots were measured from the main root apex to the crown of the plant. Fresh weight of roots and shoots were also determined.

Shoot fresh and dry weights: - The shoots of these plants were than oven dried at 70 ° C for 48 h. After which they were allowed to cool at room temperature, and were their dry weight was measured.

3.22 Water Content Analysis

The fresh shoot of each plants were weighed and recorded as WF then dried at 70 ^{o}C for 48 h. The dry matter weighed was receded WD (Jin et al., 2017). The leaf water content was calculated as the following:-

Water content (%) = (WF-WD)/WF*100

Where, WF- fresh weight

WD- Dry weight

3.23 Chlorophyll content

Chlorophyll estimation of leaves of treated and control plants was done according to the method of Warren (2008). Two hundred milligram of frizzed fresh leaf tissues of each sample were homogenized using chilled methanol in a prechilled clean glass mortar and pestle. Chlorophylls were extracted from the ground samples by adding 1mL of methanol and shaking for two min. Samples were centrifuged for 10 min at 3000 rpm and the supernatant was transferred to a second microcentrifuge tube. The pellet was re-extracted with a second 1mL aliquot of methanol by adding 1 mL of methanol to the pellet, shaking for another two min, centrifuging and removing the supernatant. The pellet was discarded while the two supernatants were pooled and used for measurement of chlorophyll. The absorbance of the solution was measured at 645 nm and 663 nm against the solvent (methanol) as blank by Agilent BioTek Synergy LX multimode reader.

The amount of chlorophyll present in the extract was calculated in mg chlorophyll per gram tissue according to the following equation (Warren et al., 2008).

Calculation of Chlorophyll Concentration

The absorbance of 200 µL of sample in a microplate (A652, microplate A665, microplate) was converted into a 1 cm pathlength corrected absorbance using the measured Pathlength:

A652, 1 cm = (A652, microplate − blank)/pathlength

Chlorophyll concentration was calculated from 1 cm corrected pathlength using recently published formulae of Ritchie (2006):

Chl a (µg/mL) = −8.0962 A652, 1 cm + 16.5169 A665, 1 cm

Chl b (µg/mL) = 27.4405 A652, 1 cm − 12.1688 A665, 1 cm

The measured pathlength of 200 µL of water was 0.51 and this was used to create a 1 cm corrected value by dividing the experimental absorbance (blanked) by pathlength:

A652, 1 cm = (A652, microplate − blank)/0.51,

A665, 1 cm = (A665, microplate − blank)/0.51

3.24 Total soluble protein

About 1.5 gm frizzed leaves was weighed. About 1 mL of protein extraction buffer per gram of leaf in a cold mortar and pestle was added and grinded the tissue until a thick paste is produced. Collect the paste was collected and placed in a 1.5 mL micro centrifuge tube and centrifuged for 20 min at 12000 rpm then transferred the supernatant to another 1.5 mL micro centrifuge tube. The blank consists of Phosphate buffer with no protein. A series of protein standards consists of a known concentration of Bovine serum albumin (BSA, A8806 Sigma), prepared from 1 mg/mL BSA source solution in the range 50-1000 µg/mL in phosphate buffer. The sample was diluted. The assay was performed using 50 µl of each protein standard or unknown sample and 200 µl of dye reagent in 96 microplate wells. The content was mixed for 60 sec by shaking in 8 shapes. The absorbance values of all the samples and controls were measured after five min incubation at room temperature at 595 nm by ELISA reader (Agilent BioTek Synergy LX multimode reader (Pour Nouroozi et al., 2015).

3.25. Molecular Identification of hyper tolerant AR *Bacillus* Species

The extraction of genomic DNA of ARB *Bacillus* was performed by phenol-chloroform assay from pure culture, and DNA amplification of the 16S rRNA gene was performed by the sets of universal primer: 8F (5′-AGAGTTTGATCCCTCAG-3′) and 1492R (5′GGTTACCTTGTTACGACTT-3′) .PCR amplification conditions were as follows: 30 cycles of denaturation at 98 ° C for 10 sec and annealing at 55 ° C for 5 sec with final elongation at 72°C for 1 min. PCR products were purified using QIAquick PCR purification kit according to the manufacturer’s instructions. Purified PCR product was sequenced at Macrogen (Seoul, Republic of Korea). All reference sequences were obtained from the NCBI. TheEzTaxon-e server was used to calculate the pairwise similarity of the nearly complete 16S rRNA gene sequences (Kim et al., 2012). Sequences were aligned using CLUSTAL W ver. 2.01 (http://clustalw.ddbj.nig.ac.jp/) (Thompson et al., 1997), and the phylogenetic analysis (neighbour-joining) was conducted using MEGA ver.XI with bootstrap values calculated from 1000 replications (Tamura et al., 2013).

3.26 Statistical analysis

Statistical Analysis Statistical analyses were performed using Graph Pad Prism (version8.0) and Microsoft Office Excel 2013. A completely randomized design was used for all experiments, with at least three replications for each treatment. Treatments were compared via ANOVA (tukey’s test) using the least significant differences test (LSD) at 5 % ($P \leq 0.05$) probability level(Mengistie & Awlachew et al., 2022).

CHAPTER IV

RESULT

4.1 Bacterial Isolation

Arsenic resistant bacteria were isolated from sixteen soil sample. In total, 62 colonies were randomly selected and subcultured on NA media supplemented with increasing concentration of sodium arsenite and arsenate.

Table 2: Isolation of *Bacillus* species from various soil sample

Location	Sample Code	Sample Type	Isolated Strain (*n*)
Nawalparasi	NS	Top soil	7
Rautahat	NP1,NP2,NP3,NP4	Top soil	12
Bara	B1,B2,NP6,NP7	Top soil	10
Saralahi	S1,S	Top soil	10
Chitwan	CS	Top soil	7
Rupandehi	RS, BH11	Top soil	8
Mahottari	M1,M2	Top soil	8
Total isolates	16		62

4.2 Minimum inhibitory concentration

Microbial resistance to arsenate [As (V)] and arsenite [As (III)] were determined by visible growth after 72h in NA plates and the minimal medium plates supplemented with varying concentrations of sodium arsenate and sodium arsenite. Among the tested strains, only two isolates M1 and S1 could tolerate higher level of arsenate and arsenite concentration. Both S1 and M1 ARB exhibited MIC at a range 1000 ppm sodium arsenite and 5500 ppm sodium arsenate in NA medium but both the isolates showed decreased growth pattern with increasing concentration of arsenite, and finally stopped growing after 1000 ppm

arsenite and 5500ppm arsenate. So only these two strains S1 and M1 considered as hyper tolerant arsenic resistant bacteria and selected for Bioremediation and PGPR.

4.3. Investigation of Oxidation and reduction of arsenic by the isolates by silver nitrate method

After the addition of silver nitrate into 72 h old culture plate containing arsenite, the media slowly turned brown (Fig. 8 a and b) which confirmed the presence of silver arsenate in the media. But when silver nitrate was mixed with the culture containing arsenate, it also turned brown confirming the presence of silver arsenate. Hence it is observed that none of the bacteria has the ability to reduce arsenate to arsenite, but both of them can oxidize arsenite to arsenate. The presence of arsenate was revealed by brownish precipitates which shows the arsenite oxidizing bacteria.

4.4 Morphological Biochemical Characterization

Both the isolates were white in colour, have smooth surface, flat colonies opaque. Both the isolates were Gram-positive, rod shaped, endospore, facultative anaerobes, (Fig. 8 c, d, e, f). Different biochemical properties of the two isolates are presented in Table 3. Isolates S1 and M1 were able to ferment sugars like glucose, fructose, lactose, sucrose, galactose, mannose, mannitol, maltose and melibiolose. Based on the sugar assimilation pattern, test isolates could be *Bacillus* species.

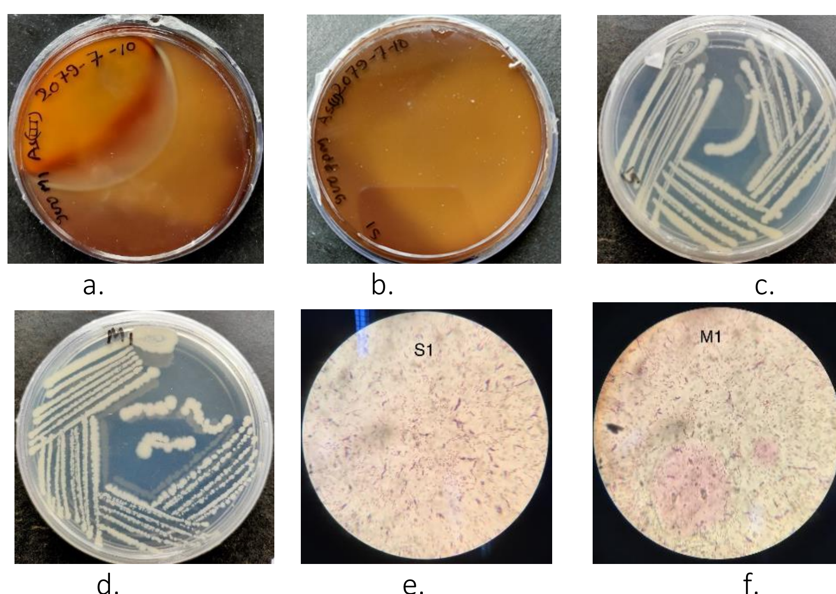


Figure: 16 Presence of brown color indicates arsenate (a & b), colon morphology(c&d) and gram staining (e &f) for S1 and M1 respectively

Table 3: Morphological and biochemical test of isolates

BIOCHEMICAL TEST	ISOLATES	
	S1	**M1**
Motility	+ve	+ve
Gram stain	+ve	+ve
Cell shape	Rod	Rod
Spore site	Central	Central
Catalase	+ve	+ve
Oxidase	+ve	-ve
Indole	+ve	+ve
MR	+ve	+ve
VP	-ve	-ve
H_2S	-ve	-ve
Citrate	-ve	-ve
TSIA	K/A	K/A
Gelatin hydrolysis	+ve	+ve
Starch hydrolysis	+ve	+ve
Glucose	+ve	+ve
fructose	+ve	+ve
Melibiose	+ve	+ve
Galactose	+ve	+ve
Ribose	+ve	+ve
Manitol	+ve	+ve

4.4 Bioremediation test or effect of arsenic on bacterial growth

The two arsenic resistance bacterial isolates M1 and S1 were grown in NB supplemented with sodium arsenite (200 ppm-1400 ppm). The growth of the isolates was monitored periodically (24h interval) by measurement of optical density. The growth rates of M1 and S1 showed significant decreased (P<0.05) with increasing concentration of arsenite (fig.9 a,b,c,d,e,f) whereas increasing time period as 24,48,72 h significantly (P<0.05) increasing the bacterial growth which indicates removal or detoxification of sodium arsenite to less toxic arsenic (fig.10a and 10b) respectively.

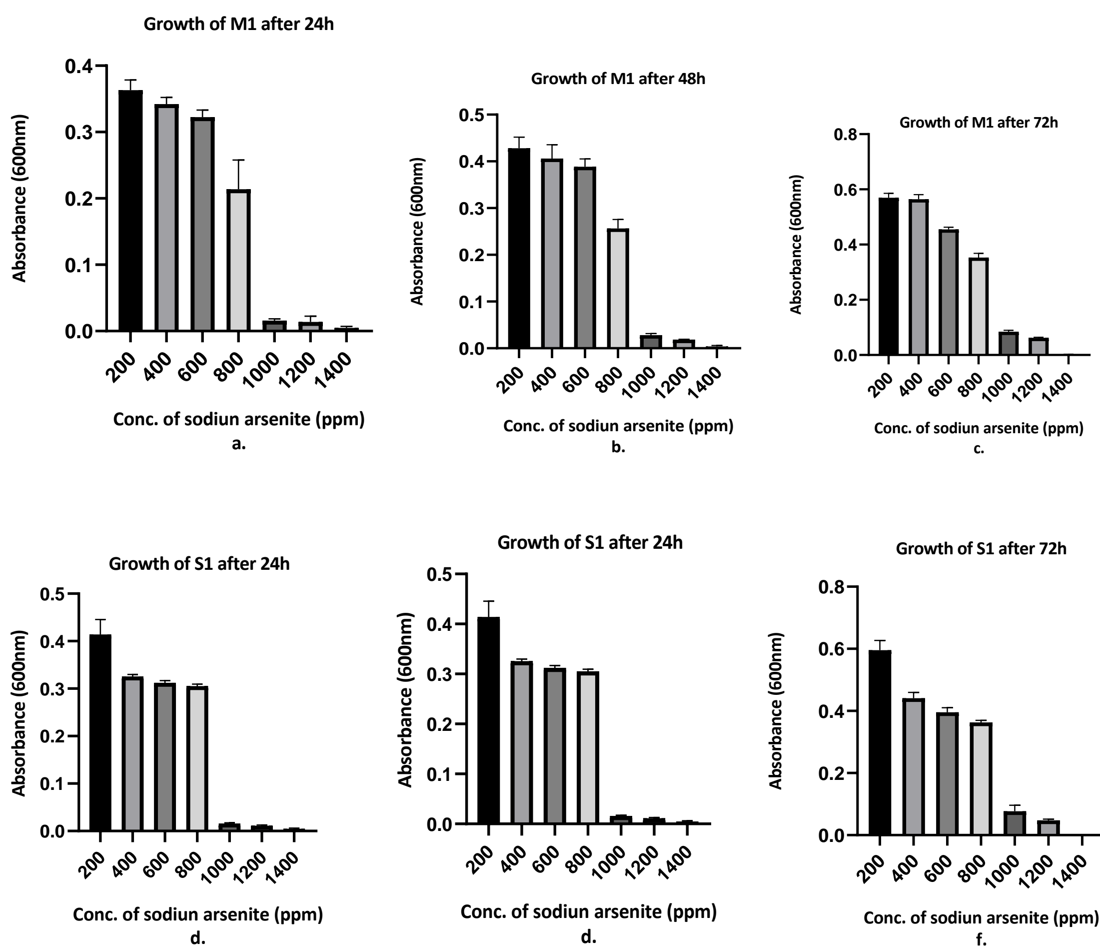
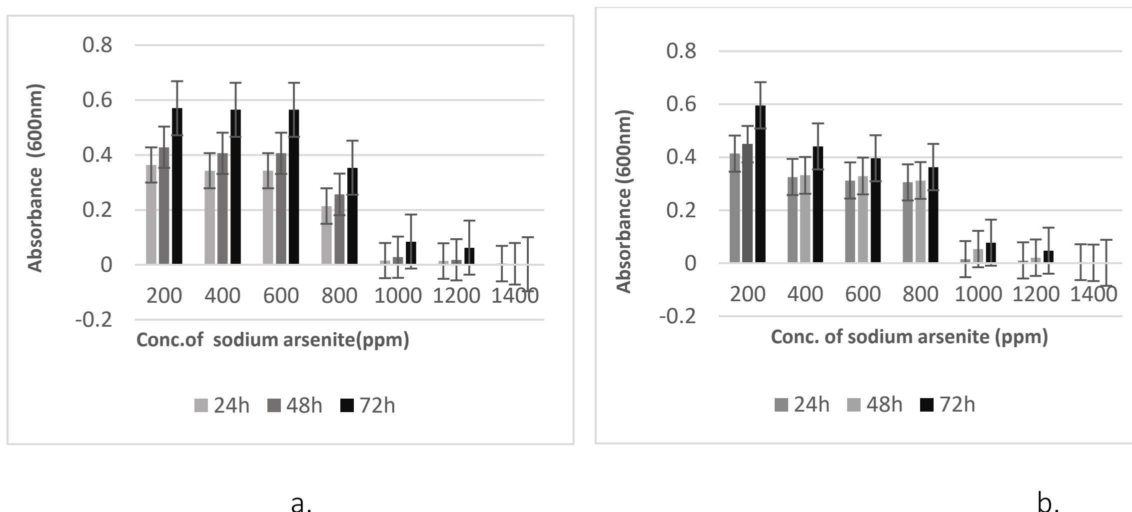


Figure: 17 Effect of sodium arsenite on M1 (a, b,c) and S1(c,d,e) after 24,48 and 72 h respectively.



a. b.

