Molecular Characterization of ACC Deaminase Producing Bacteria for Induced Systemic Tolerance in Wheat (*Triticum aestivum* L.)

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY
By

RAJNISH PRAKASH SINGH

Under the Supervision of
Prof. Prabhat Nath Jha

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI, (RAJASTHAN)-333031
INDIA
2016

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI, RAJASTHAN, INDIA

CERTIFICATE

This is to certify that the thesis entitled "**Molecular Characterization of ACC Deaminase Producing Bacteria for Induced Systemic Tolerance in Wheat (*Triticum aestivum* L.)**" submitted by **Rajnish Prakash Singh, ID No. 2011PHXF420P** for the award of Ph.D. Degree of the Institute, embodies original work done by him under my supervision.

Signature in full of the Supervisor
Name: **PRABHAT N. Jha**
Designation: Associate Professor

Date:

Acknowledgements

Foremost, I would like to express my sincere gratitude to God, for providing me the grace and privilege to successfully completing the Doctorate program.

My deeply appreciation goes out to my supervisor Prof. Prabhat Nath Jha, whose expertise, understanding, and patience added considerably to my Doctorate experience. I appreciate his patience, motivation, enthusiasm, skill in many areas and his assistance in all the time of research. I could not have imagined having a better advisor and mentor for my Doctorate study. His untiring efforts to guide me at every step and to sort out my difficulties throughout my research period are of great value. It was under his tutelage that I developed a focus and became interested in vision and human factors.

A special thanks goes to my Doctoral Advisory committee members, Prof. Jitendra Panwar and Prof. Rajesh Mehrotra, Department of Biological Sciences, BITS Pilani, for their motivation, encouragement and valuable suggestions. I must also acknowledge all the faculty members of Department of Biological Sciences, BITS Pilani for their support, direction and became more of a friend than a professor. I thank all the non-teaching staff for assisting me in department related issues.

I sincerely thank the acting Vice Chancellor Prof. Dr. Souvik Bhattacharya and Director Prof. Ashoke Kumar Sarkar of BITS Pilani, Pilani campus for providing laboratory facilities for conducting my research work.

I would also like to thank AIRF-JNU (Advanced Instumental Research Facility- Jawahar lal Nehru University), NHRDF (National Horticultural Research and Developmental Foundation, Nashik, India), Xcelris Genomic Labs Ltd. Ahmadabad, Eurofins genomics Labs Ltd. Jaipur for processing and analyzing of my research samples to generate the valuable data.

I thank Ms. Zara Bukhari, Mr. Sunil Nahata and Mr. Shreyansh Nalwaya who worked along with me as part of their project work. I would certainly be thankful to my seniors Dr. Amit Kumar Subudhi, Dr. Navin Jain and my friend Parva Sharma for their love, encouragement and support for my research study. I am also grateful to my seniors: Dr. Deepak, Dr. Purva Bhalothia, Dr. Garima Gupta, Dr. Swarna Kanchan, Dr. Hemraj Chipa, Dr. Arpit Bhargava, Pancheela, Gurpreet, and juniors: Divya, Monika Jangir, Vidushi, Manohar, Shradha, Dilip, Vikram, Poonam, Sandeep, Zaiba, Ms. Monica Sandhu, and Shahid for their cooperation and

help during my study. I wish to thank Dr. Sachi Singh and Dr. Prameela Jha, (DST-Women Scientist) for sharing scientific ideas during this period.

I duly acknowledge my friends from other department Dr. Kasiviswanadharaju Pericherla, Dr. V. Kameswara Rao, Dr. Manoj Kumar Muthyala, Dr. Ashok Penta, Dr. Mukund Pandurang Tantak, Dr. Nisar A Mir, Dr. Noorla Baig, Ms. Poonam Khedar, Mr. Devesh Agrawal, Mr. Shiv Shiman, Mr. Nitesh Kumar Nandwana, Mr. Somesh Mishra, Ms. Pallavi, and Mr. Subhash Chander.

Last but not the least, I would like to express my thanks to my parents for their love, care, support and encouragement at every stage of my life. In addition, financial assistance from DBT, Govt. of India and BITS Pilani are duly acknowledged.

Rajnish Prakash Singh

Abstract

Introduction: Plant growth and productivity are highly affected by the several environmental stress factors. Among different plant responses to stressors, increase in the pool of ACC (1-aminocyclopropane-1-carboxylic acid), an immediate precursor of ethylene, leads to the production of 'stress ethylene' that severely affects the various parameters of plant growth and yield. Plant growth promoting associative/endophytic bacteria with ACC deaminase activity can hydrolyze the ACC to α-ketobutyric acid and ammonia and maintain the optimum level of ACC inside the plants which in turn stimulates plant growth under stress conditions. These PGPR can also promote plant growth by providing nutrition (nitrogen, iron, and phosphorous), phytohormone production, and amelioration of stressors by inducing production of osmolytes and antioxidants in plants. Several PGPR producing ACC deaminase has been reported to promote plant growth under stress conditions. However, the detail characterization of these bacteria at molecular level and their molecular effect on plant health are scarce. Moreover, few studies have reported isolation of ACC deaminase producing bacteria from plants of desert region which faces extremes of stresses namely temperature and salinity. Therefore, the aim of the present study was to investigate and characterize the plant growth promoting rhizobacteria (PGPR) with ACC deaminase activity at physiological and molecular level to exploit its effect on plant (wheat) under stress conditions, particularly salt and temperature. Major objectives of the present study were: (i) isolation of associative ACC deaminase producing bacteria from plants growing in Thar desert and their characterization of plant growth promoting properties, (ii) to test the effect of selected PGPR on growth of wheat plant under salt and temperature stresses, (iii) selection of an efficient strain based on plant growth promoting effect and to test their ability to ameliorate salt stress through modulation of antioxidants and osmolytes in plants, (iv) generation of *AcdS* (gene encoding ACC deaminase) null mutant and test the role of ACC deaminase in amelioration of salt stress, (v) to test the effect of selected strain on proteomic profile of plants grown under salt stress, and (vi) genomic analysis of selected bacterial strain for studying its metabolic, plant growth promoting, colonization and virulence potential.

Methodology: Rhizospheric bacteria with ACC deaminase activity were isolated on selective media containing ACC as a nitrogen source. All the recovered isolated were subjected to basic biochemical and microbiological tests following standard protocol and identified based on partial sequence of 16S rRNA gene. The tests of plant growth promoting properties such as ACC deaminase activity, indole acetic acid (IAA) production, solubilization of mineral

phosphate, siderophore production, nitrogen fixation, ammonia production, and antagonistic activity were conducted following standard methods. Based on ACC deaminase activity and other PGP traits, twelve isolates were selected for plant growth promoting test under salt (0 to 200 mM) and temperature (25 ºC to 35 ºC) stress conditions under controlled environment of plant growth chamber. For bacterial treatment, surface sterilized seeds were bacterized, germinated and transplanted in autoclaved water. For salt treatment, experimental plants were sprayed with Hoagland medium containing appropriate concentration of NaCl. Based on the results of bacteria-inoculated plant growth under salt stress, isolate SBP-8 was further tested for its ability to protect the wheat plant by modulation the ionic balance, production of osmolytes and antioxidant activity in wheat plant following appropriate protocols. To study the changes in bacterial membrane under salt stress, fatty acid composition were analyzed by FAME analysis using gas chromatography. In addition, an ability of isolate SBP-8 to colonize wheat plants was tested by CFU count of bacteria recovered from plants after treatment, fluorescence microscopy, and ERIC-PCR-based DNA fingerprinting approach. Further, differential expression of proteins under salt stress (200 mM NaCl) in SBP-8-inoculated wheat plants was studied. For this, proteins were extracted from treated and control plants following TCA-Acetone precipitation method, and peptides were analyzed using Waters Synapt G2 Q-TOF instrument proteins. Finally, genome of the most effective strain obtained in this study was sequenced using Illumina Paired-end sequencing platform technology. The sequencing data were analyzed using various standard softwares.

Results and discussion: Overall twenty five ACC deaminase producing bacteria were obtained from the selected plants. Based on partial 16S rRNA gene sequencing, these isolates were identified as the member of different genera like *Enterobacter, Bacillus, Klebsiella, Pantoea, Stenotrophomonas, Mesorhizobium, Phyllobacterium,* and *Serratia* etc. Among 25 isolates, 12 showed appreciable plant growth promoting properties which were selected for detail physiological characterization of ACC deaminase, PCR amplification of *AcdS* gene, and their effect on plant growth under different concentration of NaCl (salt) and temperature stress. Best ACC deaminase activity was observed in isolate SBP-8 followed by SL-12, and SBP-9. Optimum activity of ACC deaminase in these isolates was observed at 6% NaCl (170 to 400 nmol α-ketobutyrate/mg protein/h), 30 ºC temperature (270 to 385 nmol α-ketobutyrate/mg protein/h), and pH 8.0 (285 to 370 nmol α-ketobutyrate/mg protein/h). The ACC deaminase activity was highest when ammonium chloride and lactose were used as nitrogen and carbon source respectively. Out of these isolates, SBP-8 showed best plant

growth in terms of root/shoot length (36-47%), chlorophyll content (45-94%), and biomass (19-24%). The GC-MS profile of cell extract at different salt concentration comprised of hydrocarbons, fatty alcohols with carbon chain length varying in range of C_{13} - C_{54}. It showed that level of unsaturation and fatty alcohols increase in membrane lipids of isolate SBP-8 at higher concentration of NaCl, former of which can increase the fluidity of membrane and modulate distribution of transporters to decrease accumulation of Na^+ in cells. Ability of the test isolate to confer induced systemic tolerance (IST) in wheat plants was confirmed by pot assay, which demonstrated minimum damages to wheat plant in presence of bacterium inoculum under abiotic stress conditions. The bacterial inoculation also significantly induced the osmolytes production like proline and total soluble sugar, and enhanced the various antioxidative enzymatic activity like superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX). The observation of florescent bacteria on treated plant roots stained with acridine orange showed large number of fluorescent bacterial cells. Further identity of colonized bacteria was checked by ERIC-PCR profile of recovered bacteria from treated plants and that of pure culture. Decreased stimulation of plant growth under salt stress by *AcdS* null mutant (*Klebsiella* sp. SBP-8) suggests direct role of ACC deaminase in amelioration of salt stress. Further, effect of the test isolate on proteomic profile of host plants under salt stress was investigated which showed that the inoculation of isolate SBP-8 positively up-regulated the various proteins expression responsible for salinity tolerance and defense response. The bacterial inoculation enhanced the expression of transporter proteins (e.g., malate transporter, and two pore calcium channel protein) and various proteins involved in cell integrity (e.g.,tubulin, profilin, casparian strip membrane protein' (CASPs) etc. It also up-regulated certain proteins related to photosynthetic machineries (e.g., RuBisCO, cytochrome f, photosystem II protein D1, cytochrome b, photosystem II CP47 reaction center protein, and chloroplast envelope membrane protein), and translational machinery (e.g., IF2, IF 1, and ribosomal protein under salt stress. As isolate SBP-8 stimulated plant growth under salt stress through various mechanisms, we analysed its genome. The isolate SBP-8 was initially identified as *Klebsiella* sp. based on partial sequence analysis of 16S rRNA. Therefore, it is described as *Klebsiella* sp. up to chapter V of this thesis. However, on whole genomic analysis, this isolate was identified as *Enterobacter cloacae* whose genomic detail has been described in chapter VI. Genome size of *E. cloacae* SBP-8 is 5.8Mb having 55% GC content with 4052 protein coding genes. In this study we examined genes required for plant growth promotion, stress response, motility, chemotaxis, adhesion, invasion, biofilm

and virulence. It revealed the presence of genes potentially involved in plant growth promotion (ACC deaminase, IAA synthesis by the indole-3-acetamide (IAM) and indole-3-acetonitrile (IAN) pathways, siderophore synthesis and transport) and various other useful genes. Phylogenetic analysis of *AcdS* genes suggested that *Enterobacter cloacae* SBP-8 showed its closest homology to the *Enterobacter* groups. In the genome of *Enterobacter cloacae* SBP-8, several genes efflux transporters were identified which can contribute to metal resistance and multi-drug resistance in the isolate. Overall, present study suggests that *E. cloacae* SBP-8 can be used as potential biofertilizer. However, its pathogenicity must be checked before its use at field level.

Contents

Certificate
Acknowledgement
Abstract
Content
List of figures
List of tables
List of abbreviations

Chapter I General Introduction **Page No.**

1.1. General introduction 1-2
1.2. Pathway of ethylene biosynthesis 2-8
1.3. ACC deaminase: An overview 8-18
1.4. Genetics and expression of ACC deaminase 19-25
1.5. Ecological significance of ACC deaminase bacteria 25-26
1.6. Transgenic plants with ACC deaminase activity 26-27
1.7. Alternative to ACC deaminase: Use of chemicals 27-27
1.8 Other mechanism of stress protection mediated by PGPR 27-28
1.9 Gaps in existing research 28-29
1.20 Objectives 29-30

Chapter II Isolation and characterization of ACC deaminase producing bacteria from desert region of Rajasthan

2.1 Introduction 33-34
2.2 Materials and methods 34-45
2.3 Results 45-68
2.4 Discussion 69-75

Chapter III Physiological characterization of ACC deaminase activity and plant growth promoting test under abiotic stress conditions

3.1 Introduction 77-79
3.2 Materials and methods 79-84
3.3 Results 85-105
3.4 Discussion 106-108

Chapter IV Evaluation of *Klebsiella* sp. SBP-8 on plant growth under abiotic stress condition: a multifunctional study

4.1 Introduction 110-111
4.2 Materials and methods 111-117
4.3 Results 118-129
4.4 Discussion 130-135

Chapter V Proteomic analysis of wheat plant following inoculation of *Klebsiella* sp. SBP-8 under salt stress

5.1 Introduction 137-138
5.2 Materials and methods 138-140
5.3 Results 141-156

5.4 Discussion 157-164

Chapter VI Genome analysis of plant growth promoting bacterium *Enterobacter cloacae* SBP-8

6.1 Introduction 166-167
6.2 Materials and methods 167-169
6.3 Results 170-190
6.4 Discussion 191-193
Summary and conclusions 194-197
Future scope of work 198-198
References 199-226
Appendix 227-232
List of publications 233-236
Biography of supervisor and student 237-238

List of Figures

S. No.	Chapter I: General introduction	Page No.
1	Fig. 1.1. A schematic diagram of ethylene biosynthesis pathway found in higher plants. The enzymes catalyzing each step are shown above the arrows. ACC=1-aminocyclopropane-1-carboxylic acid	6
2	Fig. 1.2. Reaction mechanism catalyzed by microbial ACC deaminase. Route I: direct β-H (Hydrogen) abstraction, Route II: Addition of nucleophiles followed by β-H (Hydrogen) abstraction. Modified figure adapted from the source ref. Hontezas et al. (2006)	12
3	Fig. 1.3. The phylogentic tree constructed from *AcdS* gene sequence of different bacterial strains. The sequence data obtained from IMG database of JGI were used. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed	18
4	Fig. 1.4. The regulatory circuits of *AcdS* gene expression in *Pseudomonas putida* UW4 and other related bacteria. Abbreviations AcdR: regulatory gene for ACC deaminase; AcdB: encoding for glycerophosphoryl diester phosphodiesterase; LRP: Leucine responsive protein; FNR: fumarate nitrate reductase protein; CRP: c-AMP receptor protein; *AcdS*: gene for encoding ACC deaminase	21
5	Fig. 1.5. A model for *AcdS* gene regulation in nitrogen fixing *Mesorhizobium* sp. Expression of *AcdS* is positively regulated by $NifA_2$ protein which binds to σ^{54} and switch on transcription of *AcdS* gene. $NifA_1$ is also required in regulation of *AcdS* but its role is not well understood.	23
	Chapter II: Isolation and characterization of ACC deaminase bacteria from desert region of Rajasthan	
6	Fig. 2.1. Images of selected plants that were used for isolation of ACC deaminase bacteria. Plants were collected from various region of Thar desert of Rajasthan	36
7	Fig. 2.2. Test of motility shown by the isolates *Enterobacter cloacae* ZNP-1 (1), *Enterobacter cloacae* ZNP-3 (2), *Serratia marcescens* SL-11 (3), *Serratia marcescens* SL-12 (4) and *Bacillus licheniformis* HSW-16. Panel A, B, C represent the swimming, swarming and twitching motility	58
8	Fig. 2.3 Test of siderophore production by isolate SBP-8, CDP-13 and SBP-9	61
	Chapter III: Physiological characterization of ACC deaminase activity and plant growth promoting test under abiotic stress conditions	
9	Fig. 3.1. ACC deamianse activity of selected isolates at different temperature (25-40 ºC) concentration	86
10	Fig.3.2. ACC deamianse activity of selected isolates at different ACC (1-5 mM) concentration	87
12	Fig. 3.3. ACC deamianse activity of selected isolates at different pH (5.0-9.0) concentration.	88
13	Fig. 3.4. Amplification and analysis of *AcdS* gene of *Klebsiella* sp.	93

	SBP-8. Panel 'A' shows amplicon of *AcdS* (650 bp) of test isolate in Lane 2 and DNA ladder mix (SM0331) in Lane 1. Panel 'B' represents dendrogram based on *AcdS* sequence of test isolate and other species which showed similar *AcdS* sequence. Other sequences of *AcdS* used for analysis were retrieved from NCBI Genbank data base. Neighbor-joining method was performed using the software packages Mega version 6.0, at bootstrap value of (n = 500)	
14	Fig. 3.5. Amplification and analysis of *AcdS* gene of *Bacillus lichniformis* HSW-16. Panel 'A' shows amplicon of *AcdS* (800 bp) in Lane 2 and DNA ladder mix (SM0331) in Lane 1. Panel 'B' represents dendrogram based on *AcdS* sequence of test isolate and other species which showed similar *AcdS* sequence. Other sequences of *AcdS* used for analysis were retrieved from NCBI Genbank data base. Neighbor-joining method was performed using the software packages Mega version 6.0, at bootstrap value of (n = 500)	94
15	Fig. 3.6. Amplification and analysis of *AcdS* gene of *Enterobacter cloacae* ZNP-2. Panel 'A' shows amplicon of *AcdS* (600 bp) in Lane 2 and DNA ladder mix (SM0331) in Lane 1. Panel 'B' represents dendrogram based on *AcdS* sequence of ZNP-2 and other closely related species. After retrieving the other sequences from NCBI Genbank data base, Neighbor-joining method was performed using the software packages Mega version 6.0, at bootstrap value of (n = 500)	95
	Chapter IV: Evaluation of *Klebsiella* sp. SBP-8 on plant growth under abiotic stress condition: a multifunctional study	
16	Fig. 4.1. Effect of SBP-8 inoculation on proline (Panel A), and total soluble sugar (Panel B), in wheat plants grown under different salinity conditions (0 mM, 150 mM, 175 mM, 200 mM NaCl). Values are mean of three replicates±SD (n=9). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test (p<0.05). FW: fresh weight.	123
17	Fig. 4.2. Effect of SBP-8 inoculation on malondialdehyde content under different salinity conditions. Values are mean of three replicates±SD (n=3). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test (p<0.05). FW: fresh weight.	124
18	Fig. 4.3. Effect of SBP-8 inoculation on auxin, and total protein content under different 0 mM, 150 mM, 175 mM, 200 mM NaCl salinity conditions. Values are mean of three replicates±SD (n=3). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test (p<0.05). FW: fresh weight.	125
20	Fig. 4.4. SBP-8 inoculation on anti-oxidative enzymatic activities in treated and control plants under salt stress conditions (A) SOD activity (B) CAT activity (C) POX activity. Values are mean of three replicates±SD (n=3). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test (p<0.05)	126
21	Fig. 4.5. Efficiency of colonization of *Klebsiella* sp. SBP-8 was assessed 15 days after germination of bacterized wheat plants (A; i, ii) visualization of acridine orange-stained wheat plant roots showing	127

	bacterial cells using fluorescence microscopy (B) ERIC-PCR profile of bacterium colonized in wheat plants and confirmation of its identity using pure culture (Lane M: DNA ladder SM0311, Lane C: control DNA, Lane SBP-8: DNA of *Klebsiella* sp. isolated from inoculated plant)	
22	Fig. 4.6. Evaluation of plant growth promotion test following inoculation of (A) *Klebsiella* sp. SBP-8 (*AcdS*$^{+}$) and SBP-8 (*AcdS*$^{-}$) at 200 mM NaCl stress (B; i) plant growth in terms of shoot and root length (B; ii) biomass measurement in terms of fresh wt. and dry wt.	129
	Chapter V: Proteomic analysis of wheat plant following inoculation of *Klebsiella sp*. SBP-8 under salt stress	
23	Fig. 5.1A. Pie charts showing the distribution of differentially expressed proteins based on their predicted biological process (A), molecular functions (B), and cellular process (C) in Treatment T-1	142
24	Fig. 5.1B. Pie charts showing the distribution of differentially expressed proteins based on their predicted biological process (A), molecular functions (B), and cellular process (C) in Treatment T-2	143
25	Fig. 5.1C. Pie charts showing the distribution of differentially expressed proteins based on their predicted biological process (A), molecular functions (B), and cellular process (C) in Treatment T-3	144
26	Fig. 5.2A. The major functional groups of proteins identified in treatment T1 (control vs uninoculated plants with salt stress)	146
27	Fig. 5.2B. The major functional groups of proteins identified in treatment T2 (control vs incoculated plants)	147
28	Fig. 5.2C. The major functional groups of proteins identified in treatment T3 (bacteria- inoculated vs salt stress)	148
29	Fig. 5.3A. Number of proteins up-regulated/down-regulated in each functional category in treatment T-1	149
30	Fig. 5.3B. Number of proteins up-regulated/down-regulated in each functional category in treatment T-2	150
31	Fig. 5.3C. Number of proteins up-regulated/down-regulated in each functional category in treatment T-3	151
32	Fig. 5.4. Venn diagram representing the presence and common proteins in each treatment (T-1, T-2, T-3)	152
33	Fig. 5.5. Hierarchical cluster analysis of 79 differentially expressed proteins common to the three experimental treatments (T1-T3). T1 treatment represents the untreated control plant verses salt stress (200 mM NaCl). T-2 treatment represents the control plant against the bacterial inoculated plant. T3 is the comparison against bacterial inoculated plant against salt stress in the presence of bacterial inoculum. Upregulation or downregulation is indicated by the green and red color respectively, whereas black represents no expression. The intensity of the colors increases as the expression differences increase, as shown in the bar at the bottom	156
	Chapter VI: Genome analysis of plant growth promoting bacterium *Enterobacter cloacae* SBP-8	
34	Fig. 6.1. Cicular map representation of *Enterobacter cloacae* SBP-8 complete genome. Rings show from outside (1) Nucleotide sequences (2) GC content, and (3) GC skew. GC content and GC skew were	172

	calculated using a 10-kb window in steps of 200 bp. Genomic map was constructed using the BLAST Ring Image Generator (BRIG, version 0.95).	
35	Fig. 6.2. Plasmid map representation of *Enterobacter cloacae* SBP-8 complete genome. Rings show from outside (1) Nucleotide sequences (2) GC content, and (3) GC skew. GC content and GC skew were calculated using a 10-kb window in steps of 200 bp. The genomic map was constructed using the BLAST Ring Image Generator (BRIG, version 0.95).	173
36	Fig. 6.3. Genomic islands of *Enterobacter cloacae* SBP-8 predicted by Island Viewer. The outer black circle shows the scale line in Mbps. Predicted genomic islands are colored based on the following methods: SIGI-HMM, orange; IslandPath-DIMOB, blue; Integrated detection, red. Black plot represents the GC content.	174
37	Fig. 6.4. Subsystems distribution statistic of *Enterobacter cloacae* SBP-8 based on genome annotations performed according to RAST server. The pie chart presents the abundance of each subsystem category and the count of each subsystem feature is listed in parentheses at the chart legend.	175
38	Fig. 6.5. Upstream regulatory elements of *AcdS* structural gene of *Enterobacter cloacae* SBP-8. The regulatory elements were analyzed by the SCOPE (Suite for computational identification of promoter elements, genei.dartmouth.edu/scope) (version 2.1.0) tool using default parameters.	177
39	Fig. 6.6. Phylogenetic analysis of *Enterobacter cloacae* SBP-8 based on whole genome sequences, with the available genome sequence of *Enterobacter cloacae*. The evolutionary history was inferred by the Neighbour-joining method with bootstrap value 1000.	181
40	Fig. 6.7. A phylogenetic tree of ACC deaminase (*AcdS*) gene of *Enterobacter cloacae* SBP-8 and other closely related bacterial genera. The evolutionary history was inferred by the Neighbour-joining method with bootstrap value 1000.	183
41	Fig. 6.8. Modeling of *AcdS* protein. (a) Template-4D96 (experimental, from *Salmonella*), (b) Model generated for our protein from *Enterobacter cloacae* SBP-8, (c) Superimposed proteins (our protein and 4D96 from Angel 1), (d) Superimposed proteins (our protein and 4D96 from Angel 2).	184
42	Fig. 6.9. A phylogenetic tree of *AcdS* protein sequence of *Enterobacter cloacae* SBP-8 and other closely related bacterial genera. The evolutionary history was inferred by the Neighbour-joining method with bootstrap value 1000.	185
43	Fig. 6.10. Evaluation of evolutionary distance between the 16S rRNA (A) and *AcdS* gene (B) in the selected bacterial genera. The nucleotide sequence of selected bacteria was aligned in ClustalW, and the distance was calculated in mega 6.0. Error bar represents the standard error of means of triplicates.	186
44	Fig. 6.11. Comparison of evolutionary distance between the 16S rRNA and *AcdS* gene in the selected bacterial genera. The values were relative to *Enterobacter cloacae* SBP8.	187
45	Fig. 6.12. Phylogenetic tree showing the relationship of *Enterobacter*	188

	cloacae SBP-8 to closely related bacteria. The 16S rRNA gene sequence of closely related species was obtained from NCBI GenBank database. The tree was obtained using neighbor joining method of software package Mega version 6.0 at the bootstrap value of (n= 1000).	
46	Fig. 6.13. Phylogenetic tree showing the relationship of *Enterobacter cloacae* SBP-8 to closely related bacteria. The house-keeping *rpoB* gene sequence of closely related species was obtained from NCBI GenBank database. The tree was obtained using neighbor joining method of software package Mega version 6.0 at the bootstrap value of (n= 1000).	189
47	Fig. 6.14. Phylogenetic tree showing the relationship of *Enterobacter cloacae* SBP-8 to closely related bacteria. The house-keeping *rpoD* gene sequence of closely related species was obtained from NCBI GenBank database. The tree was obtained using neighbor joining method of software package Mega version 6.0 at the bootstrap value of (n= 1000).	190

List of Tables

S. No.	Chapter I: General introduction	Page No.
1	Table 1.1 ACC deaminase mediated protection by PGPR in response to various stresses	4-5
2	Table 1.2 List of microorganism with ACC deaminase activity	14-15
3	Table 1.3 Distribution of ACC deaminase in domain Eukarya	17
	Chapter II: Isolation and characterization of ACC deaminase bacteria isolated from desert region of Rajasthan	
4	Table 2.1 Details of plants used for isolation of ACC deaminase containing bacteria	35
5	Table 2.2 Number of ACC positive bacterial isolates recovered from various plant species	46
6	Table 2.3 ACC deamianse activity of all recovered isolates	47
7	Table 2.4 Biochemical characteristics of various ACC deaminase bacteria	49
8	Table 2.5 Antibiotic sensitivity test of ACC utilizing bacterial isolates	50
9	Table 2.6 Carbohydrate utilization efficacy of ACC utilizing bacterial isolates	52-54
10	Table. 2.7 Test of hydrolytic enzymatic assay by all twenty five ACC utilizing bacteria	56
11	Table. 2.8 The closest affiliations of the representative isolate in the GenBank according to the 16S rRNA gene sequences	60
12	Table 2.9 Plant growth promoting features of isolated bacterial isolates	62
13	Table 2.10 Antifungal activity of isolates against tested fungal species	64
14	Table 2.11 Antibacterial activity of isolates against tested pathogenic bacterial strains	65
15	Table 2.12 Test for growth of various ACC deaminase bacteria at various temperatures	67
16	Table 2.13 Test for growth of various ACC deaminase bacteria at various salt concentrations	68
	Chapter III: Physiological characterization of ACC deaminase activity and plant growth promoting test under abiotic stress conditions	
17	Table 3.1 The selected isolates used for physiological characterization of ACC deaminase activity and plant growth test	80
18	Table 3.2 List of primers used in PCR amplification	82
19	Table 3.3 Physico-chemical properties of soil used for pot study	83
20	Table 3.4 ACC deamianse activity of selected isolates under different salt concentrations	85
21	Table 3.5 ACCD value of selected isolates at different time intervals	89
22	Table 3.6 ACCD value of selected isolates at different carbon sources	90
23	Table 3.7 ACCD value of selected isolates at various nitrogen sources conditions	91
24	Table 3.8 Plant growth promotion test following inoculation of selected isolates under stress conditions, Control: 0 mM NaCl; Stress;	98-101