Figure 18: Removal of aresenic by M1 (a) and S1 (b) after 24, 48 and 72 h respectively.

4.5. Optimization of growth physiochemical properties

Effect of physicochemical conditions on bacterial resistance to arsenic was assessed by observing the growth at different pH and temperature. Different temperature studied were room temperature ((20, 25, 28, 30, 35, 37, 40, 45, 50, 55, 60, 65, and 70°C), pH range used (2,2.5,3,3.5,4,4.5,5,5.5,6,6.5,7,7,5-8) and salt (2,4,6,8,10%). Bacterial culture in NB broth was provided with different physio-chemical conditions and incubated for 24 h. Effect of different parameters was observed by measuring the absorbance of culture at 600 nm.

4.5.1 Effect of Nacl

The both S1 and M1 isolates showed that the cells were able to grow maximum at 4% NaCl were 0.3785 and 0.3305 and minimum at 10 % were 0.0025 and 0.0035 respectively but unable to grow at higher concentration of NaCl, showing that the isolate was sensitive to the salt concentration (Fig. 11a).

4.5.2 Effect of temperature

The organisms were found to be temperature sensitive as at higher and lower temperatures, a low growth was observed that might be due to a hindrance in the metabolic activity. The results indicate that the optimum temperature for the growth of both S1 and M1 isolates at 37 ° C were 0.694 and 0.805 respectively. Growth was hampered at very low and high temperatures of (Fig. 11b).

4.5.3 Effect of pH

The effect of pH on growth of ARB strains were studied at different pH range (2,2.5,3,3.5,4,4.5,5,5.5,6,6.5,7,7,5-8). The results indicate that the optimum pH for the growth of both S1 and M1 isolates at 7.0 were 0.61 and 0.621. The growth was hampered at very acidic and alkaline pH as indicated by growth of the soil isolates (fig.11c).

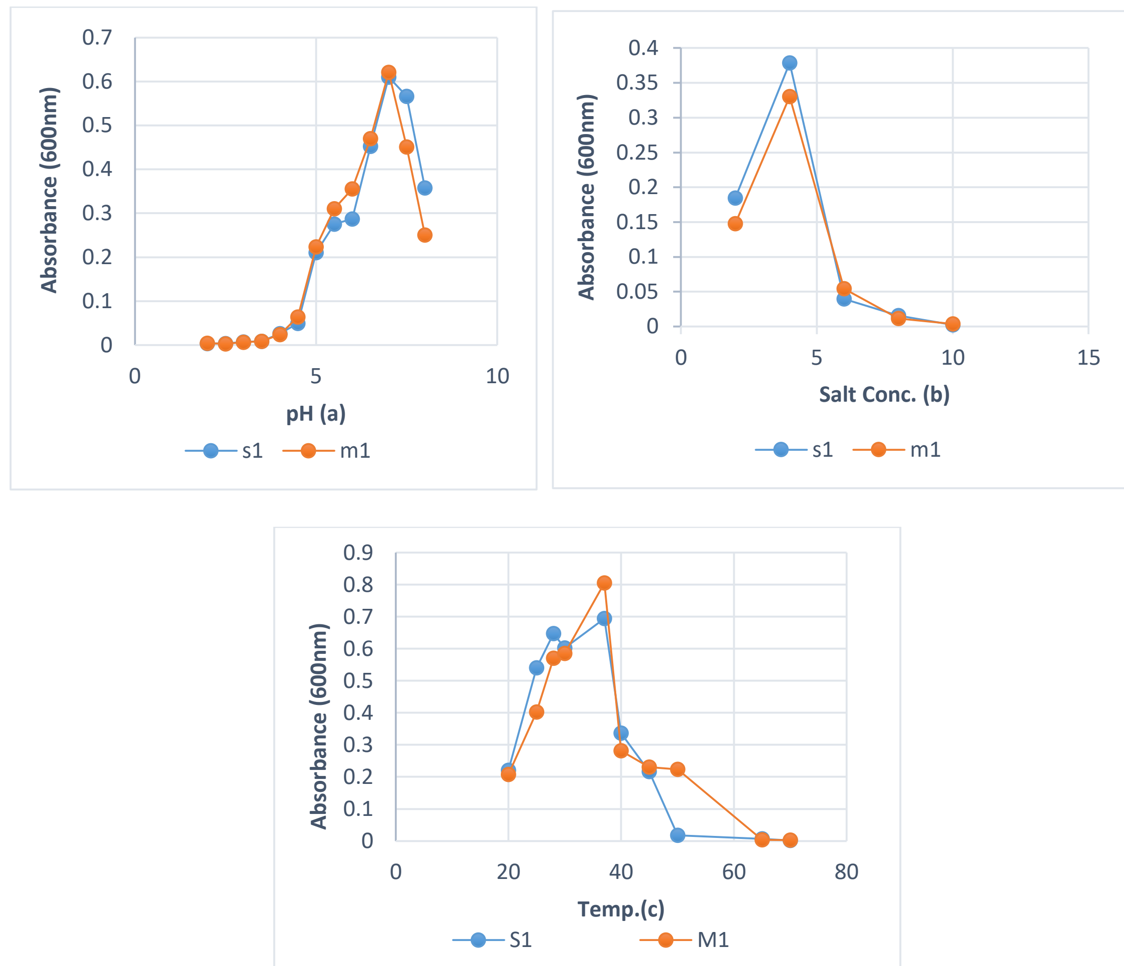


Figure: 19 Effect of salt (a), salt (b) and temp. (c) On bacteria S1 and M1

4.6. Determination of antibiotic resistance

The antibiotic susceptibility test (AST) showed that both ARB isolates were sensitive to antibiotic. Both isolates S1 and M1 were tested for the ability to grow in various antibiotic-supplemented media, (Table 4)

Table 4: Inhibition zone of bacteria against antibiotics

Antibiotic	Zone of inhibition	
	S1	m1
Ciprofloxacin CIP 30 mcg	2.7	2.6
Nalidixic acid NA30	2	2
Ceftazidime CAZ	0.7	
GentamicinGEN	2	2.1
BacitracinB	1.2	1.2
CefaclorCEC30/10	1.7	1.7
Amikacin AK	2	2.3
Ertapenem ETP10	1.8	1.9
Colistin cl30		0.5
CEPALPTHIN CEP30	0.7	0.9
Linezolid LZ 30	2.8	2.5

4.7. Estimation heavy metal resistance of bacteria

The both S1 and M1 were showed a varied level of resistance to heavy metals tested. Results showed that both M1 and S1 had MIC levels against cobalt, copper, and zinc up to 200ppm and both strains were showed the maximum resistance against lead with MIC value 400ppm (Fig12.a&b).

4.8. Biocompatibility of isolated bacteria

The growth of S1 was slightly suppressed by M1 or vice- versa so growth of both bacteria were not mixed and were considered as un-compatible. Both isolates might not suitable for bacterial consortium (Fig 12c &d).

4.9. Hemolysis test

Hemolytic activities of both S1 and M1 tested isolated were evaluated on blood agar plates. None of the tested strains showed α-hemolytic and ß-hemolytic activity when grown on Columbia blood agar plates. The both S1 and M1 strains showed γ hemolytic, i.e., negative, or no hemolytic activity (Fig.12e &f).

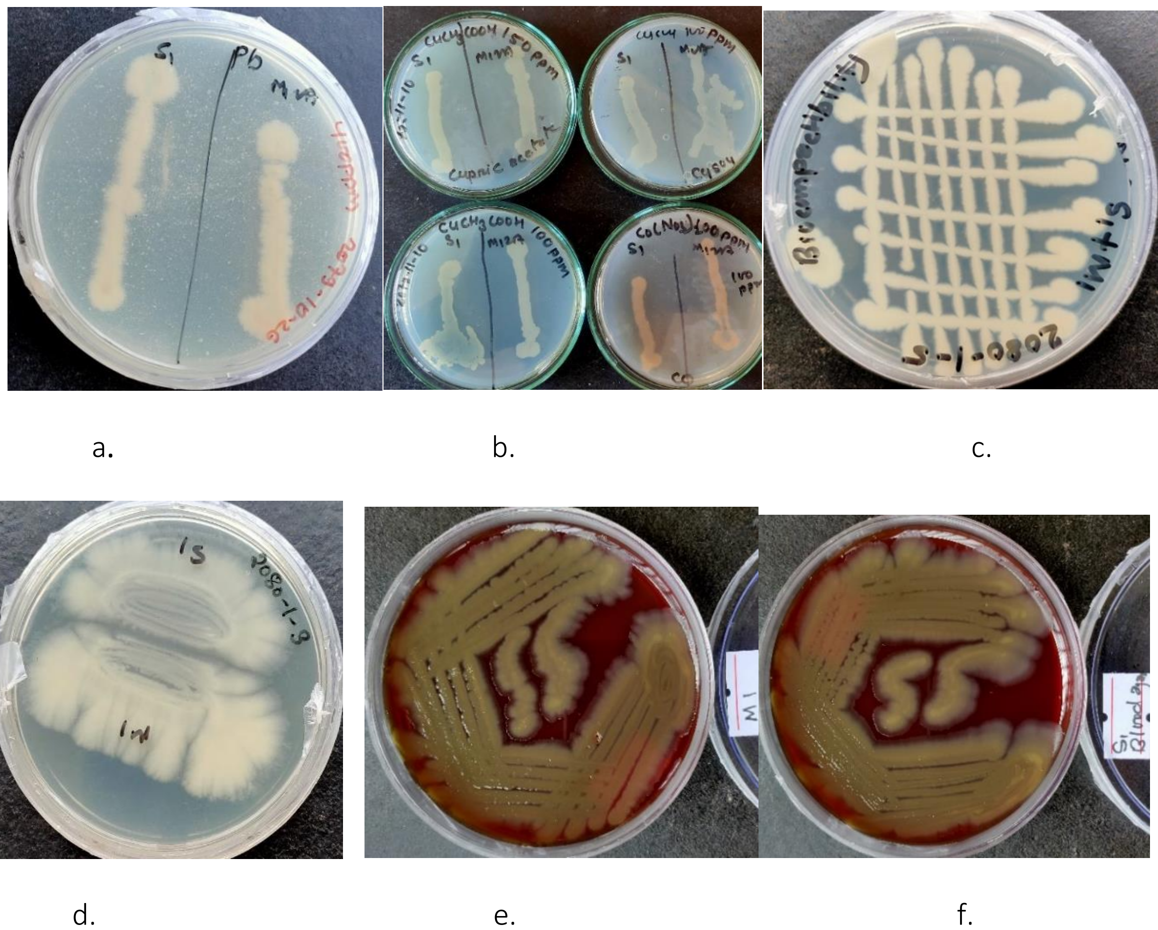


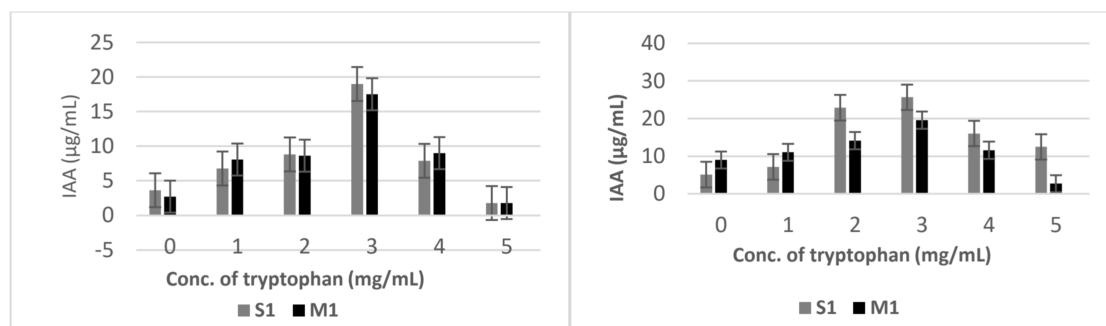
Figure: 20 : HM tolerance (a&b), biocompactibility of S1 and M1(c&d) and hemolysis test for M1 (e) &S1 (f).

4.10. IAA production Assay

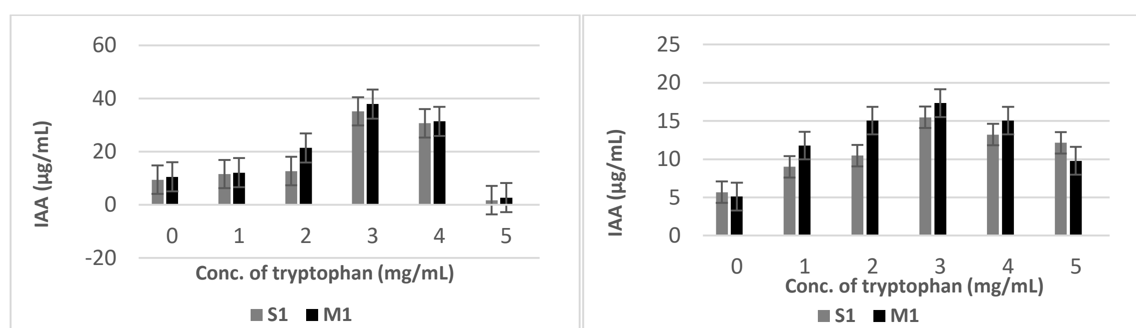
The both bacterial isolates exhibit their positive reaction by developing pink colour when reacted with Salkowski's reagent which indicates positive result for IAA production.

Observation of IAA production was carried out from 24-96 h at 24 h interval. The IAA production began 24 h after incubation and reached maximum production during the 72 hour of incubation period, then decreased after 96 h. The maximum IAA production of

35.19 µg mL^{-1} and 37.89 µg mL^{-1} were obtained when 3 mg mL^{-1} of l-tryptophan was amended in the medium for both isolates S1 and M1 respectively after 72h.



a. b.



c. d.

Figure: 21 IAA production ability of the isolates S1 & M1 after 24h (a), 48(b), 72(c) and 96(d) h of incubation at 37ºC

4.11. Nitrogen fixation assay

The both l isolates were screened for their ability to fix nitrogen on the NFb solid medium in which M1 isolate gave a maximum zone of colour than S1 isolates (Fig. 15 a).

4.12 Ammonia Producing Assay

The qualitative determination of ammonia production was recorded from both bacterial isolates, however, M1 showed the brown colour indicates maximum ammonia production (Fig.15b).