	150 mM NaCl; Stress; 175 mM NaCl; Stress; 200 mM NaCl	
25	Table 3.9 Plant growth promotion test following inoculation of selected isolates under temperature stress; A: 25 ºC; B: 30 ºC; C: 35 ºC	103-105
	Chapter IV: Evaluation of *Klebsiella* sp. SBP-8 on plant growth under abiotic stress condition: a multifunctional study	
26	Table 4.1 GC-MS analysis of FAME composition of *Klebsiella* sp. SBP-8 (0 mM NaCl)	119
27	Table 4.2 GC-MS analysis of FAME composition of *Klebsiella* sp. SBP-8 (175 mM NaCl)	119
28	Table 4.3 GC-MS analysis of FAME composition of *Klebsiella* sp. SBP-8 (200 mM NaCl)	120
29	Table 4.4 Influence of *Klebsiella* sp. SBP-8 on Na^{+}, K^{+} and Ca^{2+} ion concentrations in shoot tissue under control and salt stress	122
30	Table 4.5 Plant growth test of wild (SBP-8, $AcdS^{+}$) and mutant strain (SBP-8, $AcdS^{-}$) at 200 mM salt stress	128
	Chapter V: Proteomic analysis of wheat plant following inoculation of *Klebsiella sp*. SBP-8 under salt stress	
31	Table 5.1 The identified proteins with their UNIPROT-ID used for cluster analysis	153-155
	Chapter VI: Genome analysis of plant growth promoting bacterium *Enterobacter cloacae* SBP-8	
33	Table 6.1 General properties of genome of PGPR *Enterobacter cloacae* SBP-8	171
34	Table 6.2 Gene distribution for motility, chemotaxis, adhesion and root colonization in genome of PGPR *Enterobacter cloacae* SBP-8	176
35	Table 6.3 Presence of secretion system and their gene/protein distribution	179

Abbreviation

ACC	1-aminocyclopropane-1-carboxylate
ACCD	ACC deamianse
ANOVA	Analysis of variance
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CAS	Chrome-azurol-S
CAT	Catalase
CFU	Colony forming unit
CMC	Carboxymethyl cellulose
CTAB	N-cetyl trimethyl ammonium bromide
DF	Dworkin-Foster
DMRT	Duncans multiple range test
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphate
EPS	Exopolysaccharide
ERIC-PCR	Enterobacterial repetitive intergenic consensus sequences-PCR
FTIR	Fourier-transform infrared spectroscopy
GA	Gibberellic acid
GC-MS	Gas chromatography-mass spectroscopy
gus -β	glucosidase
IAA	Indole-3-acetic acid
IMViC	Indole, methyl red test, Voges-Proskauer test
ISR	Induced systemic resistance
IST	Induced systemic tolerance
KB	Ketobutyrate
LB	Luria-bertani
MDA	Malondialdehyde
MUC	4-methylumbelliferyl-cellobioside
MUG	4-methyllumbelliferyl-β-D-glucoside
NA	Nutrient-agar
NBRIP	National botanical research institute-phosphate
NCBI	National center for biotechnology information

OD	Optical density
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGPB	Plant growth promoting bacteria
PGPR	Plant growth promoting rhizobacteria
POX	Peroxidase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAR	Systemic acquired resistance
SOD	Superoxide dismutase
TCA	Trichloroacetic acid
TSS	Total soluble sugar

CHAPTER I

General Introduction

1. Introduction

Plant growth and productivity are limited by several physiological and environmental factors that include the availability of macro and micronutrients, physical and chemical properties of soil, plant genotype and growth conditions. Apart from these factors, plant growth and yield are detrimentally affected by diverse biotic and abiotic factors. The latter include stressors such as salt, cold and high temperature, drought, water logging, mechanical wounding, presence of heavy metals, and other organic and inorganic toxic compounds (Sharma et al., 2016). The loss incurred due to these factors are estimated to be >50% for most major crop plants (FAO, 2012).

Soil bacteria attached to the surface of roots are capable of promoting plant growth by colonizing the plant root (Kloepper et al., 1989, Cleyet-Marcel et al., 2001). The microorganisms that colonize the roots of plants following inoculation onto seeds and enhance the plant growth are termed as ‘plant growth promoting rhizobacteria’ (PGPR) (Nelson, 2004). According to their relationship with the plants, PGPR can be categorized into two groups: symbiotic bacteria capable of forming specialized structures or nodules on host plant roots, and free -living bacteria that interact with the roots in the soil (Glick, 2005). The main functions of these bacteria are to supply nutrients to crops, stimulate the plant growth via production of plant hormones, inhibit phytopathogens, improve soil structures and aid in bio-leaching of inorganics (Ehrlich, 1990). PGPR can promote plant growth directly by (i) solubilization of insoluble phosphates, (ii) lowering of ethylene level, (iii) phytohormone production, (iv) nitrogen fixation, and/or (v) synthesizing siderophores (Glick et al.,1995; Dobbelaere et al., 2001), and indirectly by production of (i) allelochemicals like antibiotics, (ii) antifungal metabolites (iii) enzymes like chitinase, cellulose, β- 1,3 –glucanase (Wheatley, 2002; Compant et al., 2005) (iv) volatile organic compounds, (VOCs), (v) toxins, (vi) competition for colonization sites and nutrients, and (vi) induced systemic resistance (Glick et al., 1999; Van Loon, 2007) .

Many aspects of plant life are regulated by modulation of ethylene levels which is subjected to environmental cues, including biotic and abiotic stresses (Hardoim et al., 2008). In many plants a burst of ethylene is required to break seed dormancy following germination and to induce stress related genes in response to biotic and abiotic stresses. However, the sustained high level of ethylene may inhibit root and shoot elongation (Jackson, 1991), which in turn affect plants growth. In these circumstances, plant growth promoting bacteria protect the plants from

various biotic and abiotic stressors to maintain the growth, so-called 'induced systemic tolerance (IST)' (Yang et al., 2009). Many PGPR having an ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase (ACCD) which confers IST to plants. The ACC deaminase producing bacteria sequester and degrade plant ACC to ammonia, and α-ketobutyrate, which provide nitrogen and energy respectively to them. By removing ACC, bacteria decrease the deleterious effect of ethylene, thereby, ameliorate plant stress and promote plant growth (Glick, 2007). Therefore, plant growth promoting bacteria equipped with ACC deaminase activity are of utmost importance in reducing the deleterious effect of environmental stressors. The role of few ACC deaminase bacteria for their ability to protect plants from various stresses has been summarized in Table 1.1. The present chapter discusses role and biosynthesis of ethylene in plants, biochemical and molecular aspects of bacterial ACC deaminase, and other bacterial properties which enable plants to tolerate abiotic stresses.

1.2 Pathway of ethylene biosynthesis

Ethylene controls the plant growth under growth limiting conditions or stress (Abeles et al., 1992). Ethylene is synthesized by amino acid L-methionine (Met) via the intermediates, S-adenosyl-L-methionine (SAM) and 1-aminocyclopropane-1-carboxylic acid (ACC) (Fig. 1.1) (Yang and Hoffman, 1984). Experimental evidence showed that ethylene is derived from carbon 3 and 4 of methionine. The enzymes involved in the ethylene biosynthesis are SAM synthase which catalyzes the conversion of methionine to SAM, ACC synthase which hydrolyze the SAM to ACC, and ACC oxidase, which metabolize ACC to ethylene, carbon dioxide and cyanide (Glick, 1998). SAM is synthesized from the methionine (Met) and adenosine triphosphate (ATP), which gets converted to 1-aminocyclopropane-1-carboxylic acid (ACC), an immediate precursor of ethylene (Taiz and Zeiger, 2006). The genes coding these enzymes and their regulatory factors have been manipulated for agricultural and commercial purposes. Experimental observation indicates that when ACC is supplied exogenously to plant tissue, ethylene production increases substantially. However, not all the ACC is converted to ethylene in tissues. Under some condition, in presence of ACC N-malonyltransferase, ACC is converted to a conjugated form N-malonyl ACC (Martin and Saftner, 1995).

Table 1.1 ACC deaminase mediated protection by PGPR in response to various stresses

Bacteria	Host plant	Stress protection	Reference
Achromobacter piechaudii ARV8	*Lycopersicon esculentum*	Salt	Mayak et al. (2004)
Alcaligenes sp., *Bacillus pumilus*	*Brassica napus*	Heavy metals	Belimov et al. (2001)
Burkholderia sp. J62	*Lycopersicon esculentum*	Lead	Jiang et al. 2008
B. phytofirmans PsJN	*Vitis vinifera*	Low temperature	Ait Bakra et al. (2006)
Enterobacter aerogenes NBRIK24	*Brassica juncea*	Fly-ash soil	Kumar et al. (2008)
E. sakazakii 8MR5	*Zea mays*	Striga-infested soils	Babalola et al. (2003)
E. cloacae CAL2	*Brassica napus*	Arsenate	Nie et al. (2002)
Kluyvera ascorbata SUD165	*Brassica napus*	Nickel	Burd et al. (1998)
K. ascorbata SUD165/26	*Lycopersicon esculentum*	Lead	Burd et al. (2000)
Pseudomonas putida UW4	*Lycopersicon esculentum*	Flooding	Grichko & Glick (2001)
P. fluorescens YsS6	*Lycopersicon esculentum*	Salt	Ali et al. (2014)
Pseudomonas putida	*Vigna radiata*	Salt	Mayak et al. (1999)
P. putida UW4	*Lycopersicon esculentum*	Salt	Yan et al. (2014)
Pseudomonas fluorescens	*Arachis hypogea*	Salt	Saravanakumar & Samiyappan (2007)
P. putida	*Lycopersicon esculentum*	Low temperature	Chang et al. (2007)

P. fluorenscens CHA0	*Solanum tuberosum*	Plant pathogen	Wang et al. (2000)
P. fluorenscens CHA96	*Cucumis sativus*	Plant pathogen	Wang et al. (2000)
P. putida UW4	*Pinus sabiniana*	Plant pathogen	Nascimento et al. (2013)
P. putida UW4, *P. putida* HS-2	*Brassica napus*	Nickel	Farwell et al. (2007)
P. fluorescens ACC-5	*Pisum sativum*	Drough	Zahir et al. (2008)
Pseudomonas sp.	*Pisum sativum*	Drough	Arshad et al. (2008)
Sinorhizobium sp. Pb002	*Brassica juncea*	Lead	Di Gregorio et al. (2006)
Variovorax paradoxus	*Brassica juncea*	Cadmium	Belimov et al. (2005)

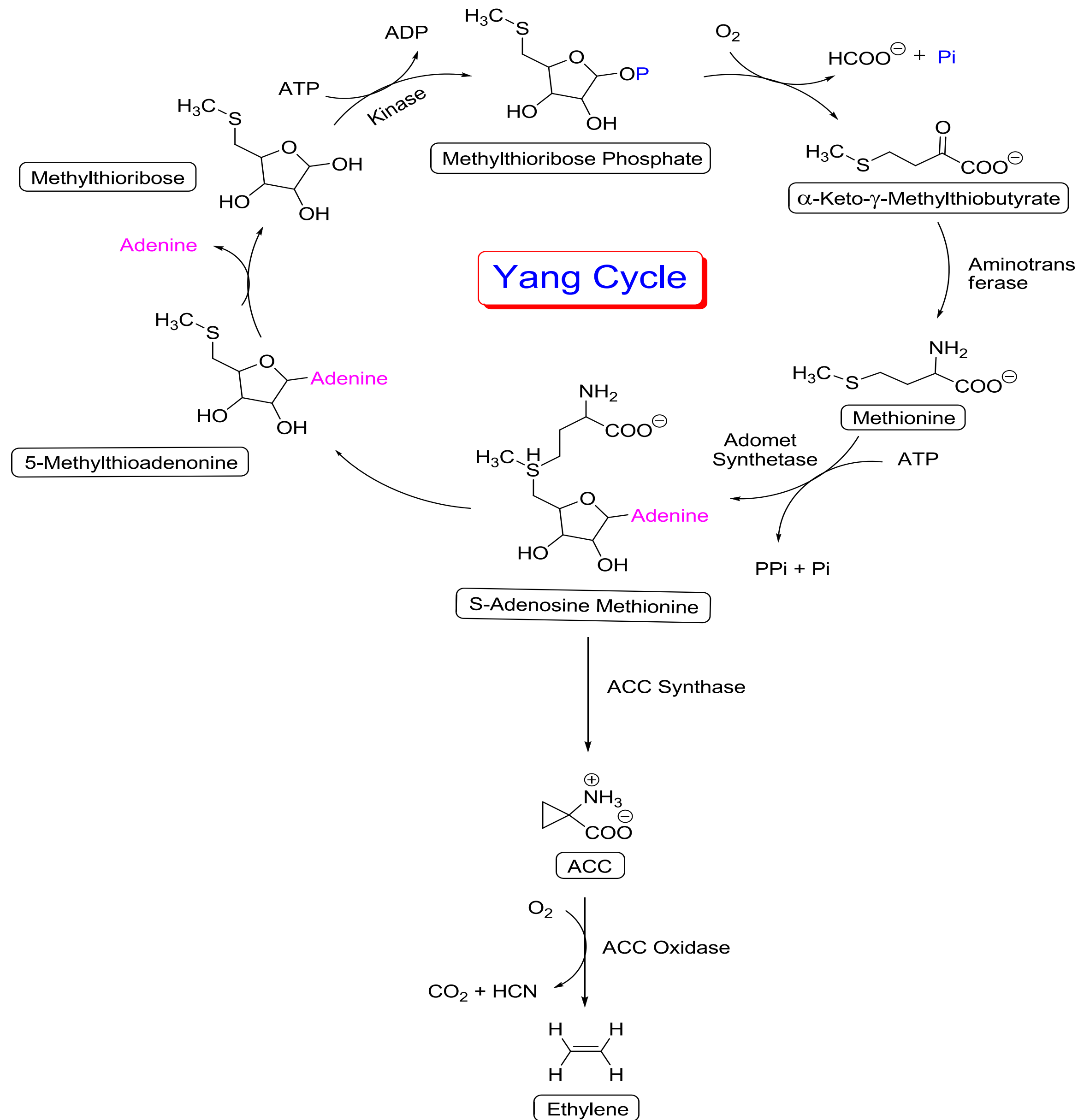


Fig. 1.1. A schematic diagram of ethylene biosynthesis pathway in higher plants. The enzymes catalyzing each step are shown above the arrows. ACC=1-aminocyclopropane-1-carboxylic acid.

The synthesis of ethylene is affected by a number of different factors including temperature, light, nutrition, gravity, and the presence of various types of biological stress to which the plant may be subjected. Regarding a plant's response to stress, an increased level of ethylene is formed in response to the presence of metals, organic and inorganic chemicals, extreme temperature, ultraviolet light, insect attack, phytopathogens (bacteria and fungi) and mechanical wounding. The continuous production of ethylene is concomitant with the appearance of visible damage to the plant such as senescence, chlorosis and abscission etc. The level of ethylene increases during different kind of stresses and show various symptoms on plant growth and development. Therefore, after a severe infection by pathogens, a large portion of damage occurs to a plant due to autocatalytic ethylene synthesis and not from direct pathogen action (Glick, 2007).

The models of synthesis of stress ethylene conclude that ethylene is being synthesized in two peaks. The first ethylene peak is typically a small fraction of magnitude of the second peak. This small peak of ethylene is believed to be responsible for transcription of genes that encode the plant defensive/protective proteins (Glick, 2007). The second much larger ethylene peak appears following the synthesis of ACC by plants in response to stress that may occur up to several days after the initial ethylene peak. The second ethylene peak is detrimental to plant growth and is often involved in initiating processes like senescence, chlorosis and leaf abscission etc.. In this case, the reduction of high level of ethylene by any chemical and biological treatment can significantly lower the magnitude of the second peak of stress ethylene that should simultaneously decrease the stress-induced damage to the plant (Van Loon et al., 2006).

1.2.1 Physiological effect of ethylene

Ethylene, at optimal concentration (10 g L^{-1}), is essential for normal growth and development in plants such as the formation of adventitious root and root hair, accelerate the seed germination, seed dormancy breaking etc. (Arshad, 1990). However, at higher concentration (25 g L^{-1}), it induces defoliation, inhibition of root elongation, inhibition of nodulation in legumes, leaf senescence, and abscission, chlorophyll destruction, and epinasty. Ethylene has been shown to inhibit the nodule development in legumes like *Pisum sativum* (Guinel and Geil, 2002), *Trifolium repens* (Goodlass and Smith 1979), *Medicago sativa* (Peters and Crist-Estes, 1989). Experimental evidence supports that low concentrations of exogenous ethylene application have

a negative effect on the formation and function of nodules observed in various legumes like *Phasulus vulgaris* (Grobbelaar et al., 1971) and *Pisum sativum* (Lee and LaRue, 1992), even complete inhibition of nodules at higher concentration. There is evidence suggesting that exogenous ethylene blocks the invasion of the infection thread in the root cortex of the plant host (Spaink, 1997). On the other hand, the application of ethylene inhibitors like aminoethoxy vinyl glycine (AVG), silver thiosulphate (STS) enhanced nodulation in leguminous plants (Nukui et al., 2000). Rhizobitoxine produced by *B. elkanii* has been observed to be an inhibitor of ethylene synthesis. However the mechanism of ethylene mediated nodule control is not fully known.

1.3 ACC deaminase: An overview

ACC deaminase, a pyridoxal phosphate-dependent enzyme, was first discovered in soil microorganism and shown to convert ACC to ammonia and α-ketobutyrate, both of which further metabolized by the microorganisms. The ability of microorganisms to grow on a minimal medium having ACC as its sole nitrogen source was attributed to ACC deaminase enzyme activity (Honma and Shimomura, 1978; Glick et al., 1995; Ma et al., 2003a). ACC deaminase was first purified from *Pseudomonas* sp. strain ACP and partially purified from *P. chloroaphis* 6G5 (Klee, 1992) and *P. putida* GR12-2 (Glick, 1994). Its presence has been detected in a number of bacterial strains by several scientific groups around the world (Babalola et al., 2003; Ghosh et al., 2003; Ma et al., 2003b; Belimov et al., 2005; Hontzeas et al., 2005; Madhaiyan et al., 2006). Following sections shed light on different biochemical and molecular aspects of bacterial ACC deaminase.

1.3.1 Biochemistry of ACC deaminase

ACC deaminase is usually present in bacteria at a low level and induced by ACC, its substrate. The induction of ACC deaminaseis a slow and complex process which results from the increased availability of ACC during environmental stresses. On continuous exposure to stresses, ACC synthase of the plants is induced and catalyze the synthesis of ACC which are secreted by roots. As the amount of ACC increases the bacterial ACC deaminase is induced and catabolize ACC (Glick, 2007). ACC deaminase activities can be quantified by monitoring the production of either ammonia or α-ketobutyrate (Honma and Shimomura, 1978).

ACC deaminase enzyme has been purified from different sources which appear to have similar molecular mass and form. Native size of 110-112 kDa has been reported from *Pseudomonas* sp. strain ACP and 105 kDa for the enzyme from *Pseudomonas putida* GR12-2. The enzyme is trimeric (homomer) in form and has an approximate subunit mass of 36,500 daltons. Km value of ACC deaminase for ACC has been estimated for enzyme extracts of microorganism at pH 8.5 (Klee, 1992). The absorption spectra of purified ACC deaminase from *Pseudomonas* sp. show the bands at 416 nm at pH 6 and a 326 nm band at pH 9. It is possible that the 326 nm band seen at pH 9.0 is the active form of ACC deaminase to which substrates and inhibitors bind (Jacobson, 1994; Honma, 1985). The Km value for binding of the ACC deaminase enzyme with the substrate ACC ranged between 1.5-17.4 mM at pH 8.5 in experiments conducted on twelve different organisms (Honma and Shimomura, 1978; Klee and Kishore, 1992; Honma, 1993; Hontzeas et al., 2004). This low Km value indicates that the enzyme does not have very high affinity for the substrate ACC (Glick et al., 1998). The optimum temperature for the activity of ACC deaminase is 30 °C and the optimum pH is 8.5 (Jacobson et al., 1994; Honma and Shimomura, 1978). The induction of ACC deaminase has been observed at a level as low as 100nM. The rate of induction is rapid with in the first few hours of induction and then the level off after approximately eight hours (Jacobson and Glick, 1994). The increase in enzyme activity has been observed approximately 10 fold over the basal level of activity although the concentration of ACC is increased up to 10,000 fold.

For the breakdown of ACC, opening of cyclopropane ring of ACC is the main feature in the reaction mechanism of ACC deaminase. Although the reaction mechanism is not fully understood, nucleophilic addition and elimination appear to be the most likely route by which the cyclopropane bond is cleaved (Castro et al., 2009; Walsh et al., 1981). ACC deaminase is competitively inhibited by L-isomers of the amino acids such as L-alanine, L-homoserine, L-α aminobutyric acid, although the strongest inhibition is seen with L-alanine and L-serine. ACC related compounds such as 2-alkyl–ACC and vinyl-ACC can also function as substrates for ACC deaminase purified from *Pseudomonas* sp. strain ACP. The enzyme shows an unusual specificity for D-amino acids and is inactive with any of the L-amino acids or their derivatives (Honma, 1985; Walsh et al., 1981). NMR studies show that a proton is eliminated from the α-carbon of D-alanine but not its L-isomer. These findings explain the deamination of D-amino acids and of several beta substituted D-alanines by ACC deaminase and are consistent with the sterospecific

cleavage of the cyclopropane ring during ACC deamination (Honma, 1985). In the presence of D-alanine ACC deaminase is inactivated more effectively by the iodoacetamide derivatives, 1,5 N iodoacetamidoethyl-1-aminonapthalene-5-sulfonic acid (1,5–I-AEDANS) than by iodoacetamide. During inactivation, two residue are modified; a thiol group in cysteine residue 162 and the aldimine bond of pyridoxal phosphate with lysine residue 51 (Honma and Yamada, 1993).

The bacterial enzyme activity is localized only in the cytoplasm (Jacobson et al., 1994). ACC deaminase activity has been induced in both *Pseudomonas* sp. strain ACP and *P. putida* GR12-2 by ACC at a level as low as 100nM (Honma and Shimomura, 1978; Jacobson et al., 1994). Both bacterial strains were grown in a rich medium and then switched to a minimal medium containing ACC as its sole nitrogen source. The rate of induction similar to the enzyme from two bacterial sources was relatively slow: complete induction required 8 to 10h.

1.3.2 Reaction mechanism catalyzed by ACC deaminase

Based on their three dimensional structures, pyridoxal phosphate enzymes has been classified into four major types, a) tryptophan synthase b) aspartate aminotransferase c) D-amino acid aminotransferase d) alanine racemase. ACC deaminase is a member of pyridoxal 5’-phosphate (PLP) dependent enzymes fitting into the tryptophan synthase family. PLP enzymes catalyze a wide range of metabolic reactions such as transamination, deamination, decarboxylation, eliminations of β and the γ substituentgroups. The reaction catalyzed by ACC deaminase differs from other PLP-dependent enzymes as the ring cleavage can not proceed through α-carbanionic intermediate due to lack of abstractable α-hydrogen atom from the substrate ACC. Two types of reactions can be catalyzed by ACC deaminase for breakdown of ACC. First, abstraction of hydrogen atom and opening of cyclopropane ring by Lys^{51} mediated by a series of hydrolytic reactions and second, opening of cyclopropane ring by nucleophilic attack on β-carbon atom of ACC followed by β-proton abstraction at the pro-R carbon by a basic residue Lys^{51}(Zhao et al., 2003).

1.3.3 Proposed mechanism

The substrate ACC (1-aminocyclopropanecarboxylate) reacts with internal aldimine [A] resulted from the reaction of PLP (pyridoxal 5’-phosphate) cofactor with Lys residue of ACC deaminase

enzyme. This leads to conversion of internal aldimine to external aldimine [C] *via* aminylintermediate [B] known as trans-aldimination process (Fig.1.2). These initial mechanistic routes are shared between both proposed mechanisms a) direct β hydrogen abstraction, and b) nucleophilic addition followed by β-hydrogen abstraction.

Ose et al. (2003) proposed that Lys^{51} residue of ACC deaminase causes an initial direct β hydrogen abstraction of the methylene proton leading to the formation of a quinonoid [1] (Fig. 1.2, Route 1). The quinonoid [1] undergoes further electronic rearrangement and protonation to form another quinonoid [2]. This is followed by nucleophilic attack by a basic residue on the protein backbone, which ultimately produces 2-ammoniobut-2-enoate and aquinoid [3]. These products reversibly undergo hydrolysis to form 2-oxobutanoate and ammonium, regenerating the internal aldimine.In this proposed mechanism, route II steps are identical to those proposed for the route I upto the production of external aldimine [C] (Fig. 1.2, Route II). Ring opening is initiated by nucleophilic attack of a basic residue of protein on the pro-S β-carbon of ACC and a nearby second basic residue located on the protein, removes a proton from the pro-R β carbon of ACC which results in the formation of a quinonoid. The remaining mechanistic steps are identical to those of the first mechanism (Fig. 1.2, Route II).

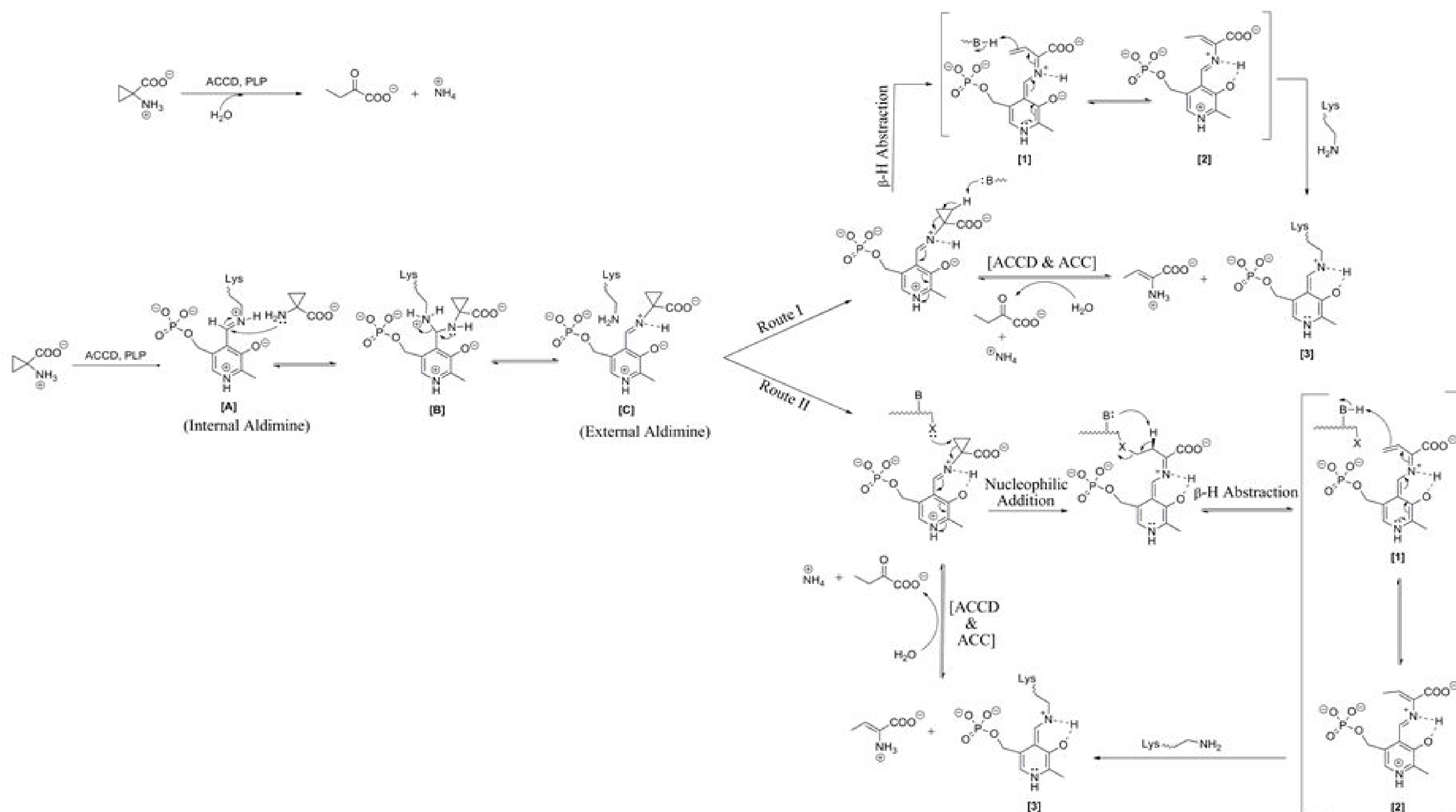


Fig. 1.2 Reaction mechanism catalyzed by microbial ACC deaminase. Route I: direct β-H (Hydrogen) abstraction, Route II: Addition of nucleophiles followed by β-H(Hydrogen) abstraction. Modified figure adapted from the source ref. Hontezas et al. (2006).