4.13 Zinc solubilizing assay

Both M1 and S1 bacterial strains were cultured on Bunt and Rovira agar plates for their potential to solubilize inorganic ZnO and ZnCO3 and both showed positive response against Zn solubilisation (Fig.15d&e). The efficiency of these bacterial strains was then checked on

the basis of colony and halozone diameter (Table 5) .Both isolates exhibited varying degree of zinc solubilization in both zinc supplemented medium (ZnO and ZnCO3).Result showed that M1 in ZnO and amended media showed maximum solubilizing efficiency and solubilizing index (S.E. = 232.14±7.29, S.i. = 3.32±0.83) followed by S1 (S.E. = 196.3±3.70, S.I. = 2.96±0.74). Similarly M1 in ZnCO3 and amended media showed maximum (S.E. = 204.17±4.17, S.I. = 3.014±0.04) followed by S1 (S.E. = 175±7.23, S.I. = 2.75±0.72).

Table 5: Zinc solubilizing and solubilizing index

isolates	Zn sources at 0.1%			
	Solubilizing efficiency (%)		solubilizing index	
	ZnO	ZnCO3	ZnO	ZnCO3
M1	232.14±7.29	204.17±4.17	3.32±0.83	3.014±0.042
S1	196.3±3.70	175±7.23	2.94±0.74	2.75±0.72

4.12 Phosphate solubilizing assay

4.12.1 Solid agar plate

The MRVK agar plate containing bromophenol pH indicator. The yellow or orange halozones were formed around phosphate solubilizing bacteria. Produced yellow or orange halozones (Fig. 15c) due to acid production presence of bromophenol pH indicator in the medium. The change in colour was found due to acidification of the medium by the p-solubilization. The decolorization of S1 greater than M1.

4.12.2 PVK agar plate

S1 was solubilized phosphate on Pikovskaya's agar medium by inducing clear zones (Fig. 15) but M1 only grow on the pvk agar plate having no any clear zone.

4.12.3 Liquid pvk medium

The bacterial strains tested differed in their ability to solubilize TCP, based on the formation of clear halos around colonies growing on Pikovskaya agar medium. Specifically, S1 presented a small zone but not M1. In contrast, the Bacillus strains did not produce any clear zone surrounding the colonies on this medium, even though these bacteria have previously been shown to dissolve TCP on Pikovskaya agar (Figueroa-López et al. 2016). To

verify that these strains had not lost their ability to solubilize TCP, we performed an experiment in liquid Pikovskaya medium. Efficiency of phosphate solubilization was further confirmed by measuring the level of solubilized P in the liquid PVK (Table 6). It is evident that S1 isolate from showed highest P solubilization (2.36±0.06 mg/L and 7.75±0.02mg/L) and M1 isolate also showed lowest P solubilization (1.43±0.1 mg/L amd 6.86±0.05mg/L) after 7 and 15 days respectively.

Table 6: Concentration of phosphate solubilizes for Both S1 and M1

Isolates	Concentration of phosphate solubilization(mg/L)	
	7 days	14 days
M1	1.43±0.1	6.86±0.05
S1	2.36±0.06	7.75±0.02

4.14 Organic acid production

4.14.1. PH of culture medium

Inoculation of medium with ARB strains caused a significant decrease in the pH as compared to un-inoculated control. The pH of the culture medium was taken after different time intervals and it was observed that pH gradually decreased with time. Maximum pH reduction (4.8, 4.2) was observed in S1 bacterial strain followed by M1 (5, 4.4) after 7 and 15 days respectively.

Table 7: pH of PSB broth after 7 and 15 days

Isolates	Initial pH	PH after 7day	PH after 15 days
M1	7.2	5	4.4
S1	7.2	4.8	4.2

4.14.2. Estimation of organic acid by HPLC

High Performance Liquid Chromatography (HPLC) was used to examine the organic acids produced ARB isolates M1 and S1 based on the retention time of citric acid, succinic acid, tartaric acid and lactic acid were found to be 7.81, 11.42, 8.34 and 12.01 min respectively by running standards of those organic acids. By comparing the retention time of standards, Organic acids detection in the pvk culture broth of the both isolates S1 and M1 were determined after 7 day of incubation. Two organic acids citric (136.82± 16.72) and

lactic acid (2173.30± 140.12) were detected from the culture medium of the isolates M1 (Fig.a) and only citric acid by (147.97± 5.33) S1 (Fig.14b). Deionized water was taken as a control.

Table 8: Organic acid concentration produced by both isolates

Isolates	Organic acid concentration(µg/mL)	
	Citric acid	Lactic acid
S1	147.97± 5.33	
M1	136.82± 16.72	2173.30± 140.12

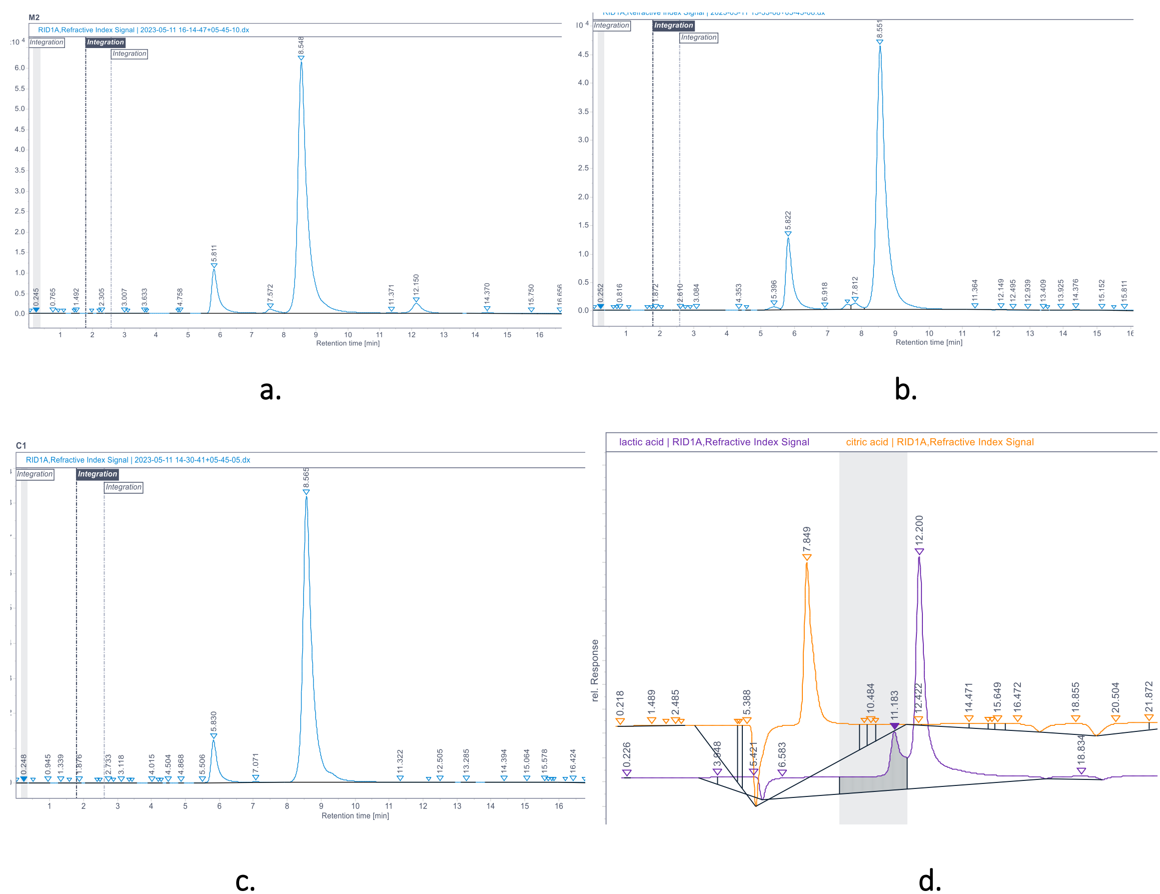


Figure: 22 Retention time of M1 (a), S1 (b), Control(c) & standard (d) of citric acid and lactic acid

4.15. Phosphatase assay

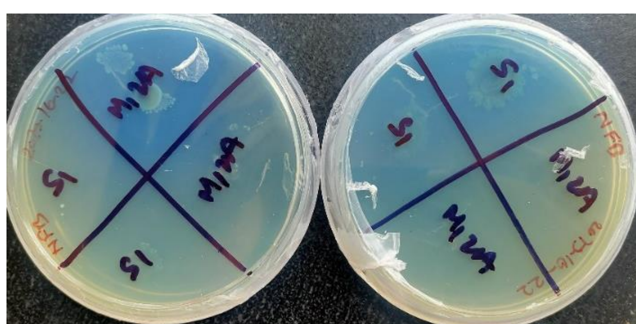
Alkaline and acid phosphatase activities for the both isolates were detected in the culture medium. Alkaline phosphatase activity was greater than that of acid phosphatase in S1 and

M1 after 7 days, acid phosphatase activity was 0.008±0.002 and 0.019±0.004 and alkaline phosphatase 0.075±0.0059 and 0.029±0.0.001 produced by S1 and M1 respectively.

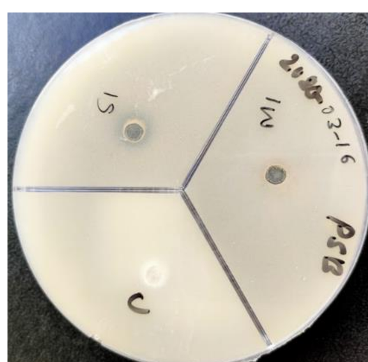
Table 9: Concentration of ALP and ACP produced by S1 and M1

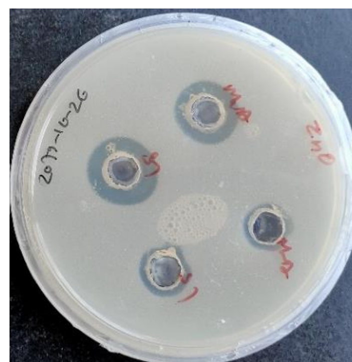
Isolates	Concentration (U)	
	ALP	ACP
S1	0.075±0.0059	0.008±0.0002
M1	0.029±0.001	0.019±0.004

*U= µmol/mL



a. b.





c. d. e.

Figure: 23 Blue colour indicating nitrogen fixation abilities of Both S1 and M1 strains (a), Brown colour indicates ammonia production of both S1 and M1 strains,phosphate solubilizing (c) and clear zone indicate zinc solubiling on ZnO(d) and ZnCO3(e) by S1 and M1

4.16 In vitro screening of bacterial isolates for their bio control properties

4.16.1 Screen for hydrogen cyanide (HCN) production

Both isolates were converted the yellow colour to deep yellow to reddish brown that indicates positive for HCN production (Fig.16a).

4.16.2. Dual plate assay for antagonism of isolates

The isolated ARB displayed excellent antifungal activity against the tested fungal pathogens. In the present study, three different fungal pathogenic strains *Fusarium oxysporum, Alternaria solani, and Rhizoctonia solanin* were used for the antifungal assay. Dual culture plate assay was used to determine the inhibition zones of the test fungal pathogen. Inhibition zones of pathogenic fungi on PDA plates were measured as percentage values. The highest percentage of growth inhibition was observed on *Fusarium oxy*. (28.75±1.2, 26.25±1.2) followed *R.solani* (18.29±1.22, 25.61±1.21 and *A. solani* were (20.93±2.33, 19.77±1.16) by S1 and M1 isolates respectively.

Table 10: Inhibition zone of fungus by S1 and M1

Isolates	inhibition ZONE (%)		
	A. solani	*R. solani*	*F. oxysporum*
M1	19.77±1.16	25.61±1.21	26.25±1.2
S1	20.93±2.33	18.29±1.22	28.75±1.2

4.16.3 Detection of Biofilm production

4.16.3.1Congo red agar assay

The isolates were screened for the biofilm formation and were confirmed by Congo red agar method. All the isolates show black colonies with a dry crystalline consistency which indicated positive for biofilm formation (Fig.16b).

4.16.3.2 Tube assay

The biofilm formation was also evaluated qualitatively by tube assay. The tubes were stained with crystal violet and the entire isolates shows adherence to the walls and bottom of the test tube (Fig. 16c).

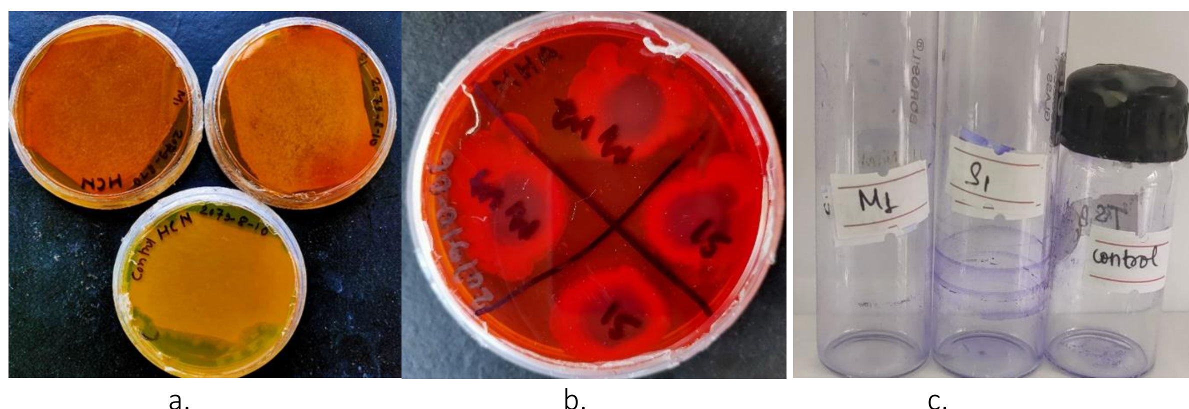
4.16.3.3 Microtiter plate assay

The quantitative estimation of the biofilm was done by microliter plate assay. Optical Density (OD) was recorded at 550nm using ELISA reader. The mean values of OD blank were subtracted from the mean values of OD of test strains. The isolates were classified into: non-adherent (OD < ODc); weakly-adherent (ODc < OD < 2xODc); moderately-adherent (2xODc < OD < 4xODc); strongly-adherent (4xODc < OD) (table 14) (Stepanovic et al., 2000).

Table 11: Detection of Biofilm by microtiter plate assay

Isolates	OD at 550nm(DC)	OD at 550nm(UDC)	Biofilm formation
S1	1.14±0.3	1.29±0.14	Strong
M1	1.35±0.22	1.3±0.19	Strong

* 2(control) =0.399±0.022,* 4(control) =0.796±0.044 DC=diluted culture, UDC= undiluted culture



a. b. c.

Figure: 24 HCN activity (a), black colonies with a dry crystalline consistency which indicated positive for biofilm formation (b), 35 stained with crystal violet and shows adherence to the walls and bottom of the test tube indicates positive for Biofilm product

4.17 Hydrolytic enzymes production

4.17.1 Protease production activity

The clear zones were formed around colony of both isolates S1 and M1 which indicated that both was positive for protease (Fig.17a)

4.17.2 Cellulose production activity

Both Bacterial isolates showed clear halo zone on cellulose medium after addition of Congo red was indicated a positive result for cellulose synthesis (Fig.17b)

4.17.3 Production of amylase (starch hydrolysis)

The plates were flooded with iodine solution, kept for a minute and then poured off. Iodine reacts with starch to form a blue color compound. This blue color fades rapidly. Hence the colorless zone surrounding colonies indicates the production of amylase (Fig.17c & 17d).

4.17.4 Laccase production activity

Laccase enzyme react with guaiacol to give reddish brown color product, both isolates S1 and M1 were found to be positive. Development of brown color zone, surrounding the bacterial growth, was indicating the production of Laccase (Fig.17e).

4.17.5 Lipase producing activity

Both isolates were showed white precipitate around colony indicated lipase activity (Fig.17f).

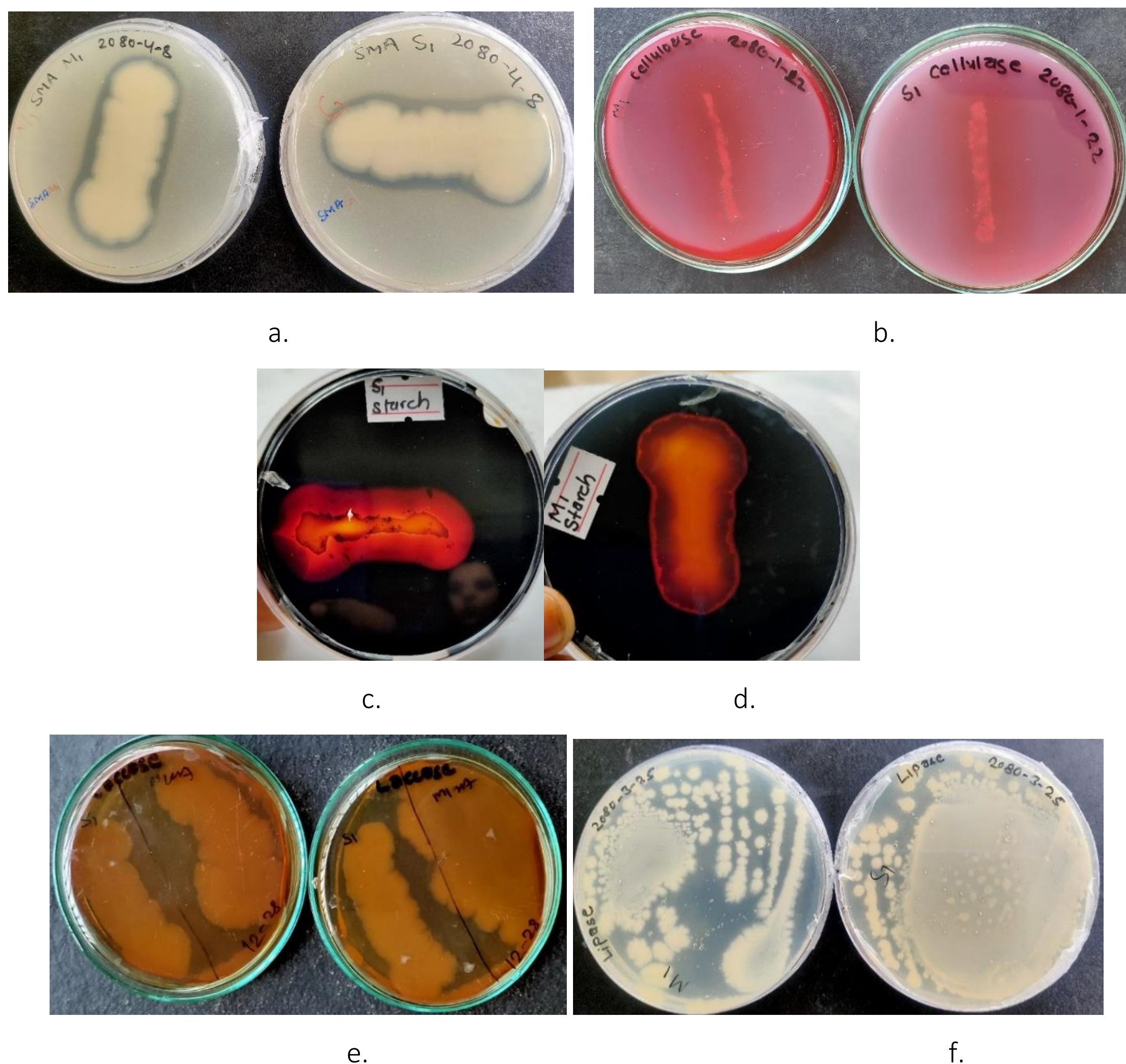


Figure: 25 clear zone formed around colony in SMA (a) and CMC (b) indicates positive for protease and cellulose, colourless zone surrounding colonies indicates the production of amylase(d), Development of brown color indicating laccase production(e) and precipitate

4.18 Growth parameter of plant

4.18.1 Shoot length (SL)

Our results indicated that bacterial inoculation enhanced the shoot length in both arsenic amended soil and without arsenic amended soil (fig.18 a&b). The treatments had significantly ($P < 0.05$) enhanced the plant shoot height over untreated plants grown in arsenic amended soil. The maximum shoot length was showed by strain S1 20.67±2.35

(38.95%) in half dose fertilizer fertilizer+ARB +arsenic amended soil followed by 19.62±0.34 (35.70 %) in S1(ARB)+arsenic amended soil while the maximum shoot length was showed by strain M1 19.50±1.54 (35.30 %) in fertilizer+ARB +arsenic amended soil followed by 17.73±3.59 (28.85 %)in S1(ARB)+half dose fertilizer+ normal soil which increased shoot length as compared to the un-inoculated control in both arsenic amended and normal soil.

4.18.2 Leaf Area (LA)

Both bacterial treatment significantly (P < 0.05) influenced leaf area of plant over untreated plants (control) grown in arsenic amended soil (fig.18c &d). The maximum leaf area was showed by strain S1 91.00±0.00 (59.78 %) in half dose fertilizer+S1ARB +arsenic amended soil followed by 82.00±0.00 (55.37 %) in S1(ARB)+normal soil while the maximum leaf area was showed by strain M1 61.81±5.87 (40.78 %) in M1(ARB) +arsenic amended soil followed by 52.95±4.00 (30.89 %) in M1(ARB)+ normal soil which increased leaf area as compared to the un-inoculated control in both arsenic amended and normal soil.

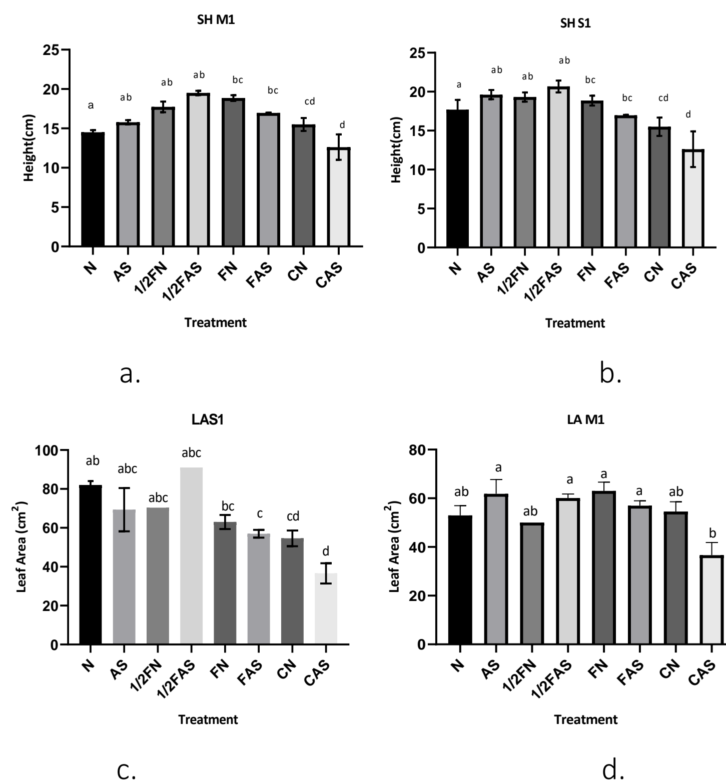


Figure: 26 : Effect of sodium arsenite on shoot height (a&b) and leaf area(c & d) of Brassica juncea with and without S1 and M1 PGP ARB. Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM

Table 12: Efficacy of strains S1 and M1 against shoot lenght and leaf area

	Efficacy of isolates (%)			
	SL		LA	
Treatment	S1	M1	S1	M1
N	28.72	12.10	55.37	30.88
AS	35.70	20.06	47.21	40.78
1/2FN	34.69	28.85	47.97	26.80
1/2FAS	38.95	35.10	59.78	39.08
Un-inoculated Treatment				
FN	33.09		41.91	
FAS	25.64		35.74	
CN	18.60		32.95	
CAS	0.00		0.00	

4.18.3 Root length (RL)

The M1 bacterial inoculation did not significantly ($P > 0.05$) affect root length parameter (fig.19a) with maximum root length was showed by strain M1 7.50±0.29 (29.78 %) in M1ARB +arsenic amended soil followed by 6.33±0.24 (16.84 %) in half dose fertilizer +M1 (ARB) +normal soil. While The analysis of data in (Figure 19b) showed significantly ($p \leq 0.05$) higher length promotion over un-inoculated plant (control) for S1 strain with maximum root length was showed by strain S1 8.50±0.67 (38.04 %) in S1 (ARB) +arsenic amended soil followed by 7.53±0.55 (30.09 %) in S1 (ARB) + normal soil which increased leaf area as compared to the un-inoculated control in both arsenic amended and normal soil.

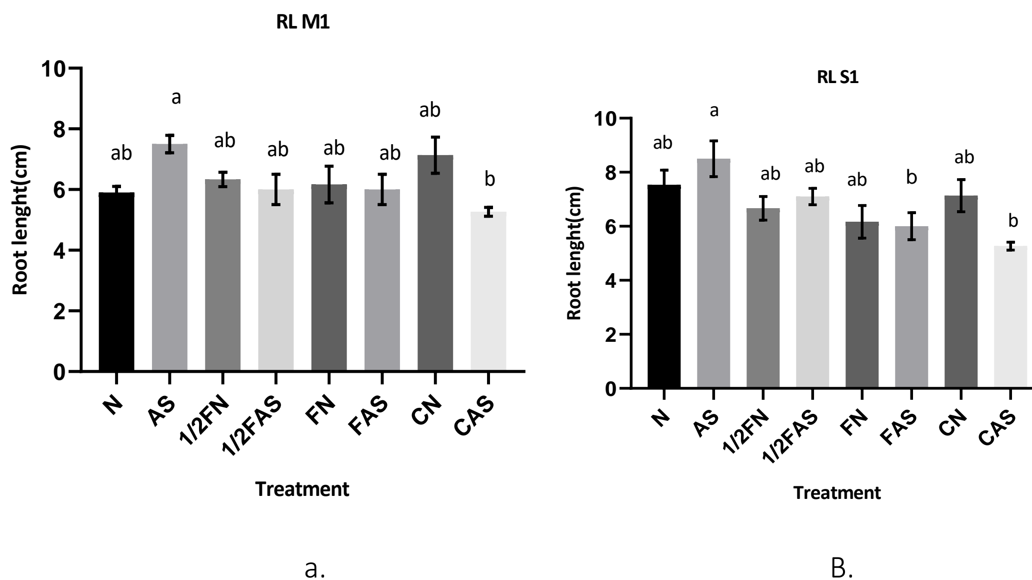


Figure: 27 Effect of sodium arsenite on Root length of Brassica juncea with and without S1 (b) and M1 (a) PGP ARB. Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM.

Table 13: Efficacy of strains S1 and M1 against Root lenght

	Efficacy (%) RL	
Treatment	S1	M1
N	30.09	10.73
AS	38.04	29.78
1/2FN	21.00	16.84
1/2FAS	25.82	12.22
Un-inoculated	**Treatment**	
FN	14.60	
FAS	12.22	
CN	26.17	
CAS	0.000	

4.18.3 Fresh shoot and dry shoot weight

The statistical analysis of data showed that bacterial inoculation M1 strain treatments had not significantly (P < 0.05) enhanced the plant fresh shoot weight and dry shoot weight over untreated and treated plants grown in arsenic amended soil (Fig.20) but bacterial inoculation S1 strain treatments had significantly (P < 0.05) enhanced the plant fresh shoot

weight and dry shoot weight over untreated and treated plants grown in arsenic amended soil (Fig. 20).

Fresh shoot (FW)

The maximum fresh weight was showed by strain M1 4.96±0.64 (32.642 %) in M1ARB + arsenic amended soil followed by 4.77±1.32 (30.19 %) in full dose fertilizer +normal soil (Fig.20a) while the maximum fresh weight was showed by strain S1 7.09±0.944 (52.87 %) half dose fertilizer+S1(ARB) +normal soil followed by 4.92±0.71 (32.02 %) in S1(ARB)+ arsenic amended soil (Fig.20b) which increased fresh weight as compared to the un-inoculated control in both arsenic amended and normal soil.

Dry shoot weight (DW)

The maximum dry weight was showed by strain M1 0.62±0.04 (37.21 %) in 50 %+ M1ARB +arsenic amended soil followed by 0.51±0.06 (22.764 %) in M1 ARB+ normal arsenic amended soil (Fig.20b) while the maximum dry weight was showed by strain S1 0.77±0.09 (49.17 %) in half dose fertilizer+S1(ARB) + normal soil followed by 0.58±0.09 (32.24 %) in half dose fertilizer+S1ARB + arsenic amended soil (Fig.20d) which increased dry shoot weight as compared to the un-inoculated control in both arsenic amended and normal soil.

Table 14: Efficacy of strains S1 and M1 against Fresh weight and dry weight of plant

	Efficacy (%)			
Treatment	FW		DW	
	S1	M1	S1	M1
N	27.38	27.26	1.41	6.71
AS	32.03	32.64	1.91	22.76
1/2FN	52.87	6.40	49.17	21.76
1/2FAS	20.81	23.23	32.24	37.21
Uninoculated	Treatment			
FN	30.19		20.49	
FAS	22.08		7.01	
CN	19.77		16.81	
CAS	0.000		0.000	