1.3.4 Prevalence of ACC deaminase

The presence of ACC deaminase has been reported in all three domains, i.e., eukarya, bacteria, and archaea. However, ACC deaminase activity is known to be present majorly in different species of bacteria (Table 1.2). ACC deaminase activity has been found in a wide range of gram positive and gram negative bacteria (Nascimento et al., 2014). It has also been reported in strains of *Pyrococcus horikoshii* (Fujino et al., 2004), a hyperthermophilic archaeon. Among eukaryotes, production of ACC deaminase is well evident in some fungi, which include a few species of yeast such as *Hansenula saturnus* (Minami et al., 1998), *Issatchenkia occidentalis* (Palmer et al., 2007), other fungal species namely *Penicillium citrinum* and *Trichoderma asperellum,* and a stramnopile, *Phytophthora sojae* (Jia et al., 1999; Singh and Kashyap, 2012; Viterbo et al., 2010). Recently, ACC deaminase activity has also been observed in certain plants such as *Arabidopsis thaliana,* poplar, and a tomato plant (McDonnell et al., 2009; Plett et al., 2009). The presence of ACC deaminase has been confirmed at the molecular level by amplification and sequence analysis of *AcdS*, a structural gene encoding ACC deaminase. The *AcdS* gene is commonly found in Actinobacteria, Deinococcus-Thermus, three classes of Proteobacteria (α, β, and γ), various fungi belonging to Ascomycota and Basidiomycota, and in some Stramenopiles. Although, the presence of ACC deaminase activity has been demonstrated in bacteria belonging to phyla Chlorobi, Bacteroidetes, and Firmicutes but the genes corresponding to ACC deamianse have not been reported yet (Nascimento et al., 2014). On the contrary, putative *AcdS* genes have been reported in *Meiothermus* and *Phytophthora* based on the sequence similarity but there is no record of ACC deaminase activity in these thermophilic strains.

Table 1.2 List of microorganism with ACC deaminase activity

Strain	ACCD activity (nmol α-KBmg^{-1}h^{-1})	References
Achromobacter xylosoxidans A551	400	Belimov et al. (2005)
Acidovorax facilis	0.0007	Belimov et al. (2005)
A. facilis 4p-6	3,080	Belimov et al. (2001, 2005)
A.xylosoxidans AF302097	151	Belimov et al. (2001)
A. xylosoxidans Bm1	90	Belimov et al. (2005)
Alcaligenes sp. AF288728	1172	Belimov et al. (2001)
A. xylosoxidans AF302096	555	Belimov et al. (2001)
A.xylosoxidans AF288734	305	Dell' Amico et al. (2008)
Bacillus pumilus AF288735	760	Belimov et al. (2001)
Burkholderia caryophylli	598	Shahroona et al. (2007b)
Enterobacter cloaceae	295	Nadeem et al. (2010)
E. aerogenes	341	Nadeem et al. (2007)
Escherichia coli DH5α/p4U2	285	Shah et al. (1998)
Flavobacterium ferrugineum	405	Nadeem et al. (2007)
Methylobacterium sp.	94	Madhaiyan et al. (2006)
Mycobacterium sp.	1.14	Dell'Amico et al. (2008)
Pseudomonas aeruginosa	153	Zahir et al. (2009)
P. bathycetes	501	Nadeem et al. (2007)
P. brassicacearum AY007428	972	Belimov et al. (2001)
P. chlororaphis	456	Nadeem et al. (2007)
P. fluorescens	421	Nadeem et al. (2007)
P. fluorescens ATCC 17400/pkK-ACC	157	Shah et al. (1998)
P. fluorescens TDK1	349	Zahir et al. (2009)

P. fluorescens biotype G	490	Shaharoona et al. (2006a)
P. fluorescens biotype F	342	Zahir et al. (2008)
P. oryzihabitans AF288732	890	Belimov et al. (2001)
P. syringae	440	Nadeem et al. (2007)
P. tolaasii	1.16	Dell'Amico et al. (2008)
Rhizobium hedysari ATCC 43676	20	Ma et al. (2003)
*R. leguminosarum*128C53K	5	Belimov et al. (2001, 2005)
R. leguminosarum 99A1	430	Ma et al. (2003)
Rhodococcus sp. AF 288731	833	Belimov et al. (2001)
Rhodococcus sp. strain Fp2	7,320	Belimov et al. (2001, 2005)
Rhodococcus sp. strain 4N-4	12,970	Belimov et al. (2001, 2005)
Serratia ficaria	326	Nadeem et al. (2010)
S. proteamaculans	276	Zahir et al. (2009)
S. quinivirans SUD165	12	Belimov et al. (2001, 2005)
Variovorax paradoxus 3P-3	3,700	Belimov et al. (2001, 2005)
V. paradoxus 5C-2	4,322	Belimov et al. (2001, 2005)
V. paradoxus 2C-1	3,588	Belimov et al. (2001, 2005)
V. paradoxus sp.	1805	Belimov et al. (2005)

We analyzed the prevalence of *AcdS* gene in IMG (Integrated microbial genomes) database (http://img.jgi.doe.gov/) from Joint Genome Institute (JGI) using locus tag search corresponding to *AcdS* of *Pseudomonas putida* UW4. The *AcdS* sequences having more than 1000 bp were chosen for further analysis (Fig. 1.3). Altogether, 485 strains belonging to different genera including *Acidovorax, Bordetella, Brenneria, Burkholderia, Collimonas, Cupriavidus, Curvibacter, Dickeya, Herbaspirillum, Halomonas, Lonsdalea, Methylibium, Pantoea, Phytophthora, Polaromonas, Pseudomonas, Ralstonia, Serratia, Tatumella, Variovorax,* and *Xenophilus* showed the presence of *AcdS* gene. Important species belonging to these genera are listed in Table 1.3. These data were extracted from the genomic sequences of organisms from different ecological niches including ahuman host, bulk soil, plants, and water. For the presence of a gene encoding ACC deaminase in other domains (eukarya and archaea), gene search was conducted using the product name as a criterion for the search. It revealed that very few members of archea showed the presence of ACC deamianse gene. It includes strains of *Archaeoglobus fulgidus, Pyrococcus abyssi, Pyrococcus furiosus* and *Thermococcus nautili.* Analysis of metagenomic database revealed the presence of genes encoding ACC deamianse in various kingdoms, i.e., animalia, chromalveolate, fungi and plantae of domain Eukarya (Table 1.3). It suggested that among domain Eukarya, ACC deamianse gene is prevalent in members of phylum Ascomycota and Basidiomycota. The IMG database extends our knowledge of the existence of ACC deaminase gene in other higher plants from kingdom plantae which include soybean, potato, maize and castor oil plants. The presence of ACC deaminase encoding genes in several members of kingdom Animalia seems to be intriguing as no obvious role of ACC deamianse in animals is known. The presence of ACC deaminase gene in some pathogenic bacteria associated with human and other animals indicates that ACCD may be required for some other unknown function or these bacteria may earlier be plant-associated which later on evolved to colonize animals and other kingdoms as well. Moreover, both bacterial and fungal ACC deaminase shares a common origin and belongs to pyridoxal phosphate-dependent enzyme related to the tryptophan-synthase family.

Table 1.3 Distribution of ACC deaminase in domain Eukarya

Kingdom	Phylum	Species
Animalia	Porifera	*Amphimedon queenslandica*
	Chordata	*Branchiostoma floridae, Ciona intestinalis, Oikopleura dioica*
	Annelida	*Capitella teleta*
	Cnidaria	*Nematostella vectensis*
	Placozoa	*Trichoplax adhaerens Grell-BS-1999*
Chromealveolata	Haptophyta	*Emiliania huxleyi*
	Stramnopile	*Saprolegnia parasitica, Phytophthora ramorum*
	Apicomplexa	*Theileria annulata*
Fungi	Ascomycota	*Arthroderma benhamiae**, *Aspergillus clavatus** , *A. flavus**, *A. fumigatus, Beauveria bassiana, Cercospora sojina, Coccidioides posadasii**, *Fusarium oxysporum, Neosartorya fischeri**,
		*Penicillium marneffei**, *Talaromyces stipitatus**, *Trichophyton verrucosum**
	Basidiomycota	*Cryptococcus neoformans, Gymnopus luxurians,*
		Schizophyllum commune
Plantae	Lycopodiophyta	*Selaginella moellendorfii*
	Magnoliophyta	*Ricinus communis**, *Zea mays, Glycine max*
	Anthophyta	*Solanum tuberosum*
	Chlorophyta	*Volvox carteri*

*Putative proteins have been found.

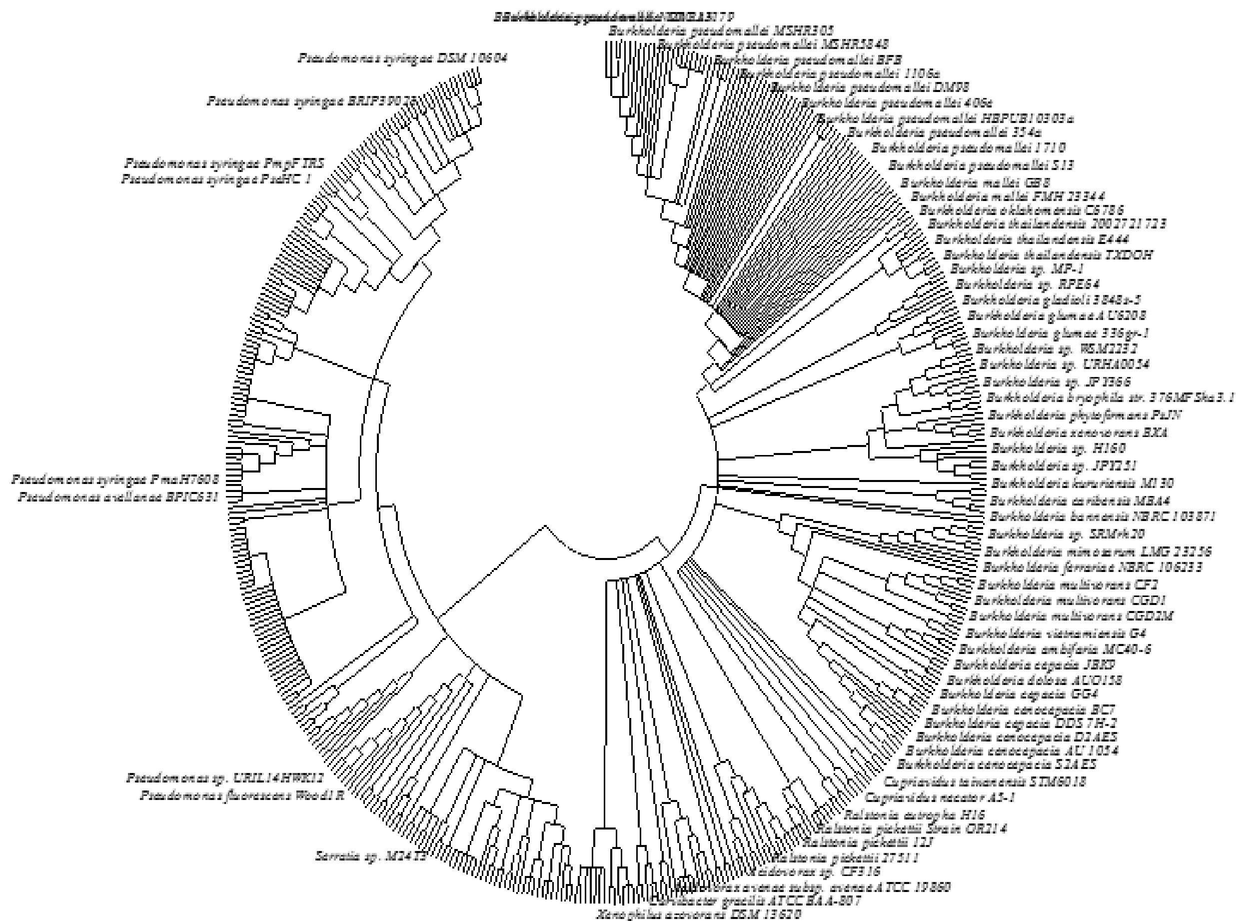


Fig. 1.3. The phylogentic tree constructed from *AcdS* gene sequence of different bacterial strains. The sequence data obtained from IMG database of JGI were used. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The evolutionary distances were computed using the maximum composite likelihood method and were in the units of the number of base substitutions per site. The analysis involved 465 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 958 positions in the final dataset. Label of bacterial strains/species showing values lower than 1% was hidden. Evolutionary analyses were conducted in MEGA6.0

1.4 Genetics and expression of ACC deaminase

1.4.1 ACC deaminase gene

Gene *AcdS* encoding ACC deaminase have been detected in several bacterial and fungal genera as discussed above. More recently, ACC deaminase has been found in wide range of gram-negative bacteria (Wand et al., 2001; Belimov et al., 2001; Babalola et al., 2013; Hontzeas et al., 2004), Gram-positive bacteria (Belimov et al., 2001; Timmusk et al., 2011), rhizobia (Ma et al., 2003b; Uchiumi et al., 2004), endophytes (Rashid et al., 2012; Sessitsch et al., 2002), and fungi (Jia et al., 1999). Putative ACC deaminase gene has also been reported several species including *R.leguminosarum bv. trifoli* (Itoh et al., 1996), and *Mesorhizobium loti* MAFF303099 (Kaneko et al., 2002). However, the expression level of ACC deaminase varies from one organism to another. Using a universal pair of primers, a segment of *AcdS* gene has been amplified and analyzed in several environmental isolates (Hontzeas et al., 2005). Several pair of primers has been designed by various researchers to detect the presence of *AcdS* gene in bacteria (Duan et al., 2009; Jha et al., 2012). Complete genetic make up and function of ACC deaminase gene has been well characterized in only a few bacterial species (Duan et al., 2013). We observed from the data recovered from IMG that nucleotide sequences of *AcdS* gene is very close to other genes namely dcyD and yedO which encode for another PLP-dependent enzyme D-cysteine sulfhydralase. This observation is supported by previous reports where some genes previously identified to encode ACC deaminase were found to encode D-cysteine desulfhydrase activity (Riemenschneider et al., 2005). To differentiate sequences of D-cysteine desulfhydrase from ACC deaminase, Nascimento et al. (2014) analyzed *AcdS* sequences for key protein residues namely Lys51, Ser78, Tyr295, Glu296 and Leu322, known to be important for ACC deaminase activity using *Pseudomonas* sp. UW4 as a reference. Any change in residues at given locations was considered likely to represent D-cysteine desulfhydrase.

Except few, *AcdS* gene in the majority of bacterial species is chromosomal DNA-borne. In symbiotic bacteria *Mesorhizobium loti* (symbiont of *lotus* spp.), ACC deaminase gene is associated with the nitrogen fixation genes and might be regulated by NifA which is known to activate *nif* gene expression in association with the product of *rpoN* gene (Ma et al., 2003a). Moreover, only a small fraction of putative *AcdS* gene has been shown to encode active enzyme (Glick et al., 2013).