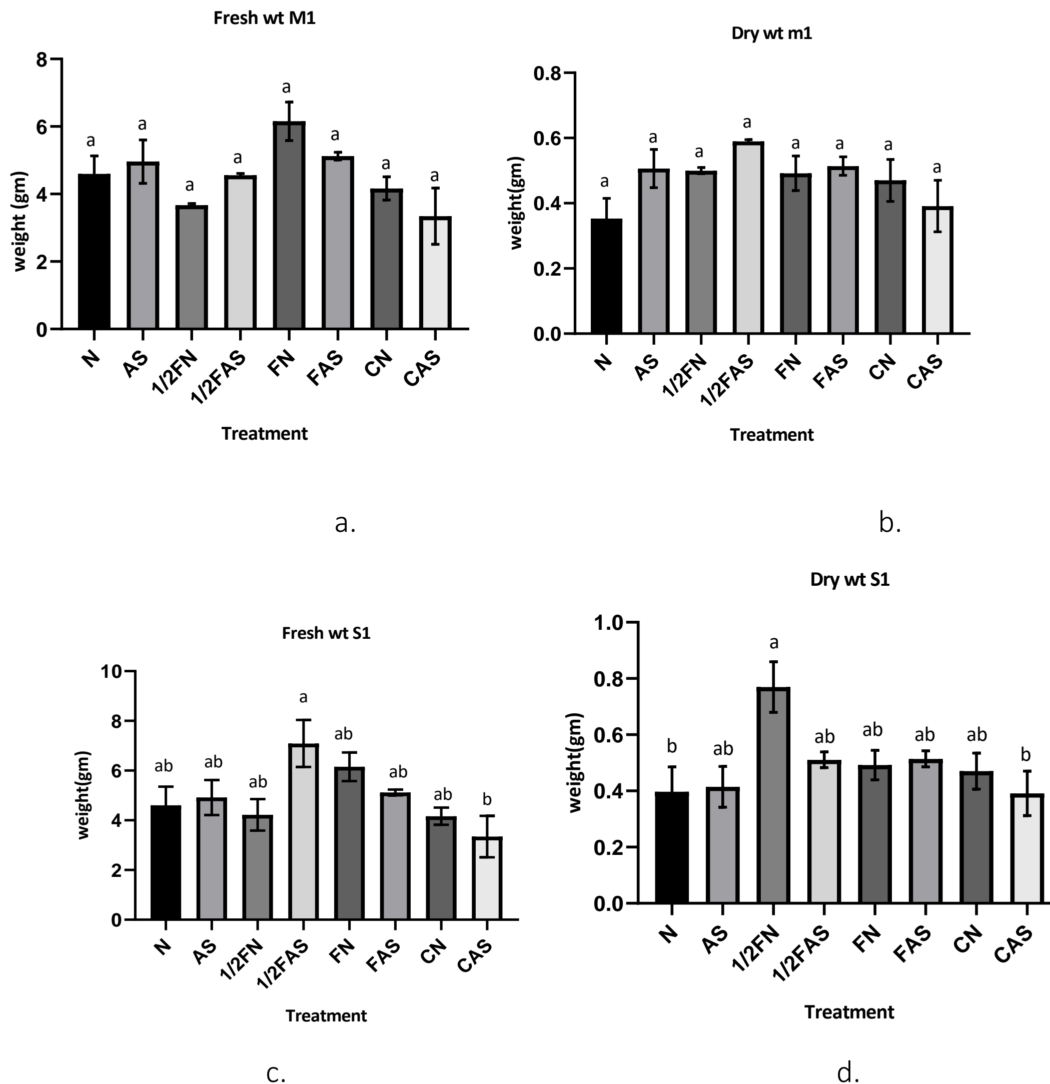
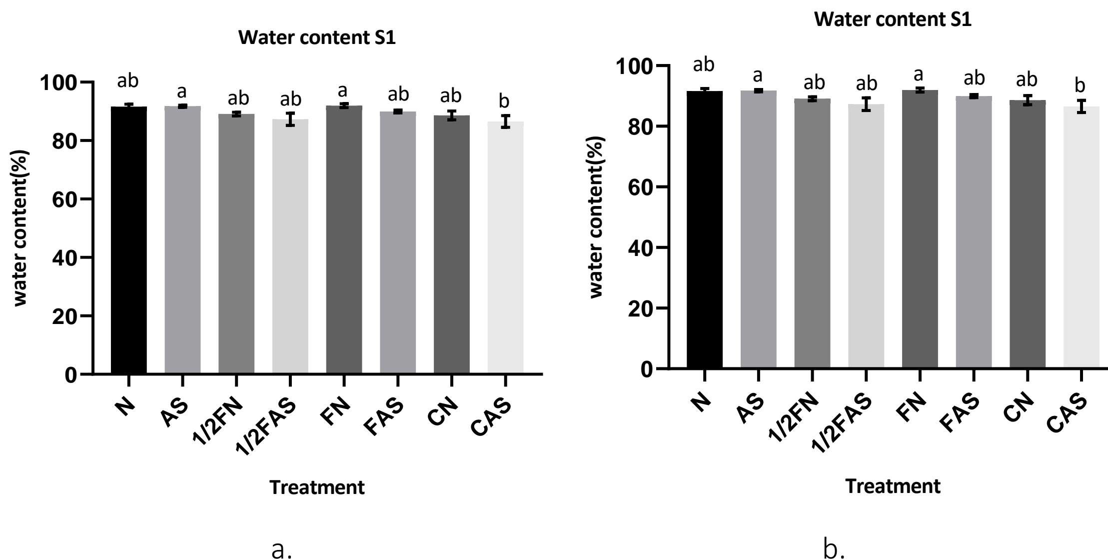


Figure: 28 Effect of sodium arsenite on fresh weight and dry weight of Brassica juncea with and without M1 (a&b) & S1(c&d) ARB. Different letters on top of bars indicate significant differences (tukey test, $P < 0.05$). Vertical bars indicate the mean ± SEM

4.19. Water content (WC)

The water content of plant treated by both isolates were significantly ($P<0.05$) increase as compared to both controls treated with arsenic and without arsenic soil. The maximum water content was (91.78±0.39 %) in arsenic amended soil followed by (91.58±0.88 %) normal soil treated with S1 isolate(Fig.21a) while M1 isolate has maximum water content was (92.40±0.70 %) in normal soil as compared to arsenic amended soil was (89.64±0.79 %) (Fig.21b).



a. b.

Figure: 29 water content of Brassica juncea with and without M1 (a) and S1 (b) PGP ARB. . Different letters on top of bars indicate significant differences (tukey test, P < 0.05). Vertical bars indicate the mean ± SEM.

Table 15: Efficacy of water content of Brassica juncea with and without M1 and S1 PGP ARB

Treatment	Water Content Eficacy (%)	
	S1	M1
N	5.52	6.36
AS	5.72	3.47
1/2FN	2.87	6.36
1/2FAS	0.87	0.59
Un-inoculated Treatment		
FN	5.91	
FAS	3.82	
CN	2.38	
CAS	0	

4.20. Chlorophyll Content

In comparison to untreated control plants grown in arsenic amended soil chlorophyll a, b and total chlorophyll content were significantly (P<0.05) improved in all the treatments by both S1 and M1 isolates (Fig.22). The highest chlorophyll 'a', chlorophyll 'b', total chlorophyll for S1 38.59±5.80 (61.42 %), 12.29±2.80 (61.44 %), and 50.88±6.08 (61.43 %) µg/mL fresh wt. in arsenic amended soil (Fig22.c,d &e) respectively whereas The highest chlorophyll 'a', chlorophyll 'b', total chlorophyll for M1 (39.17±2.24 (61.10 %) in half dose of fertilizer+M1ARB+Normal soil, 13.43±1.27(64.71 %) in M1+normal soil, 52.57±2.59

(62.667) µg/mL fresh wt. in half dose of fertilizer+M1ARB+Normal soil (Fig.22a,b&e) respectively were recorded in 24 days old crop plants . The lowest chlorophyll 'a', chlorophyll 'b', total chlorophyll (14.89±0.51, 4.74±0.05, 19.63±0.39) µg/mL fresh wt. were recorded in crop grown without plant growth promoting ARB in arsenic amended soil.

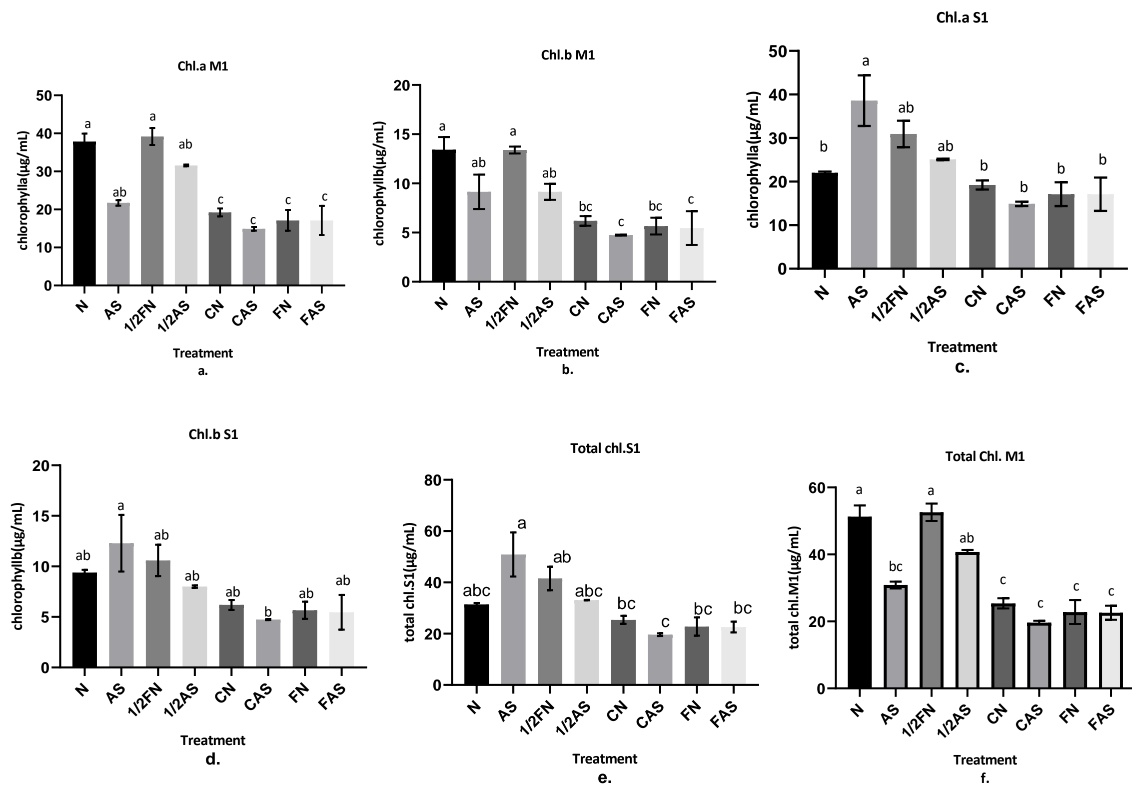


Figure: 30 Effect of sodium arsenite on chlorophyll a, b and total chlorophyll content of Brassica juncea with and without M1 (Fig.a, b &f) and S1(c, d &e PGP ARB. Different letters on top of bars indicate significant differences (tukey test, $P < 0.05$). Vertical bars indicate the mean mean ± SEM

Table 16: Efficacy of chlorophyll a and b of Brassica juncea with and without M1 and S1 PGP ARB

Efficacy (%)				
	chla		Chlb	
Treatment	**S1**	**M1**	**S1**	**M1**
N	32.44	60.69	49.55	64.71
AS	61.42	36.10	61.44	48.17
1/2FN	51.88	61.10	55.29	64.62
1/2AS	40.66	52.85	40.78	48.13
Un-inoculated	**Treatment**			
FN	13.07		16.26	
FAS	12.96		13.27	
CN	22.52		23.36	
CAS	0.00		0.00	

Table 17: Efficacy of total chlorophyll content of Brassica juncea with and without M1 and S1 PGP ARB

Total chlorophyll efficacy (%)		
Treatment	**S1**	**M1**
N	37.54	61.74
AS	61.43	22.60
1/2FN	52.75	62.67
1/2AS	40.69	51.79
Un-inoculated	**Treatment**	
FN	13.86	
FAS	13.04	
CN	22.72	
CAS	0.00	

4.21. Total protein content

The result showed that both inoculation S1 and M1 had significantly ($P<0.05$) enhanced the leaf protein content over uninoculated untreated plants grown in arsenic amended soil fig.The results on the effect of plant growth promoting ARB on protein content in leaf of Brassica juncea after 24 days . The highest protein 603.52±206.73 (109.07 %) µg/mL fresh wt. in arsenic amended soil followed by 483.53±46.73 (111.32 %) µg/mL in normal soil with half (50%) fertilizer dose were recorded in leaf of Brassica juncea grown with M1 ARB treatment of PGPR(Fig.23a) whereas the highest protein content 1493.53±146.73 (103.66%) in 1/2FNS1 treatment followed by 541.53±31.26 (110.11%) µg/mL in arsenic amended soil was recorded in leaf of Brassica juncea grown with S1 ARB treatment of PGPR (Fig.23b). The lowest protein contents -54.73±7.53 µg/mL fresh wt. in arsenic amended soil were recorded in the in leaf of Brassica juncea crop grown without plant growth promoting ARB.

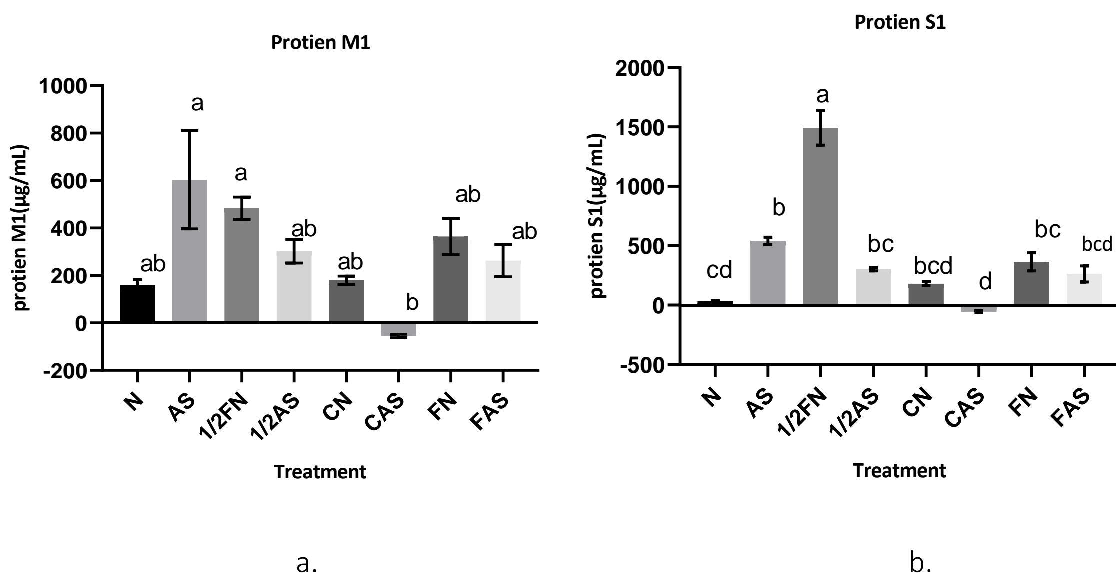


Figure: 31 Effect of sodium arsenite on total protein content of Brassica juncea with and without M1 (a) and S1 (b) PGP ARB. Different letters on top of bars indicate significant differences (tukey test, $P < 0.05$). Vertical bars indicate the mean ± SEM.

Table 18: *Efficacy of total protein content of Brassica juncea with and without M1 and S1 PGP ARB*

Treatment	Soluble protein production efficacy (%)	
	M1	S1
N	134.04	302.50
AS	109.07	110.11
1/2FN	111.32	103.66
1/2AS	118.07	118.05
Un-inoculated treatment		
FN	115.02	
FAS	120.85	
CN	130.36	
CAS	0.000	

4.21 Molecular Characterization

Based on 16S rRNA gene sequencing, isolates S1 and M1 showed highest similarity with *Bacillus cereus* (99.77%) and *Bacillus weidmanii* (99.69%) respectively. Based on based on 16S rRNA gene sequences, neighbourjoining phylogenetic tree showing the position of ARB isolates and the closely related species of the genus *Bacillus* (Fig.24).