1.4.2 Regulation of ACC deaminase

AcdS is highly regulated and expresses differentially depending on presence or absence of oxygen, concentration of substrate, and accumulation of products. Except few, mechanism of regulation of *AcdS* gene in different bacterial genera is not well understood. A model for the regulation of ACC deaminase gene in *Pseudomonas putida* UW4 (earlier known as *Enterobacter clocae* UW4) have been proposed by Glick et al. (2000). Regulatory elements for the expression of ACC deaminase gene consist of regulatory gene *AcdR* located 5' upstream of ACC deaminase structural gene (*AcdS*), promoter regions for binding of regulatory proteins like Lrp box for binding of Lrp protein, AcdB box for binding regulatory protein *AcdB*, FNR box for binding of fumarate and nitrate reductase protein and, CRP box for binding of cAMP receptor protein. In the presence of ACC, LRP forms an active octamer that binds to a complex of ACC and another protein *AcdB* (Cheng et al., 2008). *AcdB* encodes for the glycerophosphoryl diester phosphodiesterase and form complex with ACC. This triparental complex activates transcription of *AcdS* by binding to its promoter region (Li and Glick, 2001). The role of *AcdB* in *AcdS* expression has not been observed in other bacteria characterized for *AcdS* gene expression. ACC deaminase gene is negatively regulated by leucine which is synthesized from α-ketobutyrate, a breakdown product of ACC deaminase catalyzed the reaction. As the concentration of leucine increases, it favors formation of inactive LRP dimer form which leads to switching off the transcription of *AcdS* gene (Fig. 1.4).

Regulatory machinery for *AcdS* expression varies in different species. Results of IMG database analysis showed that presence of *AcdR* encoding LRP or its homologous sequences is present in the majority of bacteria. In *Bradyrhizobium japonicum* USDA 110 and *Rhizobium leguminosarum bv. Viciae* 128 C53K also, LRP like protein and σ70 promoter are involved in regulation of *AcdS* gene (Kaneko et al., 2002; Ma et al., 2003a). The phylogenetic analysis of *AcdR* gene suggested that *AcdS* and *AcdR* were evolved in a similar manner. In *Burkholderia* sp. CCGE 1002 and *Burkholderia phymatum* STM 815, there is no *AcdR* gene but it has two copies of *AcdS* gene instead, one on the megaplasmid and other on the second chromosome. These shreds of evidence suggest the genomic rearrangement events or gene insertion event in smaller replicons.

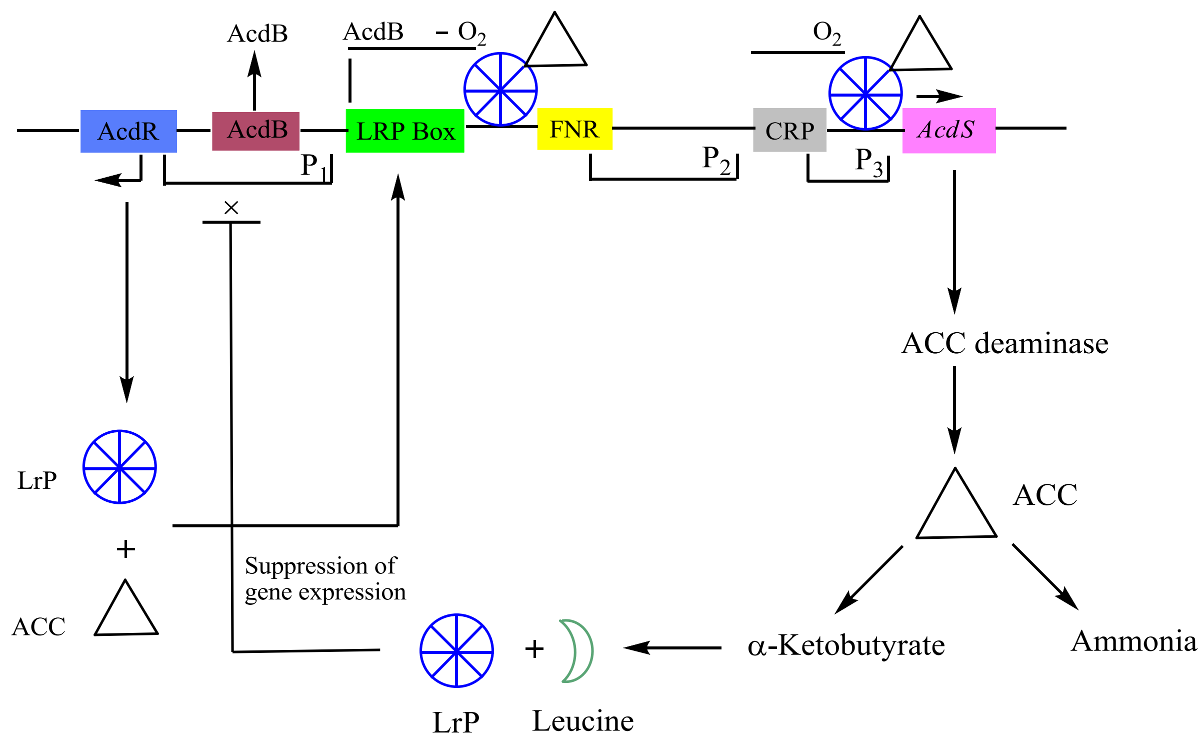


Fig. 1.4. The regulatory circuits of *AcdS* gene expression in *Pseudomonas putida* UW4 and other related bacteria. Abbreviations AcdR: regulatory gene for ACC deaminase; AcdB: encoding for glycerophosphoryl diester phosphodiesterase; LRP: Leucine responsive protein; FNR: fumarate nitrate reductase protein; CRP: c-AMP receptor protein; *AcdS*: gene for encoding ACC deaminase.

In *Mesorhizobium loti*, the upstream elements of *AcdS* and *nifH* contain *nifA1* and *nifA2* (regulatory N_2 fixing unit) and σ^{54} RNA polymerase sigma recognition site. It was assumed that expression of ACC deaminase in *Mesorhizobium loti* required the symbiotic nitrogen fixing regulatory gene *nifA2* (Nukui et al., 2006). The *nifA2* encoded protein NifA2 interact with σ^{54} RNA polymerase favoring *AcdS* transcription. The *nifA1* also affect the transcription of *AcdS* gene upto some extent, however, its role in the expression of *AcdS* is not properly understood (Nukui et al., 2006) (Fig. 1.5). The expression of *AcdS* gene in root nodules reduces the harmful effect of ethylene induced senescence and elevates the concentration of fixed nitrogen in nodules. ACC deaminase activity is generally assayed in free-living conditions but in *Mesorhizobium loti*, the activity was detected only in symbiotic nodules (Uchiumi et al., 2004). Uchiumi et al. (2004) have reported that mlr 5932, an up-regulated gene in bacteroides, encodes ACC deaminase, which is involved in enhancing nodulation in *Lotus japonicus* plants. It is to be noted that unlike free-living bacteria, ACC deaminase produced by nodule forming *Rhizobia* does not lower the ethylene level throughout the plant and may not be used to protect plants from various stress. Also, the level of ACC deaminase produced in the nodule is only 2-10% of ACC deaminase produced by free-living bacteria.

In many *Actinobacteria* and *Meiothermus,* GntR protein coding gene is found next to *AcdS* gene. This evidence indicates a possibility that some downstream elements are also involved in the regulation of ACC deaminase expression. In some members of these genera, the absence of promoter region strongly suggests that the interaction of *AcdS* gene and some downstream element next to *AcdS* gene is involved in the regulation of *AcdS* gene transcription. In certain species of *Actinobacteria* and *Proteobacteria* like *Brenneria* sp. EniD312, *Burkholderia xenovorans* LB4000 and *Pantoea* sp. At-9B, LysR family of transcription regulatory elements, are found in close vicinity of *AcdS* gene. However, the exact mechanism of regulation of ACC deaminase in these organisms is still poorly known. Additional genetic and biochemical studies are necessary to understand the mechanism of ACC deaminase regulation and functioning in different bacterial genera. Putative ACC deaminase gene in *Mesorhizobium loti* MAFF303099 does not contain any regulatory elements nor display any enzyme activity when it is induced by ACC (Ma et al., 2003b).

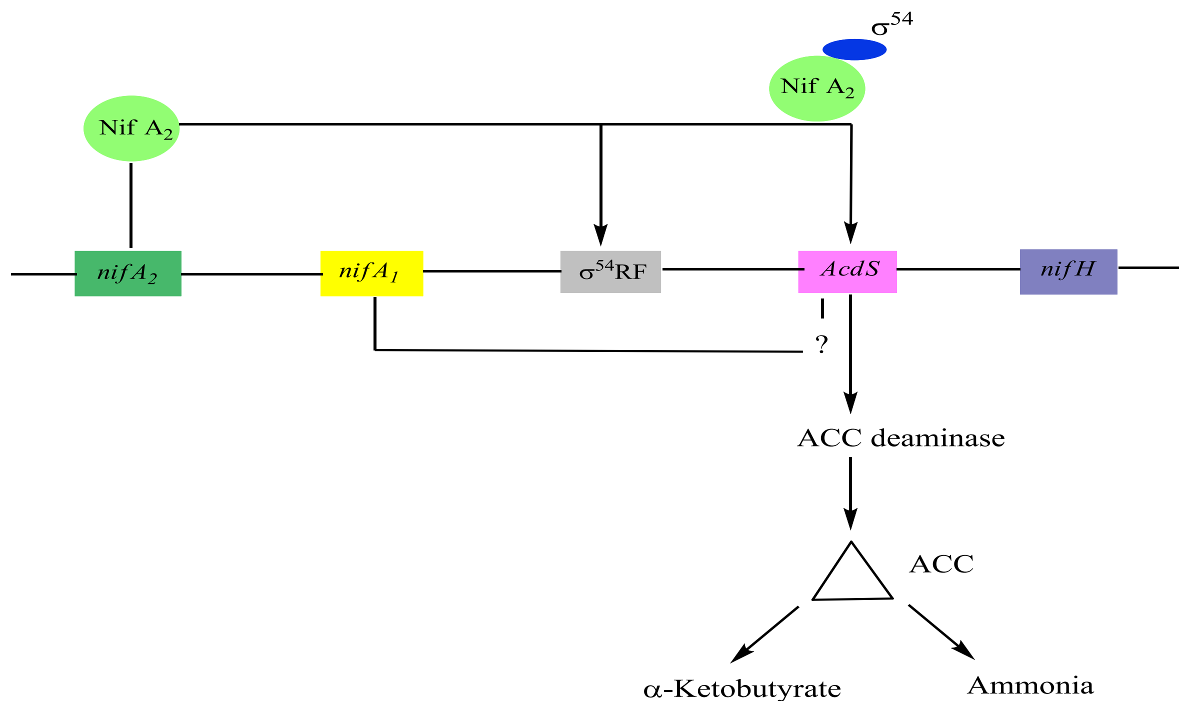


Fig. 1.5. A model for *AcdS* gene regulation in nitrogen fixing *Mesorhizobium* sp. Expression of *AcdS* is positively regulated by $NifA_2$ protein which binds to σ^{54} and switch on transcription of *AcdS* gene.$NifA_1$ is also required in regulation of *AcdS* but its role is not well understood.

1.4.3 Evolution of ACC deaminase gene

Phylogenetic analysis based on *AcdS* gene and protein sequence from different microbial species has been conducted to study the evolution of ACC deaminase gene. Available sequence data suggested that most of the ancient bacteria belonging to Actinobacteria and Deinococcus-Thermus possess *AcdS* gene in their primary and unique chromosome. The *AcdS* gene is also found in the chromosome of many α-Proteobacteria. However, extensive gene transfer between the members of α-Proteobacteria suggested that *AcdS* was transferred from the primary chromosome to a plasmid in some of the α-Proteobacteria. The presence of *AcdS* gene in the second chromosome of *Burkholderia* and in plasmids of *Pseudomonas* reflects the intragenomic transfer of *AcdS* genes from primary chromosome to plasmids. The presence of *AcdS* gene sequence in some fungi like *Fomitopsis pinicola* FP-58527 belonging to Basidiomycota also suggested having a bacterial origin. The conservation of *AcdS* protein sequences from fungi, Actinobacteria and α-Proteobacteria signifies the monophyletic origin of ACC deaminase. The presence of *AcdS* gene in some fungal classes and in members of Stramenopiles suggest that horizontal gene transfer (HGT) occurs not between the bacteria only but also between distantly related organisms. The presence of *AcdR* in juxtaposition to *AcdS* gene in most proteobacteria acquired through a coupled evolution and transmission of these genes. There is need of more research for proteins in the vicinity of *AcdS* gene which can focuses on a different mechanism of ACC deaminase regulation. To gain additional knowledge for the evolution of ACC deaminase gene, multiple *AcdS* gene sequences and protein sequences were aligned. Followed by phylogenetic studies it was assumed that ACC deaminase belongs to a broader group of pyridoxal phosphate dependent enzymes that share a common ancestor. It might be possible that this enzyme was originated as a consequence of specific mutation in its ancestral enzyme gene (Nascimento et al., 2014). The continuous vertical transmission of *AcdS* genes might responsible for the presence of *AcdS* gene in bacteria that are not associated with ACC producing organism. Intragenomic *AcdS* transfer might play a role in HGT transfer as well as divergence of *AcdS* gene. Furthermore, these intra-genomic transfers may also leads to loss of gene in many organisms. Phylogenetic analysis of several *AcdS* gene from ACC deaminase bacteria such as *Variovorax paradoxus* 5C-2*, Variovorax paradoxus* 3P-3, and *Achromobacter* sp. suggested that these genes evolved through horizontal gene transfer (Hontzeas et al., 2005).

Prigent-combaret et al. (2008) have suggested that like *AcdS*, *AcdR* might have evolved through horizontal gene transfer. Moreover, the presence of *AcdS* gene in symbiotic islands of many *Mesorhizobium* sp. like *M. loti* R7A, *M. australicum* WSM2073T, *M. opportunistum* WSM2075T confirms the horizontal transfer of *AcdS* gene (Nasciment et al., 2012). Even though most of the phylogenetic studies of *AcdS* gene has been done on Proteobacteria, presence of ACC deaminase enzyme have been reported in Actinobacteria (Hontzeas et al., 2005), *Firmicutes* (Ghosh et al., 2003), and *Bacteroidetes* (Maimaiti et al., 2007). Based on horizontal (lateral) gene transfer, the juxtaposition of *AcdS* and *AcdR* gene has been observed in *Agrobacterium tumefaciens* d3, *Bradyrhizobium japonicum* USDA 110, *Rhizobium leguminosarum bv. Viciae* 128C53K, *Achromobacter xylosoxidans* A551 (Hontzeas et al., 2005; Trott et al., 2001; kaneko et al., 2002). However, it is not clear that *AcdR* gene was inherited along with the *AcdS* gene. The current knowledge about the phylogeny of evolution of the *AcdS* and *AcdR* gene is incomplete.