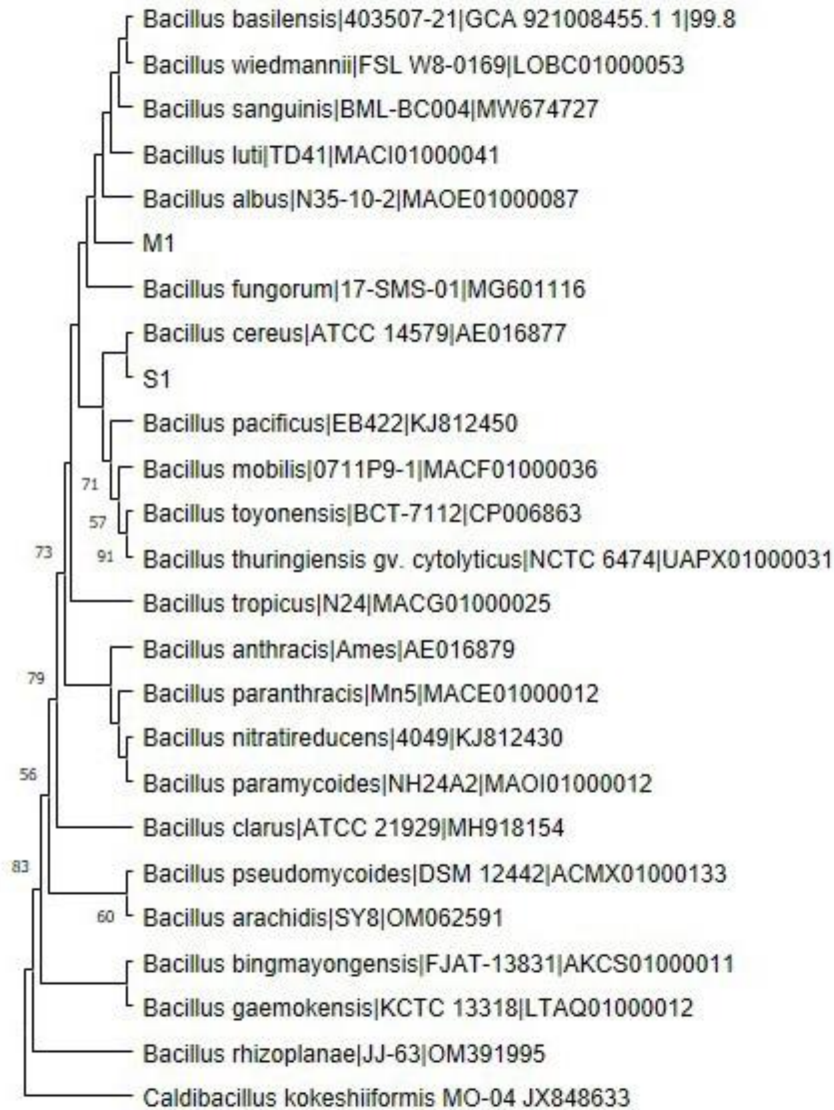


Figure: 32 Neighbor-joining tree based on 16S rRNA gene sequences, showing the position of isolates S1 and M1, and their closely related reference strains inclusive of other *Bacillus* species. Bootstrap values (expressed as percentages of 1000 replications)

4.23 Summary

Hence arsenic resistant Bacillus was isolated from soil and were assayed for MIC of arsenic which could tolerate sodium arsenite (1000 ppm) and sodium arsenate (5500 ppm) and isolated arsenic resistant *Bacillus* were Arsenic oxidizing in nature and were confirmed by silver nitrate test and In addition, both isolates showed heavy metal resistant against cobalt, copper, and zinc up (200 ppm) and lead (400 ppm). The isolated ARB exhibited plant growth-promoting activities such as auxin and hydrogen cyanide production, phosphate solubilization, and nitrogen fixation zinc solubilization, biofilm production,

heavy metal resistant, organic acid production, phosphatse production, amylase ,HCN, protease ,Laccase ,lipase producers. Bio control activities were also observed *against F. oxisporum, R. solani* and *A. solani* etc. The effect of ARB analyzed in green on growth of Brassica juncea L. Czern, both in presence and absence of arsenic where significantly increase in shoot length, root lengths, leaf area, water content ,chlorophyll a & b,total chlorophyll, soluble protein in leaf were observed as compared to uninoculated control in the presence and absence of arsenic on plant.

CHAPTER V

DISCCUSSION

The species of Bacillus belong to the bacteria kingdom; Phylus Firmicutes; Class Bacilli; Order Bacillales and Family Bacillaceae (Maughan & Van der Auwera et al., 2011). Currently, the genus includes over 427 species (Alcaraz et al., 2010); https://www.bacterio.net/genus/bacillus). The greatest concentration of heavy metals, including arsenic and its derivatives is found in soil. These substances might be detrimental to plants and animals as well as other soil constituents. But microbes that can use and break down these dangerous metals can also be found in soil, offering a potential solution to this issue such as *Bacillus* species (Radhakrishnan et al., 2017; Schallmey et al., 2004) . In our study ARB were isolated from sixteen soil sample. In total, 62 colonies were randomLy selected and subcultured on NA media supplemented with increasing concentration of sodium arsenite and arsenate only two isolates M1 and S1 could tolerate higher level of arsenate and arsenite concentration. Isolation of arsenic resistance *Bacillus* species from soil have been reported previously (Shakya et al. 2012; Dey et al. 2016). Both S1 and M1 ARB exhibited MIC at a range 1000 ppm sodium arsenite and 5500 ppm sodium arsenate in NA medium but both the isolates showed decreased growth pattern with increasing concentration of arsenite, and finally stopped growing after 1000 ppm arsenite and 5500 ppm of arsenate. So only two isolates S1 and M1 considered as hyper tolerant arsenic resistant bacteria and selected for Bioremediation and PGPR. This reults indicated that presence of hyper tolerant arsenic *Bacillus* in arsenic contaminated agricultural soil and water of Nepalease is abudent. It was also reported that large number of *Bacillus* were found in arsenic contaminated soil and water (Bista & Shakya et al., 2017b; Magar et al., 2022a; Poudel et al., 2019).

For examination of oxidative and reductive properties in media plates supplemented by arsenic and flooded with AgNO3 brown precipitation seen that indicated both strains S1 and M1 oxidized arsenite i.e. arsenic oxidizing bacteria. Both ARB isolates M1 and S1 were grown in NB supplemented with sodium arsenite (200 ppm-1400 ppm). The growth of the isolates was monitored periodically by measurement of optical density. The growth rates of M1 and S1 showed significant decreased with increasing concentration and growth rate

increasing while increasing time period as 24,48,72 h increasing the bacterial growth which indicates removal or detoxification of sodium arsenite to less toxic arsenic. *Bacillus* species isolated were identified as arsenic oxidizing *Bacillus* (Anguita et al., 2018; Laha et al., 2021). Bacillus are arsenic resistant bacteria can be used as a novel pathway for the bioremediation of arsenic (Dey et al., 2016). The bacterial isolates (FAs 1, 4 and 9) were exhibited tolerance against sodium arsenite at 100mM (Iqtedar et al., 2019). In Nepal, highly arsenic-resistant strain of *Bacillu*s species has been reported by (Poudel et al., 2019) which can tolerate up to 1,000 ppm As(III) and 15,000 ppm As(IV) and also reported by (Magar et al., 2022a) in which *Bacillus* tolerant upto 600 ppm As(III) but in our study both arsenic resistant bacillus can 1400 ppm arsenite and 5500 ppm arsenate.This is first ever reported arsenic resistant bacteria in Nepalease agricultureal soil which have highest arsenic tolerant capabilities.

Both the isolates were white in colour, have smooth surface, flat colonies opaque, gram-positive, rod shaped, endospore, facultative anaerobes. Isolates S1 and M1 were able to ferment sugars like glucose, fructose, lactose, sucrose, galactose, mannose, mannitol, maltose and melibiolose. Based on the sugar assimilation pattern, test isolates could be *Bacillus* species.

The both S1 and M1 isolates were able to grow maximum at 4% NaCl were 0.3785 and 0.3305 and minimum at 10% were 0.0025 and 0.0035 respectively but unable to grow at higher concentration of NaCl, showing that the isolate was sensitive to the salt concentration.The results indicate that the optimum temperature for the growth of both S1 and M1 isolates at 37°C were 0.694 and 0.805 respectively. The effect of pH on growth of ARB strains were studied at different pH range 2-8. The results indicate that the optimum pH for the growth of both S1 and M1 isolates at 7.0 were 0.61 and 0.621. The growth was hampered at very acidic and alkaline pH as indicated by growth of the soil isolate.The data graph experienced a decrease in growth along with changes in the pH of the highest and lowest conditions.In a study *Bacillus subtilis* was able to tolerate pH in arrange of 5.0-10 with optimal growth at 7.0-7.5 (Satapute et al., 2012). According to the findings, the soil's pH was slightly acidic, which is ideal for the majority of plant growth (Kumar et al. 2019). Furthermore, several beneficial bacteria are suitable for crop promotion and can withstand an acidic pH (Kumar et al. 2019).

The both were showed a varied level of resistance to heavy metals tested. Results obtained revealed a great deal of variation in resistance pattern of the promising isolate to each metal ion studied. Both isolates S1 and M1 have resistant against cobalt, copper, and zinc (200 ppm) and lead (400 ppm) .This results indicated that both S1 and M1 isolates have abilities to tolerant against arsenic and heavy metal which make it ideal for bioremediation. A bacterium was could tolerate 36.49 mM arsenic (III), 280.44 mM arsenic (V) and 63 mM chromium (III) (Mandal et al., 2022). *Bacillus cerus* was removed cd(III) (Huang et al., 2013) and Psychrotrophilic *Bacillus* sp. PZ-1 effectively removes Pb(II) (Ren et al., 2015) from from aquous solution. Heavy-metal ions are known to co-regulate genes responsible for antibiotic resistance and decrease antibiotic susceptibility (Liang et al., 2022).

It has been observed that all both isolated strains were resistant to Ciproflaxin cip, Nalidixic acid, ceftazidime, Gentamicin, Bacitracin, cefaclor, Amikacin, Ertapenem, Colistin, Cepalpthin, and Linezolid . Environmental pollution not only triggers heavy-metal co-selection processes but also increases the level of tolerance to some antibiotics due to co-regulation of resistance genes (Seiler & Berendonk et al., 2012). The combined expression of antibiotic and heavy-metal resistance by *B. cereus* and *P. aeruginosa* may not be a chance phenomenon but rather a result of selection by heavy metal present in an environment [(Shammi & Ahmed, 2016), (Arifiyanto et al., 2017), (Nath et al., 2018)]. The present study focused on screening antibiotic-producing *Bacillus* sp. that could be used as an alternative to commercial antibiotics and pesticides against a broad range of microbes, especially those responsible for the production losses in agriculture and fisheries.

IAA production was measured highest when the concentration of tryptophan was 3 mg/mL. At higher concentration of tryptophan, there was low accumulation of IAA, this may be because tryptophan inhibits the growth of certain *Bacillus* species. According to (Ahmad et al., 2005), there was a difference between the generation of IAA and the increase in tryptophan concentration in the medium. The spectrophotometric analysis revealed that both in S1 and M1 bacteria, there was a progressive rise in IAA synthesis with corresponding L-tryptophan concentrations .Increasing the amount of IAA released when l-tryptophan (0–5 mg mL^{-1}) is added to the medium demonstrates that these bacterial strains used L-tryptophan as a precursor for IAA production. For isolates S1 and M1 respectively. The maximal IAA production of 35.19 µg mL^{-1} and 37.89 µg mL^{-1} were

obtained upon adding 3 mg mL^{-1} of L-tryptophan to the medium. Subsequently, the generation of IAA was dramatically reduced by an increase in the concentration of L-tryptophan. These findings may be explained by a potential suppression of the ARB Bacillus population caused by the increased L-tryptophan concentrations. Most likely, the microbial community is stressed by the increased L-tryptophan dosage. When l-tryptophan was added to the culture media, IAA release from bacteria increased several times (Patten & Glick et al., 2002). The data collected indicate that IAA production was growth linked, with maximal production seen during the fourth day of incubation during the stationary phase of bacterial growth. After a 96 h incubation period, IAA-degrading enzymes including peroxidase and indole acetic acid oxidase may be involved in the fall of IAA production (Jeyanthi amd Ganesh et al., 2013). The production of IAA by the *B. licheniformis* isolate in presence of L-tryptophan indicates that the strain utilized l-tryptophan as a precursor for IAA production and rise in IAA production as L-tryptophan with incubation time increased (Biswas et al., 2023).

Both isolates exhibited varying degree of zinc solubilization in both zinc supplemented medium (ZnO and ZnCO3).Result showed that M1 in ZnO and amended media showed maximum solubilizing efficiency and solubilizing index (S.E. = 232.14±7.29, S.i. = 3.32±0.83) followed by S1 (S.E. = 196.3±3.70, S.I. = 2.96±0.74). Similarly M1 in ZnCO3 and amended media showed maximum (S.E. = 204.17±4.17 , S.I. = 3.01±0.04) followed by S1 (S.E. = 175±7.22, S.I. = 2.75±0.72). A variety of microorganisms that can solubilize various zinc sources have been obtained from various habitats (Dinesh et al., 2018). Zinc solubilization in soil depends on the continued metabolic potential of the indigenous bacteria which might be infuence by the geo-physicochemical property of the soil (Dinesh et al. 2018). In the rice felds, especially in the Bengal Delta, where mostly arsenic laden ground water is used for irrigation, arsenic toxicity might limit the metabolic activity necessary for zinc solubilization (Chakraborty et al., 2017) .Therefore, it might be assumed that to perform Zn solubilazation in contaminated sites microorganisms must be resistant to As. Genus *Bacillus* is one of the most studied genera as they are found to be ubiquitous in nature with multiple growth promoting traits (Ramírez & Kloepper et al., 2010; Zhao et al., 2015) .

Both isolates S1 and M1 were capable of producing ammonia. The qualitative determination of ammonia production was recorded from both bacterial isolates, however,

M1 showed the brown color indicates maximum ammonia production and both isolates were screened for their ability to fix nitrogen on the NFb solid medium in which M1 isolate gave a maximum zone of colour than S1 isolates. Ammonium is only assimilated by the GS-glutamate synthase route; *Bacillus subtilis* lacks assimilatory glutamate dehydrogenase activity. Since ammonium-grown cells exhibit a partial repression of glutamine synthesis expression, ammonium is also a good source of nitrogen for *Bacillus subtilis* (Atkinson & Fisher, 1991)). The ability of the investigated bacterial strains to solubilize TCP varied, as evidenced by the development of a distinct halos zone surrounding colonies growing on Pikovskaya agar medium. In particular, M1 lacks any visible zone while S1 solubilizes a very limited hydrolysis zone .On the other hand, no distinct zone surrounding the colonies on Pikovskaya agar was formed, even though it has been shown that the Bacillus strains may dissolve TCP on this medium (Figueroa-López et al., 2016). We conducted an experiment in liquid Pikovskaya media to confirm that these strains were still able to solubilize TCP. For seven and fifteen days, the amount of soluble Pi and pH variations in Pikovskaya broth were observed.The non-inoculated control remained at the initial pH, and no P solubilization was recorded. The S1 isolated from Sarlahi had solubilized up to *(2*.36 ± 0.06 mg/L and 7.75±0.02 mg/L) medium and decreased the pH 5 to 4.39 and M1 isolate from mahotari district also showed lowest P solubilization (1.43±0.1 mg/L amd 6.86 mg/L) and decreased pH 4.78 to 4.17 after 7 and 15 days respectively. The same outcomes were also shown by (Sridevi & Mallaiah et al., 2009) ,who showed that a specific strain of *Rhizobium* sp. raised P solubilization and lowered the pH of the growth medium from 7 to 4.05. In liquid cultures of *Bacillus sp*. strains, soluble P concentration levels of 200 µg mL^{-1} (final pH 4.46; (Oliveira et al., 2009) and 96.73 µg mL^{-1} (final pH 5.8; (Yu et al., 2011)) have been recorded. By lowering the medium content and slightly raising pH, secreted organic acids bind insoluble P to make it soluble (Marciano Marra et al., 2012). We found that there is an inverse association between pH and P solubilization. This finding is consistent with studies by (Bianco & Defez et al., 2010) and suggests that P solubilization is facilitated by medium acidification.

HPLC analysis indicates the presence of two organic acids citric (136.82± 16.72 µg/mL) and lactic acid (2173.30± 140.12 µg/mL) were detected from the culture medium of the isolates M1 and only citric acid by S1 (147.97± 5.33 µg/mL) . Glucose is the principal element of

phosphate solubilizing medium and phosphate solubilizing microorganisms prefer glucose as a carbon source to produce organic acids (Nautiyal et al., 1999). Both in plants and microorganisms, the primary mechanisms of P solubilisation are H^+ excretion, organic acid production, and acid phosphatase biosynthesis (Arcand & Schneider et al., 2006).

Both bacteria able to produces ACP and ALP. In contrast ALP was produced maximum than ACP by both S1 and M1 strain. ACP is more effective in mineralizing organic phosphorus in acidic soils with pH values less than 7 (Luo et al., 2019). Acid phosphatase had maximal activities under acidic conditions in forest and farmLand soils (Tazisong et al., 2015).However, recent evidence indicates that compared with acid phosphatases, there is a higher abundance of genes encoding alkaline phosphatases in acidic soils (Bergkemper et al., 2016). *B. amyloliquefaciens*, which had an alkaline phosphatase activity of 2594.73 U/L (Bo et al., 2023) and *Bacillus* sp. (PSB-29), which produced alkaline phosphatase and solubilized phosphates (Odeniyi & Turaki et al., 2022). Additionally, a proteolytic strain identified *as Bacillus* sp. Mar64 was found to produce alkaline protease (Kotb et al., 2023) and a novel *Bacillus* species named *B. salipaludi*s was isolated from soil and found to produce alkaline phosphatase (Xue et al., 2021).

In general, *Bacillus spp*. are well-known for their HCN (Swiontek Brzezinska et al., 2020) and catalase production (Fibriarti et al., 2021). HCN is known for its capacity to counter the development and growth of plant pathogens (Nandi et al., 2017). Both ARB S1 and M1 were strong biofilm producing *Bacillu*s .It is reported that sometimes microbial community gain protection against toxic agents through the formation of microbial biofilms (Mah & O'Toole et al., 2001). Hence it may be possible that through the formation of biofilms the isolated bacterial strains can resist the toxicity of arsenic. The arsenic resistance mechanism of bacteria can be plasmid associated (Assessment, 2009), or by ars operon, containing the genes a*rsRBC* (Carlin et al., 1995). (Adrien et al., 2021) reported that biofilm formation helps *Bacillu*s strains to occupy space and acquire nutrients as the mobility of nutrients is enhanced within the rhizosphere by the extracellular matrix, resulting in a reduction in the colonization of plant pathogens. According to (Qiao et al., 2023) restricted production of the matrix by *Bacillus subtilis* resulted in decreased biocontrol efficacy against the *Rhizoctonia solanacearum* and *Fusarium oxysporum* pathogen .

Both ARB S1 and M1 were inhibited the growth of fungus with highest percentage of growth inhibition was observed on *F. oxysporum* (28.75±1.2, 26.25±1.2 %) followed *R.solani* (18.29±1.22, 25.61±1.21 % and *A. solani were* (20.930±2.3255, 19.767±1.163 %) by S1 and M1 isolates respectively. *Bacillus* strains have ability to produce a wide variety of secondary metabolites with antimicrobial properties like iturin, fengycin and bacillomycin, which have an inhibitory effect against several types of phytopathogens (H. Lee et al., 2008) .

The following parameters of plant morphology were assessed: the shoot height, leaf area, root length and fresh and dry weight. Statistical analysis showed significant difference between the studied traits and the treatment options of bacterial suspensions. The growth parameters of PGPR-treated plants differed significantly ($P \leq 0.05$) from those of non PGPR-treated plants, as assessed by One-way ANOVA and tukey's test.

In the case *of Brassica juncea*, the highest plant shoot height increased by 38.95 % in half dose fertilizer fertilizer+ARB +arsenic amended soil followed by 35.70 % in S1(ARB)+arsenic amended soil while the highest shoot length was showed by strain M1 35.30 % in fertilizer+ARB +arsenic amended soil followed by 28.85 % in S1(ARB)+half dose fertilizer+ normal soil which increased shoot length as compared to the un-inoculated control in arsenic amended soil. ARB-PGP enhanced leaf area by highest leaf area was showed by strain S1 59.78 % in 50 % fertilizer+S1ARB +arsenic amended soil followed by 55.37 % in S1(ARB)+normal soil while the maximum leaf area was showed by strain M1 40.78 % in M1(ARB) +arsenic amended soil followed by 30.88 % in M1(ARB)+ normal soil which increased leaf area as compared to the un-inoculated control in both arsenic amended and normal soil.

The M1 bacterial inoculation did not significantly ($P > 0.05$) affect root length parameter but increased with maximum root length by 29.78 % in M1ARB +arsenic amended soil followed by 16.84 % in half dose fertilizer +M1 (ARB) +normal soil While The analysis of data showed significantly ($p \leq 0.05$) higher length promotion over un-inoculated plant (control) for S1 strain which enhanced highest root length by 38.04 % in S1 (ARB) +arsenic amended soil followed by 30.09 % in S1 (ARB) + normal soil which increased leaf area as compared to the un-inoculated control in both arsenic amended and normal soil.

Brassica juncea, the fresh weight increased by 32.64% in M1ARB +arsenic amended soil followed by 30.19 % in full dose fertilizer +normal soil whereas the maximum fresh weight increased by 52.87 % half dose fertilizer+S1(ARB) +normal soil followed by 32.03 % in S1(ARB)+ arsenic amended soil which increased fresh weight as compared to the un-inoculated control in both arsenic amended and normal soil and dry shoot weight of strain M1 increased by 37.21 % in 50 % F + M1ARB +arsenic amended soil followed by 22.76 % in S1 ARB+ normal arsenic amended soil whereas the maximum dry weight was showed by strain S1 49.17 % in half dose fertilizer+S1(ARB) + normal soil followed by 32.24 % in half dose fertilizer+S1ARB + arsenic amended soil which increased dry shoot weight as compared to the un-inoculated control in both arsenic amended and normal soil. However, in the control soils, the chlorophyll contents did not change significantly ($P > 0.001$).

In our experiments, plant growth parameter like shoot height, leaf area and root length were significantly increased in that plants those were treated with arsenic with bacterial inoculation as well half dose (50 %) of fertilizer. This results were indicated that the both bacterial strain had showed strong pgpr activities in arsenic stress. The role of *Pseudomonas fluorescens* and *P. tolaasii* in facilitating plant growth in the presence of cadmium in *Brassica napus* (Dell'Amico et al., 2008) and that of *Ni-resistant Bacillus subtilis* in *Brassica juncea* has also been reported (Zaidi et al., 2006).

When assessing the phytoremediation efficacy of plant species in heavy metal-contaminated soils (such as As), shoot and root dry biomass yields are thought to be crucial plant development characteristics (Niazi et al., 2011; Salido et al., 2003). Compared to *B. napus*, a greater decrease in the dry biomass yields of shoots and roots was seen in *B. junce*a, particularly with high As (Niazi et al., 2017).

Due to a decrease in cell turgor in *B. juncea*, the suppression of root growth at high As concentrations may also be linked to lower mitotic activity in the root meristematic zone or a reduction in cell enlargement in the elongation zone (Niazi et al., 2011). The inoculation of mustard and Chinese cabbage seedlings with *Acinetobacter calcoaceticus* SE370 significantly increased the shoot and root lengths, fresh weight, and chlorophyll content (Kang et al., 2016). Inoculation of Cr^{6+} resistant *Pseudomonas sp.* PsA4 and *Bacillus sp*. Ba32 promoted the growth of plants at 95.3 and 198.3 µg of Cr^{6+} g^{-1} soil and maximum growth was observed in the plants inoculated with strain PsA4 (Rajkumar et al., 2006).

Both *P. mexicana* S254 and *S. maltophilia* S255 enhanced the growth of *V. radiata* and a statistically significant increase in shoot and root lengths was observed both in the presence and absence of arsenic (Huda et al., 2022). The height, leaf area, number of leaves and shoot and root dry weight significantly ($P < 0.05$) decreased for plant Brassica juncea species with increasing levels of As (Niazi et al., 2017). Application of cadmium resistant *Ochrobactrum sp*., a lead resistant *Bacillus sp*. and an arsenic resistant *Bacillus sp.* the germination percentage, relative root elongation (RRE), amylase and protease activities were increased (Pandey S. et al., 2013).The overall biomass and root/shoot ratio were also enhanced by arsenic resistant *Bacillus sp*. Inoculation (Pandey S. et al., 2013).

Decreases in chlorophyll contents were found when the *B. juncea* were grown in As amended soil without the inoculation of strain S1 and M1. With the bacterial inoculation, the chlorophyll contents increased in As amended soils, compared to the uninoculated treatments ($P < 0.1$). The chlorophyll 'a', chlorophyll 'b', total chlorophyll for S1 increased by 61.42 % ,61.44 %, and 61.43 % of fresh wt. in arsenic amended soil whereas the chlorophyll 'a', chlorophyll 'b', total chlorophyll for M1 (61.10 %) in half dose of fertilizer+M1ARB+Normal soil (64.71 %) in M1+normal soil (62.67) µg/mL of fresh wt. in half dose of fertilizer+M1ARB+Normal soil were uninoculated control in arsenic amended soil.

The total protein content increased by 109.07 %, µg/mL fresh wt. in arsenic amended soil followed by 111.32 % µg/mL in normal soil with half (50%) fertilizer dose were with M1 strain whereas 103.66 % in 1/2FNS1 treatment followed by 110.11 % µg/mL in arsenic amended soil with S1 strain. Inoculation of *Agrobacterium radiobacter* catalase were increased contents of chlorophyll, contents of soluble protein,soluble sugar, and the activities of superoxide dismutase and catalase (Wang Q. et al., 2011).

Decrease in chlorophyll content in the leaves of chickpea due to drought stress however; inoculation with PGPR amended the adverse effects of drought on chlorophyll content (Khan N. et al., 2018). The co-inoculation of soybean with *B. japonicum* and *Serratia species* increased grain yield, protein yield, and total plant protein content (Dashti et al., 1997). PGPR induced increase in leaf protein content of wheat (Afzal & Bano et al., 2008; F. Islam et al., 2014). After conducting the experiments and analyzing the data, it can be concluded that the presence of arsenic-resistant bacteria in arsenic-amended soil has a significant

impact on chlorophyll levels. The results consistently showed that the bacteria played a crucial role in mitigating the adverse effects of arsenic on chlorophyll within the soil. The presence of arsenic-resistant bacteria in arsenic-amended soil positively affects chlorophyll levels.

CHAPTER IV

6.1 CONCLUSION

Generally Arsenic is available in much higher concentration in groundwater compared to surface water. The groundwater resource is adequate to support of agricultural irrigation development in the Terai. The agricultural land of Terai irrigated through shallow tube wells and deep tube wells which contaminated with arsenic above the limit of 50ppm. Thus, agricultural and horticultural products are being grown in this region from arsenic contaminated water which get contaminated with arsenic that in soil poses significant threats to crop productivity and human health so isolation of hyper tolerant ARB bacillus that transform toxic As(III) to less toxic As(IV) and finally in organic form of arsenic which is less toxic than inorganic form. Several *Bacillus* species were isolated from the soil samples. Amoung these isolates, only two M1 and S1 have hyper As tolerant ability and converted high toxic As [III] to less toxic As [IV]. Both bacteria have abilities to detoxify the sodium arsenite (1200 ppm), arsenate(5500 ppm) , cobalt, copper, and zinc up (200 ppm) and lead (400 ppm) which make it hyper tolorent and tentatively identified *Bacillus* isolates have also abilities for IAA production, phosphate solubilization, zinc solubilization, ammonia production, nitrogen fixation abilities, organic acid production, strong biofim production, antagonistic abilities, HCN production, cellulase, amylase protease, Laccase and lipase.

The application of both bacteria significantly increased in germination, plant biomass, chlorophyll, protein content water content. Hence both isolates bacterial have best performance in presence of arsenic soiland the application both isolates significantly enhanced the plant growth parameter.

In conclusion, this research work deals with isolation and characterization of hyper tolerant arsenic resistant bacteria and its plant growth efficacy on green mustard. The effect of arsenic in plant can accumulate in crops, leading to food chain contamination and adverse health effects upon consumption. Therefore, addressing arsenic contamination in agricultural soils is essential for ensuring food security and safeguarding public health in Nepal.

These both S1 and M1 hyper tolerant ARB are first bacteria isolated from Nepalease agriculture soil showing bioremediation of arsenic as well as PGPR efficacy which might be

an innovative strategies for addressing the dual challenges of environmental contamination and agricultural sustainability. Move toward a future where arsenic-contaminated soils are remedied and agricultural output is increased in a sustainable and environmentally friendly way as arsenic resistant bio fertilizer by utilizing the natural powers of bacteria.

6.2 RECOMMENDATION

Field trials were carried out to validate the efficacy of the isolated ARB PGPR and lower arsenic uptake in crops under actual agricultural conditions. These results offer useful insights into the viability and scalability of bacterial-based remediation techniques

Work towards the development of commercial microbial inoculants containing arsenic-resistant bacteria for easy application by farmers as bio fertilizer, bicontrol, nanobiofertilizer and biofilm fertilizer to facilitate the production and distribution of these inoculants to arsenic-affected regions.

Integrate the use of ARB into integrated pest management (IPM) practices to promote holistic approaches to crop protection and soil management.

Continue research efforts to explore the genetic diversity of arsenic-resistant bacteria in different geographical regions and soil types and biotransformation in targeted plant.