1.5 Ecological significance of ACC deaminase bacteria

The ACC deaminase activity is one of the most common traitsamong plant growth promoting rhizobacteria (Glick, 2005; Honma and Shimomura, 1978). ACC deaminase bacteria exert its beneficial effect by protecting from the deleterious effect of environmental stressors (Glick, 2014), delay senescence (Ali et al., 2012), exhibit biocontrol activity against variety of phytopathogens in certain plants (Hao et al., 2011), and favor nodulation in legume plants (Nascimento et al., 2012). Role and importance of bacterial ACC deaminase in plant growth have been described in previous sections.Variation of ACC deaminase activity among microbial species at extreme environmental conditions might be useful in phytoremediation at unusual environmental sites or conditions (Glick, 2005). ACC deaminase bacteria assist associated plants in phytoremediation by biotransformation of toxic elements, rhizodegradation mediated by root exudates, and/or detoxification of heavy metals that allow host plants to survive under adverse conditions. Rhizospheric bacterial community with ACC deaminase can enhance the rate of rhizo-remediation by increasing the root system of the plant as well as increased accessto soil by roots. It results in enhanced uptake of inorganic contaminants through modification of root architecture and root uptake system of the plant. Belimov et al. (2005) reported a positive correlation between the increment of bacterial ACC deaminase activity following the

accumulation of cadmium in plant tissue and enhanced root growth. Similarly, Rodriguez et al. (2008) observed theenhanced growth of tobacco plants and substantial accumulation of metals from nickel contaminated soil following inoculation of *Pseudomonas putida* HS-2.

The presence of ACC deaminase in human pathogenic *Burkholderia cenocepacia* J 2315 as well as in plant pathogenic fungi like *Aspergillus* sp.and *Myceliophthora thermophile* suggests that ACC deaminase might play a role in the ecological fitness of these micro-organisms. Role of ACC deaminase in endophytic fungi *Penicillium citrinum* was investigated by Jia et al. (2000) who found an accumulation of ACC during mycelial growth and subsequent degradation of ACC by ACC deaminase when the mycelial growth reached the maximum. The importance of ACC deaminase in human pathogenic bacteria is not known, but its role in thepathogenesis of plant pathogens has been studied to some extent. For plant pathogenic microorganisms, it is assumed that production of ACC deaminase may help microbe to overcome ACC mediated plant responses. The presence of ACC deaminase bacteria in the close vicinity of fungal strains might have a role in increased fungal primordial proliferation by reducing ACC levels. Thus, anassociation of ACC deaminase bacteria play a significant role in fungal colonization in the extreme soil. Additional advantage of these bacteria is the ability to degrade ACC providing extra nutrients to plant (Nascimento et al., 2014).

1.6 Transgenic plants with ACC deaminase activity

The growth enhancement of plant by ACC deaminase bacteria has motivated scientists to transfer this gene into plants as a future approach to minimize the deleterious effect of ethylene in plants subjected to adverse environmental conditions (Grichko and Glick, 2001; Nie et al., 2002; Robinson et al., 2001). A transgenic Petunia hybrid with ACC deaminase gene maintains a significantly reduced amount of ACC in pollen cells (Lei et al., 1996). Similarly, the transgenic canola plants (*Brassica napus*) with ACC deaminase perform better growth under salinity stress compared to non-transgenic plant (Sergeeva et al., 2006). In a premier study, Reed et al. (1995) transformed two tomato cultivars with *AcdS* gene of *Pseudomonas chlororaphis* which resulted in lengthened duration of fruit ripening as well as significant reduction of stress ethylene production compared to parental line. Klee and Kishmore (1992) also observed a significant reduction of ethylene production (77%) and delayed in senescence in tobacco and tomato plants transformed with bacterial *AcdS*. The tomato plant (*Lycopersicon esculentum*) expressing *AcdS*

gene of *Pseudomonas* sp. 6GS exhibited reduced ethylene synthesis up to 90% (Klee et al., 1991). A large number of transgenic plants with foreign *AcdS* gene have been genetically engineered to reduce the deleterious ethylene levels in plants (Farwell et al., 2007; Grichko et al., 2000; Robinson et al., 2001; Sergeeva et al., 2006; Zhang et al., 2008). However, there is a limited report of the performance of transgenic plant containing *AcdS* gene under field condition (Farwell et al., 2006, 2007). Furthermore, future research should focus on (i) field performance of transgenic plant (ii) their survival and yield under the diverse condition and (iii) genetic re-arrangement for target gene identification for gene insertion and deletion.

1.7 Alternative to ACC deaminase: Use of chemicals

Various chemicals have been used to control ethylene levels in plants. Rhizobitoxine secreted by some microorganism and its synthetic analog, aminoethoxy vinyl glycine (AVG) and silver thiosulphate (STS) can inhibit ethylene synthesis in many plants (Sugawara et al., 2006). But AVG and STS are found to be highly toxic in food. Ethephon (2-chloroethyl phosphoric acid) is called “liquid ethylene” in agriculture and it can release ethylene that promotes early fruit ripening (Abeles et al., 1992). Cyclopropane acting as blocking agents for ethylene perception in plants is potentially valuable for extending the display life of potted plants and vase life of cut flowers (Sisler and Serek, 1997). Mizutani et al. (1998) isolated tropolone compounds from wood that can inhibit the growth of wood-rotting fungi and are reported to inhibit the synthesis of ethylene in excised peach plants.

1.8 Other mechanism of stress protection mediated by PGPR

Certain PGPR can also improve the plant growth through one or more mechanisms including production of allelochemicals and hydrolytic enzymes, disruption of quorum-sensing required for pathogenesis, induction of osmolyte production in plants, EPS production, increased antioxidant activity, and differential regulation of HKT1 etc. (Lugtenberg and Kamilova, 2009). The mechanism of plant growth promotion by these PGPR differ among bacterial strains and most commonly depend on the release of various metabolites assisting the plant growth under biotic and abiotic stress conditions. In addition, PGPR having ability to degrade organic contamination are more effective in field condition than non-PGP bacteria as they can colonize rhizosphere or rhizoplane where they obtain sufficient source of energy from root exudates for

primary metabolism which in turn enable them to and degrade xenobiotic compounds efficiently. Few volatile organic compounds (VOCs) other than HCN, such as 2,3-butanediol, or blends of volatiles produced by *Bacillus* sp. or by fungi have also been assumed to be involved in plant protection (Strobel et al., 2007). Application of genetic engineering could prove an asset for efficiency increment and better adaptability of microbes through alterations in molecular pathways leading to detoxification.

1.9 Gaps in existing research

ACC deaminase producing bacteria have been isolated from diverse crop plants such as maize, canola, rice etc. and found to increase the plant growth under controlled conditions (Mayak et al., 2004, Glick et al., 2014). However, very few studies have been conducted on isolation and characterization of ACC deaminase containing bacteria from the desert region of India (Sahay et al., 2012; Ramadoss et al., 2013). Therefore, present work was aimed to characterize the efficient ACC deaminase bacteria from the various plants growing in the desert region of Rajasthan. In addition few isolates were recovered from the high saline water of Sambhar salt lake. Based on literature survey, following gaps was identified in the present study.

- Despite several reports of ACC deaminase producing bacteria, there is a lack of information about the efficient bacteria recovered from the desert region of Rajasthan (India). The bacteria recovered from the desert region might have the ability to survive under high stresses like salt and temperature. Therefore, in the present study selected high ACC deaminase containing bacteria were tested for the plant growth promoting test on the wheat plant under controlled conditions. Ability to enhance the plant growth under salt and temperature stress can be useful to exploit them as suitable biofertilizer agent.
- Very less work has been conducted for the colonization and motility behaviour of ACC deaminase bacteria. Therefore, in present study an effort was made to test the motility pattern and colonization ability both by microscopy and molecular approach in the host plant. The selected isolates were also tested for the production of osmoprotectants, and their antioxidant activity which can ameliorate stressors. In earlier studies, the amplification and sequence analysis of ACC deamianse gene *(AcdS*) has been reported in some of the bacterial species especially belonging to *Pseudomonas* sp. We have made an attempt to detect and analyze ACC deaminase genes in environmental isolates which

were not previously reported. In addition, the *AcdS*⁻ mutant of selected isolate was generated to ensure the role of ACC deaminase in promoting plant under stress condition.

- Understanding the proteomic response in the presence of rhizobacteria could provide an insight into the differential expression of proteins responsible for enhanced stress tolerance. In the present study, an attempt was made to characterize the proteins responsible for transcriptional control, plant growth, osmoregulation, defense response, metabolic pathways and various other biosynthetic pathways.
- Genomic studies of very few ACC deaminase producing PGPR have been carried out. The genomic analysis of selected rhizobacteria can be helpful for identification of PGPR genes and other genes responsible for stress tolerance. It will also highlight on physiological potential and colonization details of the test. Moreover, the genomic analysis will also be helpful in gathering information about the regulatory mechanism of the *AcdS* gene. The detailed analysis of regulatory subunits of *AcdS* gene can be helpful in the optimal exploitation of ACC deaminase bacteria for enhancing plant productivity.

1.20 Objectives

This research work addresses the role of different mechanism like phytohormones, ACC deaminase enzyme activity in the isolated bacterial strains that strongly affect the competitiveness of organism in the environment and its plant growth promotion activity. Moreover, experimental evidence supports that bacteria with ACC deaminase enzyme activity might be a good alternative to biofertilizer to the plants growing in the stress conditions. Thus the objectives of this research work are

1. Isolation of ACC deaminase producing bacteria from the plants growing in the desert of Rajasthan (India), and their characterization of plant growth promoting properties.
2. To test and analyze the effect of selected bacterial species/strain on the growth of wheat plants based on different growth parameters.
3. Generation of *AcdS*⁻ mutant of selected bacterial species/strain to analyze the plant growth as compared to wild type.

4. Identification of proteins responsible for plant growth, stress protection, defense response, and osmoregulation following inoculation of selected bacterial strain to wheat plants employing proteomic approach.
5. Whole genome sequence analysis of selected bacterial strain to gather the information about the presence of PGPR genes, genes for the establishment in host plant as well as those responsible for colonization, motility, chemotaxis, etc.

CHAPTER II

Isolation and characterization of ACC deaminase producing bacteria from desert region of Rajasthan

2.1 Introduction

Continuous increase in World human population, changes in lifestyle, urbanization and industrialization have shrunken the total arable land for agriculture. In addition to this, exposure to abiotic stressors such as salinity, drought, temperature, flooding, air pollution, heavy metals, organic contaminants and ultraviolet light reduce the yield of the field-grown crops at large scale (Bray et al., 2000). Presently, more than 6% of the total land area of the world is salt affected especially in arid and semiarid zones (Bui, 2013). The salinization of land, a serious threat to agriculture, has increased up to 800 million hectares of land throughout the world suppressing plant growth and productivity (FAO 2008; http://www.fao.org/ag/agl/agll/spush/). Similarly, it is estimated that total land area affected by drought will increase two fold and water resource will decline upto 30% by the year 2050 (Falkenmark, 2013). Moreover, there are evidences of yield declines in wheat and paddy crops in many parts of South Asia due to increasing water stress and increased air temperature. The average temperature in the Indian sub-continent has risen by 0.57 °C in the last 100 years and likely to increase by a maximum of 2.5 °C by 2050 and 5.8 °C by 2100 (Venkateswarlu et al., 2009).

One of the most common mechanisms displayed by plants to evade harmful effects of abiotic stressors involves the production of ethylene, which induces expression of proteins that can enable plants to tolerate the stressors. Ethylene is an important hormone which helps in the formation of the root, fruit ripening, seed germination, biosynthesis of other phytohormones, and stress signaling (Abeles et al., 1992). However, production of ethylene beyond an optimal limit (termed as 'stress ethylene') inhibits plant growth and development by promoting abscission, chlorosis, and senescence leading to the death of the plant. Few reports have suggested that plants growing under continuous stressful environment die directly because of increased level of stress ethylene and not from the direct effect of stress (Van Loon, 1984; Stearns and Glick, 2003). Under these circumstances, use of chemical inhibitors of ethylene biosynthesis such as silver thiosulphate (STS), cyclic olefin norbornadiene (NBD) (Reid and Wu, 1992), and L-α-(aminoethoxyvinyl)-glycine (AVG) (Nayani et al., 1998) have been reported to prolong the shelf life of ethylene-sensitive flowers. However, the use of these chemicals has a number of environmental drawbacks. Generation of genetically modified crops is a significant step forward in the production of agriculturally important crops. Genetically-modified (GM) seeds have been

modified to contain specific characteristics such as resistance to herbicides and pesticides. Although this method is more efficient, it may have health or environmental impacts that are not being adequately addressed. As a result, the technology is surrounded by significant controversy. In the present scenario of several biotic and abiotic stresses to which agriculture is confronted, the role of PGPR could be crucial for sustainable crop production (Farwell et al., 2006).

Plant growth promoting bacteria having ACC deaminase activity could help plants to cope with the stress conditions by maintaining the stress ethylene concentration below the growth inhibitory point (Glick, 1995). The importance of these beneficial bacteria has been shown under various stress conditions. For example, *Achromobacter piechaudii* ARV8 exhibiting the ACC deaminase activity provided resistance against water stress in tomato and pepper plants (Mayak et al., 2004); *Enterobacter cloacae* UW4 protected tomato plants under flooding stress (Grichko and Glick, 2001); *Burkholderia phytofirmans* PsJN enhanced resistance to chilling stress in *Arabidopsis* (Ait Barka et al., 2006) etc. Besides, there are also few reports suggesting the importance of PGPB with ACC deaminase activity in phytoremediation and protection against organic toxico-chemicals (Huang et al., 2004; Dell'Amico et al., 2005; Reed and Glick, 2005). Therefore, the understanding of plant growth promoting bacteria with different level of ACC deaminase activity and their function in the associated plant can be utilized to ameliorate the stress conditions as well as for enhancing the growth and yield of the plant. Isolation of ACC deaminase bacteria and their beneficial effect on plant growth under various environmental stressors have been reported by several research groups (Ahmad et al., 2011; Cheng et al., 2007; Shaharoona et al., 2007). However, very few ACC deaminase producing bacteria are available which can be used as growth stimulant under abiotic stressors in field conditions. Thus, an extensive exploration of the bacteria possessing ACC deaminase activity is required to obtain the efficient and suitable bio-inoculants for protecting plants from deleterious effects of various environmental stressors to a certain extent.