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Appendices

Appendix I Media Composition

A. Media composition

1. Nutrient agar

Ingredients	g / L
Peptone	5.000
Sodium chloride	5.000
Peptone	1.500
Yeast extract	1.500
Agar	15.000
Final pH (at 25°C)	7.4±0.2

2. Nutrient broth

Ingredients	g / L
Peptone	10.000
Beef extract	10.000
Sodium chloride	5.000
PH after sterilization	7.3±0.1

3. Peptone water

Ingredients	g / L
Peptone	10.0
Sodium chloride	5.0
Final pH (at 25°C)	7.2±0.2

4. Trypticase soy broth

Ingredients	g / L
Tryptone	17.000
Soya peptone	3.000
Sodium chloride	5.000
Dextrose (Glucose)	2.500
Dipotassium hydrogen phosphate	2.500
Agar	15.000

Final pH (at 25°C) 7.3±0.2

5. NFb medium

Ingredients	g/L
DL-Malic acid	5.0,
KOH	4.0,
K_2HPO_4	0.5,
$FeSO_47H_2O$	0.05,
$MnSO4H_2O$	0.01,
$MgSO_47H_2O$	0.1,
NaCl	0.02,
$CaCl_2.2H_2O$	0.01,
$Na_2MoO_4.2H_2O$	0.002,
Bromothymol blue	2mL (0.5% in alcohol)
Agar-agar	15,
Distilled water	1000 mL,
pH	6.8

6. Pikovskaya medium

Ingredient	g/L
Ca_3 (Po_4)	0.5
Glucose	10
Yeast	0.5
$MgSo_4.7H_2o$	0.1
$Mgcl_2$	0.5
Kcl	0.2
Nacl	0.2
Agar	1.5%
PH	7.2

7. Zinc solubilizing medium (Bunt and Rovira medium)

Ingredients	g / L
Dextrose (Glucose)	10.000
Ammonium sulphate	1.000
Potassium chloride	0.200
Dipotassium hydrogen phosphate	0.100
Magnesium sulphate, heptahydrate	0.200
Zinc sources	1.000

Appendix II calculation

1. Stock solution arsenic

Preparation: - the stock solution of arsenic and heavy metals are prepared by using formula

Amount of metal salt = molecular weight of metal salt /molar mass of target metal

Stock (1000ppm) = Mo. Wt. Of metal salt/atomic wt. of target metal

2. Salkowski reagent

Preparation: - 50 mL, 35% of per chloric acid, 1 mL 0.5 M FeCl3 solution

1. Bradford Reagent (100mL)

Comassie Brilliant blue G250	0.001%
Absolute ethanol	5%
Phosphoric acid (85%)	8.5%

2. Protein extraction buffer (100mL)

Tris Hcl(8.1)	10mM
EDTA	10mM
B-mercaptoethanol	5mM
PMSF	0.1mg/mL
PH	8.0

3. Phosphate buffer

$Na_2HPo_4.7H_2O$	10.107g
$NaH_2HPO_4.H_2O$	1.697g

Procedure: - dissolve in 400mL and adjust PH 7.4 and adjust final volume 500mL by adding D/W

4. Fertilization calculation

Recommended dose of Broad Leaf Mustard: 120: 80: 60 N: P_2O_5: K_2SO_4 kg ha^{-1}

Available urea =46%, single DAP= 16% and potassium sulphate= 50%

Required fertilizer for 3 kg soil Urea=2.891gm, DAP= 2.609gm and potassium sulphate= 0.18gm

Appendix III standard curve

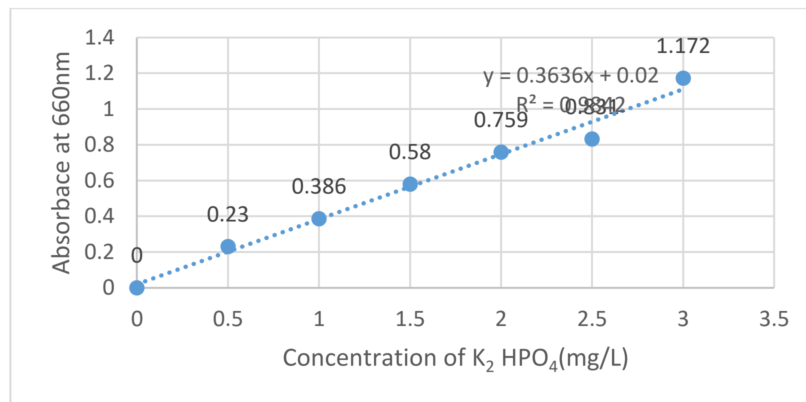

Figure 33 standard curve for p estimation

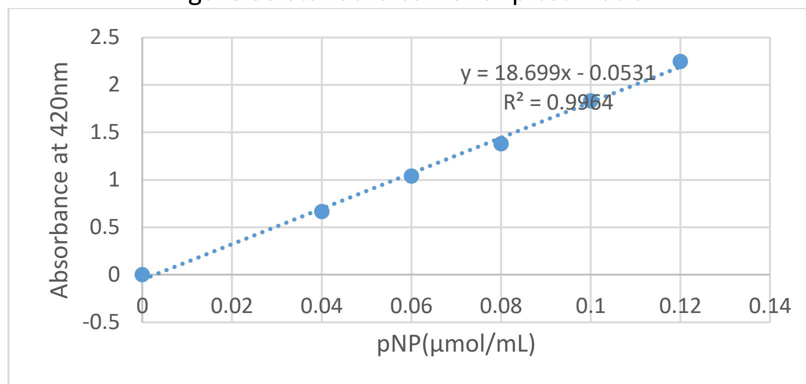

Figure 34 standard curve of pNP

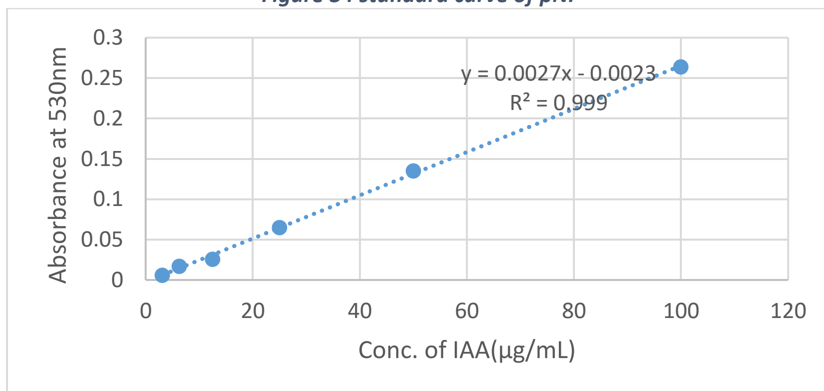

Figure 35 Standard curve of IAA

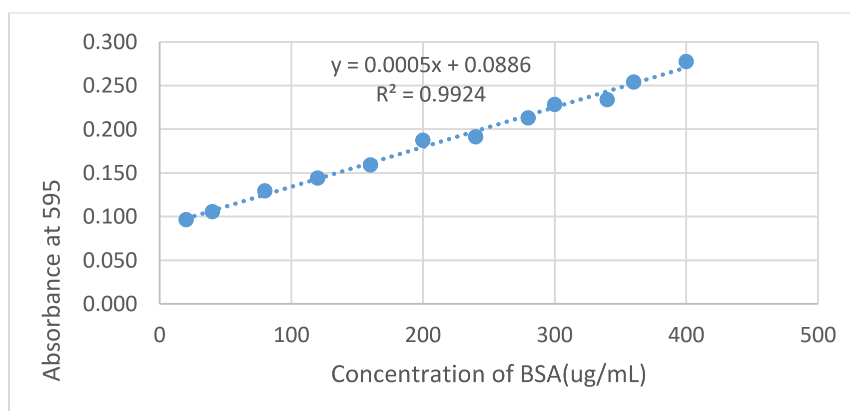

Figure 36 standard curve of BSA

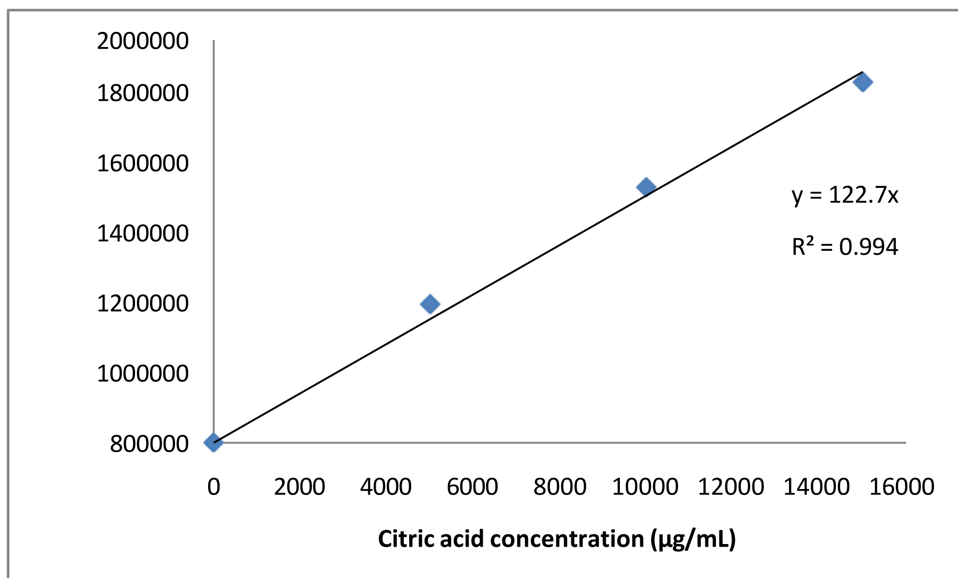

Fig 5: Standard curve for Citric acid production assay

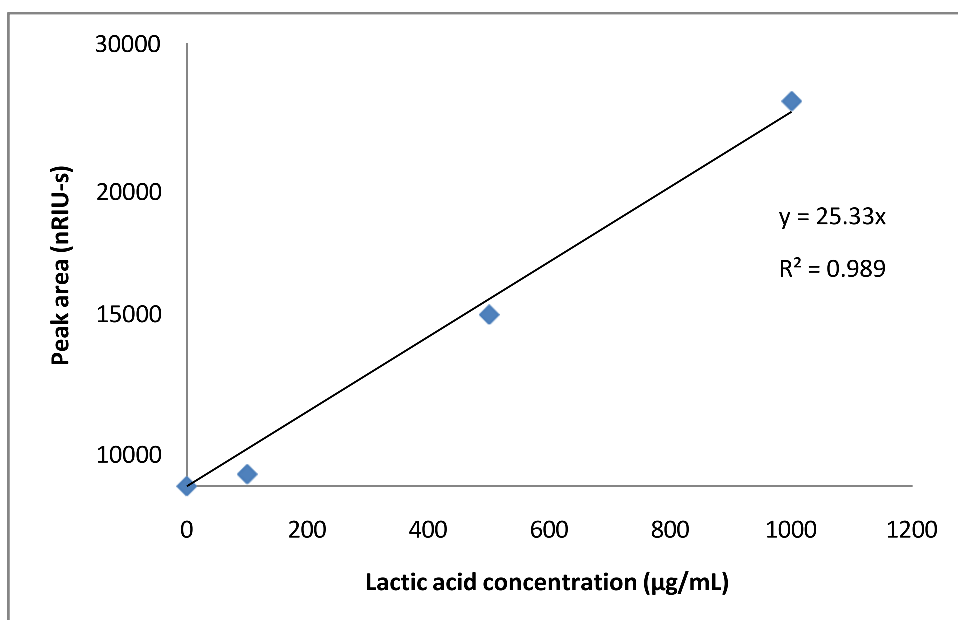

Fig 6: Standard curve for Lactic acid production assay

Fig 8 rRNA gene sequence of M1 and S1

>M1

GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCAT
GGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCA
AGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTG
TTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGA
AATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAG
TTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTC
GGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCA
TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCAT
AAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAG
CCAGCCGCCTAAGGTGGGACAGATGATTGGGGTG

>S1

GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCTTGCTCTTATGAAGTTAGCGGCGGACGGG
TGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCAT
GGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCA
AGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTG
TTAGGGAAGAACAAGTGCTAGTTGAATAAGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAG
CCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGTGA
AATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGTGGGGA
GCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAAG
TTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGG
AGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCTTC
GGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
TGATCTTAGTTGCCATCATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCA
TCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCTGCAAGACCGCGAGGTGGAGCTAATCTCAT
AAAACCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGGGGTAACCTTTTTGGAG
CCAGCCGCCTAAGGTGGGACAGATGATTGGGGTG

Appendix IV photograph of treatment

Fig.9 Fresh plant of green mustard after removal of soil with or without inoculated isolates

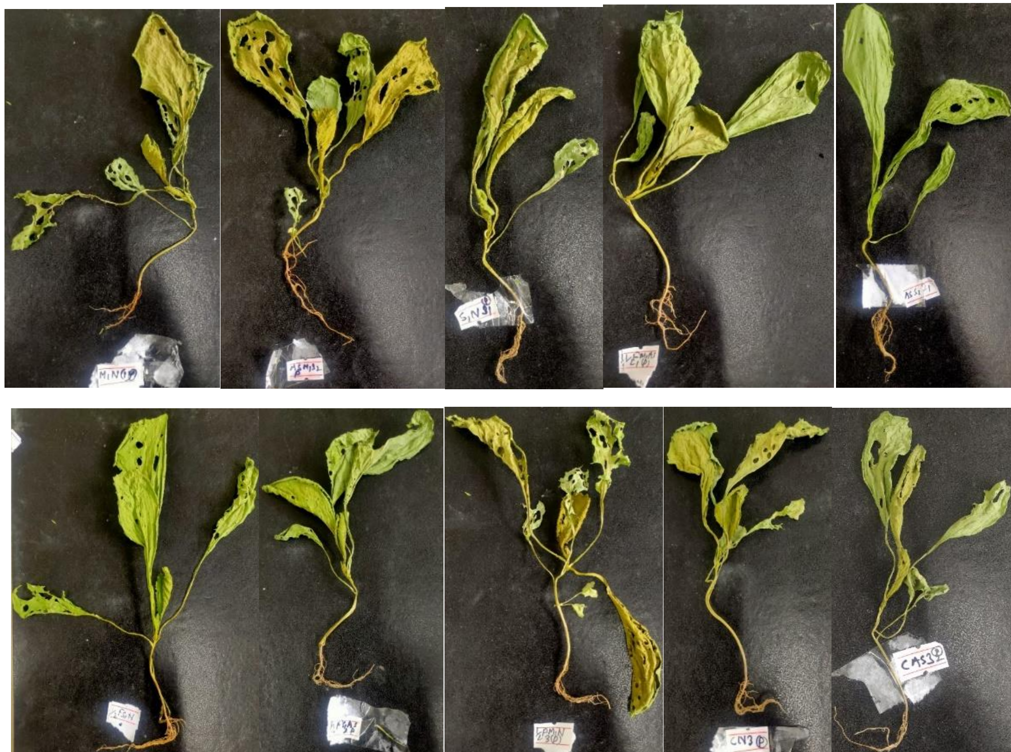

Fig.10 Dry plant of green mustard after removal of soil with or without inoculated isolates

Fig.13 sample collection , soil preparation and plant growth in green house

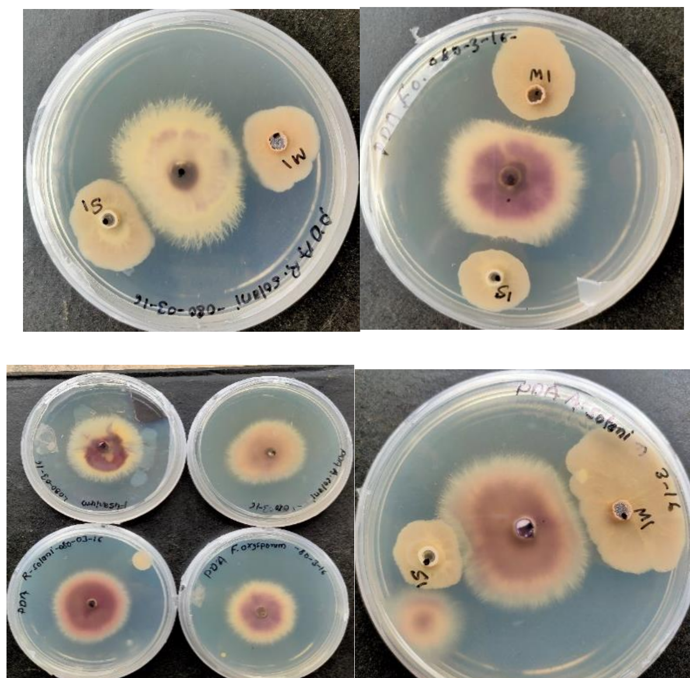

fig.14 Biocontrol of S1 amd M1 against Fungus.

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