The soil of Rajasthan faces extreme stressors including high salinity and fluctuating temperatures which can largely affect plant growth and productivity (Singh et al., 2010). Consistent exposure of plants to such stressors also indicates selection of its rhizospheric microbiome which can help them tolerate these stressors. Therefore, the present work aimed to isolate efficient ACC deaminase bacteria from plants growing in the saline soil of desert of

Rajasthan, India and to characterize them for their plant growth promoting activities. The isolates were also tested for antagonistic activity and ability to survive under various salt (NaCl) and temperature stress.

2.2 Materials and methods

2.2.1 Collection of soil and plant samples

The rhizospheric soil of various plants such as *Sorghum bicolor, Ziziphus nummularia, Capparis decidua, Calligonum polygonoides, Pennisetum glacum, Sorgastrum nutans,* and *Aerva javanica* growing in Shekhawati region (27°N & 75°E) of Thar desert of Rajasthan was used for isolation of bacteria. Detail about sampling site and geographical location has been summarized in Table 2.1. Plants were uprooted, kept in polythene bag containing moist soil and brought to the laboratory immediately for isolation. In addition to plant roots, non-rhizosphere soil from Sambhar salt lake was also taken for isolation of bacteria.

Selection of plants for the study was based on the ability of plants to grow profusely in the soil of desert. *Calligonum polygonoides* commonly known as ‘Phog’ is a small shrub found in arid and semi-arid areas of Thar desert (India). The size of the plant is usually 4 to 6 feet high, but occasionally may reach even 10 feet in height. It commonly grows on dry sandy soils and on sand dunes. It is very hardy and capable of growing under adverse conditions of the soil. *Ziziphus nummularia* also known as Jharber is a shrub up to 2 meter (6.6 ft) and is a species of *Ziziphus* native to the Thar desert of India. *Sorghum bicolor,* locally known as Jowar, is a grass species and is typically an annual but some cultivars are perennial. It grows in clumps that may reach over 4 m high. The grain is small, ranging from 3 to 4 mm in diameter. The plants used in the experimental work for isolation of bacteria have been depicted in Fig. 1.

Table 2.1 Details of plants used for isolation of ACC deaminase producing bacteria

Plant local name	Scientific name	Site of collection	Geographical location	Isolate's name
Jowar	*Sorgrum bicolor*	Chirawa	28.25° North 75.63° East	SBP-6,7,8,9,10
Jharber	*Ziziphus nummularia*	Jhunjhunu	28.13° North 75.4° East	ZNP-1,2,3,4,5
Kair	*Capparis decidua*	Jhunjhunu	,, ,,	CDP-13,14
Phog	*Calligonum polygonoides*	Churu	28.30° North 74.95° East	CPC-20,CPC-21,CPC-22,CPC-23,CPC-24,CPC-25
Bajra	*Pennisetum glacum*	Churu	,, ,,	PGP-19,
Green grass	*Sorgastrum nutans*	Churu	,, ,,	SNP-17,SNP-18
Loonia root	*Aerva javanica*	Sambhar lake	26° 54"North 75° 12"East	AJS-15
Salt lake	--	Sambhar lake	26° 54"North 75° 12"East	HSW-16, SL-11, SL-12

2.2.2 Culture media

PAF media (Composition: per litre, protease peptone 10 g, casein hydrolysate 10 g, anhydrous $MgSO_4$ 1.5 g, K_2HPO_4 1.5 g, and glycerol 10 ml) and DF (Dworkin and Foster) minimal salt medium (Composition: per litre, KH_2PO_4 4.0 g, Na_2HPO_4 6.0 g, $MgSO_4.7H_2O$ 0.2 g, glucose 2.0 g, gluconic acid 2.0 g, citric acid 2.0 g, Trace elements: $FeSO_4.7H_2O$ 1 mg, H_3BO_3 10 µg, $MnSO_4.H_2O$ 11.19 µg, $ZnSO_4.7H_2O$ 124.6 µg, $CuSO_4.5H_2O$ 78.22 µg, MoO_3 10 µg, pH 7.2 (Dworkin and Foster, 1958) were used for the isolation of ACC deaminase containing bacteria. ACC (1-aminocyclopropane-1-carboxylic acid) was added to the medium to attain its final

concentration of 3 mM for enriching ACC deaminase bacteria. 1.8 % agar was added in DF medium for preparation of DF-agar plate. All reagents used were of analytical high purity grade and were prepared using distilled water obtained from a Milli-Q system (Merck Millipore, USA).

Ziziphus nummularia

Sorghum bicolor

Calligonum polygonoides

Capparis decidua

Pennisetum glacum

Sorgastrum nutans

Aerva javanica

Sambhar salt lake

Fig 2.1. Images of selected plants that were used for isolation of ACC deaminase bacteria. Plants were collected from the various region of Thar desert of Rajasthan.

2.2.3 Enrichment and isolation of ACC deaminase containing bacteria

For the isolation of ACC deaminase containing bacteria, one gram of rhizospheric soil adhered to the roots was added to 50 ml sterile PAF media and incubated at 30 °C for 24 h on a shaker set at 200 rpm. The soil was allowed to settle, and a 1 ml aliquot was removed from growing culture and transferred to 50 ml sterile PAF medium at the same condition as that of first incubation. 1 ml aliquot was removed from the second culture and transferred to 50 ml sterile DF salt medium. Following the incubation at the same condition, a 1 ml aliquot was removed from this culture and transferred to 50 ml sterile DF salt medium containing 3.0 mM ACC (Sigma-Aldrich, USA) as the source of nitrogen. Serial dilutions of this final culture were plated onto solid DF-agar medium containing ACC. The inoculated plates were incubated at 30 °C for 4 days. Bacteria growing on above selective medium were sub-cultured several times on DF-ACC plate for confirming the ability of isolates to utilize ACC as a nitrogen source. After sub-culturing, bacterial colonies were counted and grouped on the basis of their morphological characteristics such as the difference in colony size, elevation, color etc. Each morphotype was maintained as pure culture and the glycerol stock (15% w/v) were prepared to store at -70 °C until further use.

2.2.4 Confirmation and quantification of ACC deaminase activity

The selected bacterial isolates were used for the confirmation and quantification of ACC deaminase activity following method of Honma and Shimomura (1978) with slight modifications. Enzyme extract was prepared and used for the assay as described below.

2.2.4.1 Preparation of enzyme extract

Bacterial isolates were cultured in Tryptic soy broth (Himedia Laboratories, Mumbai, India) and grown up to late log phase in a shaking water bath at 200 rpm at 30°C. The accumulated biomass was harvested by centrifugation at 8,000 g for 10 min at 4°C. Bacterial pellets were washed with DF salt minimal medium and suspended in 15 ml of DF salt minimal medium containing 3.0 mM ACC. The bacterial cells were returned to shaking water bath for induction of ACC deaminase at 200 rpm for 24 h at 30 °C. Then, cells were harvested by centrifugation, washed with 0.1 M Tris-HCl (pH 7.6), and the collected bacterial cells were resuspended in 0.1 M Tris-HCl (pH 8.5). For lysis of the cells, thirty microlitre of toluene was added to cell suspension and vortexed at highest

setting for 30 s. At this point, 100 µl of toluenized cells were set aside for protein assay at 4 °C. The remaining toluenized cell suspension was used immediately for ACC deaminase assay.

2.2.4.2 ACC deaminase assay

20 µl of 0.5 M ACC was added to 200 µl toluenized cells, briefly vortexed, and incubated at 30 °C for 15 min. Following this, 1 ml of 0.56 M HCl was added, mixed by vortexing, and spun for 5 min at 8,000 g at room temperature. One ml of resulting supernatant was mixed with 800 µl of 0.56 M HCl. Then, 300 µl of 2, 4- dinitrophenylhydrazine was added to the above mixture in a glass tube and incubated at 30 °C for 30 min. Following the addition and mixing of 2 ml of 2 N NaOH, the absorbance was measured at 540 nm in a spectrophotometer (Jasco Corporation, Japan). The quantification of ACC deaminase was expressed in terms of µmol α-ketobutyrate/mg protein/h. The µmol of α-ketobutyrate (α-KB) produced was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutyrate ranging between 0.1 to 1.0 µmol.

2.2.4.3 Estimation of protein

The protein concentration of toluenized cells was determined by the method of Bradford (1976). Different concentrations of bovine serum albumin (BSA) were used for the preparation of a standard curve. A stock solution of BSA (10 mg/mL) was diluted with distilled de-ionized water to final concentration of 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mg/ml respectively. The values of the absorbance at 595 nm versus the BSA concentration were used to construct a standard curve.

2.2.5 Biochemical characterization

Biochemical tests such as Gram staining, starch agar test, IMViC (Indole, Methyl Red, Voges Proskauer, Citrate utilization) test, and catalase assay were performed following standard protocol (Harley and Prescott, 2002). For qualitative estimation of urease production, bacterial isolate were grown in urea-agar base (Himedia, India) supplemented with 5 ml of 40% urea solution in 100 ml medium and change in color from yellow to pink indicated urea hydrolysis (Collins et al., 1995).

2.2.5.1 Antibiotic resistance profiling

The bacterial isolates were tested for its resistance to standard antibiotics namely gentamicin (30 µg), ampicillin (10 µg), erythromycin (10 µg), kanamycin (5 µg), tetracyclin (10 µg), streptomycin (25 µg), and chloramphenicol (10 µg) by the antibiotic sensitivity assay using antibiotic discs (HTM 002, Himedia). Briefly, the bacterial culture was swabbed onto NA media plates. The standard antibiotic disc (6 mm) was placed on the media surface, and the plates were incubated at 37 °C for 24 h. The experiment was performed in triplicate. The results were interpreted on the basis of the diameter of inhibition zone using the zone size interpretative chart supplied by the manufacturer (Himedia, India).

2.2.5.2 Carbohydrate utilization test

An ability of the isolates to utilize different carbohydrates such as xylose, maltose, fructose, dextrose, galactose, raffinose, trehalose, sucrose, L-arabinose, mannose, glycerol, salicin, inositol, sorbitol, mannitol, adonitol, α-Methyl-D-glucoside, rhamnose, cellobiose, ONPG, esculin hydrolysis, D-arabinose, sorbose, inulin, and mannose was tested using carbohydrate utilization test kit (KB 009, Himedia) as per instruction of the manual.

2.2.5.3 Test of hydrolytic activity

2.2.5.3.1 Cellulolytic activity: endoglucanase activity

Production of endoglucanase was tested employing Gram's iodine method with slight modifications (Kasana et al., 2008). Bacterial cultures were point inoculated onto minimal-agar medium supplemented with 0.2% carboxymethyl cellulose (CMC) and 0.3% tryptone and incubated for four days at 30 °C. Gram's Iodine solution was poured onto the bacterial growth and kept for 5 min at room temperature till the appearance of clear halo zone. Different cultures were scored as poor (+), average (++) and good (+++) for endoglucanase activity on the basis of the diameter of clear zone.

2.2.5.3.2 Cellobiohydrolase activity

The exponentially grown cultures of bacterial isolates were spot inoculated on $JNFb^-$ agar plates containing 0.5 mM ammonium chloride and ethanol (6 ml l^{-1}) as a nitrogen and carbon sources respectively. The cultures were grown for 3 days at 30 °C and then incubated at 37 °C following

the application of overlay containing 8 ml of 0.05 M potassium phosphate (pH 7.0), 0.7% agarose and 0.5 µg/ml 4-methylumbelliferyl-β-cellobioside (MUC). After 4 to 10 h, plates were illuminated to 302 nm of UV light on a UV-trans-illuminator and the active colonies were identified by the appearance of violet fluorescence (Reinhold-Hurek et al., 1993).

2.2.5.3.3 Glucosidase activity

For evaluation of glucosidase activity, bacterial cultures were grown and assayed as described for the test of cellobiohydrolase activity with slight change. For glucosidase activity, 4-methyllumbelliferyl-β-D glucoside (MUG) was used instead of 4- methyllumbelliferyl-β-cellobioside (MUC) in the overlay (Reinhold-Hurek et al., 1993).

2.2.5.3.4 Test for pectinolytic and chitinolytic activity

For pectinase test, exponentially grown cultures were spot-inoculated on NA plates containing 0.5% pectin and incubated for 72 h at 30 °C. Colonies appearing on plates were overlaid with 2% CTAB (N-cetyl-N,N,N trimethyl-ammonium bromide) and kept for 30 min at 30 °C. This was followed by washing three times with 1 N NaOH for 10 min each. Plates were then screened for the appearance of clear zones around the colonies. For chitinase test, selected isolates were streaked on colloidal chitin agar plates containing (g/l): chitin 5.0; yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_2O$, 0.3; KH_2PO_4, 1.36; agar 20, pH 7.2 at 30 °C for 72 h. Isolates showing clear zone around the streaked colony on the chitin agar plate were considered as positive for chitinase activity. Colloidal chitin was prepared by slowly adding twenty grams of chitin powder (RM1356-500G, Hi-media, India) into 350 ml of concentrated HCl and left at 4 °C overnight with vigorous stirring. The mixture was added to 2 litres of ice-cold 95% ethanol with rapid stirring and kept overnight at -20 °C. The precipitate was collected by centrifugation at 5,000 g for 20 min at 4 °C. The precipitate was washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0).

2.2.5.3.5 Test for lipase and amylase activity

For determination of lipase activity, bacterial isolates were streaked on Luria-Bertani agar (LB-agar) plates supplemented with 0.5% tributyrin plate and incubated at 30 °C in an incubator shaker. Presence of halo zone around the streaked colony was considered as lipase production. For amylase assay, isolates were spot-inoculated on the 1% starch-agar plates. After 2 days of

incubation at 28±2 °C, colonies surrounded by clear halo upon addition of iodine solution were considered positive for amylase production.

2.2.6 Test of motility

Considering that motility is required for colonization, different types of motility of the isolates were tested using standard protocol (Connelly et al., 2004).

(i) Swimming: Tryptone swim plates (1% tryptone, 0.5% NaCl, 0.3% agar) were inoculated with a sterile toothpick and incubated for 16 h at 25 °C. Motility was then assessed qualitatively by examining the circular turbid zone formed by the bacterial cells migrating away from the point of inoculation.

(ii) Swarming: For the test of swarming motility, the bacterial isolates were spot inoculated on swarm plates and grown at 30 °C for 24 h. The swarm plates were prepared by adding 0.5% Bacto-agar, 8 g of powdered NB medium (both from Difco, USA), and 5 g dextrose per liter. After autoclaving, media was poured in Petridish and dried overnight at room temperature before use.

(iii) Twitching: Cells were stab inoculated with a toothpick through a thin (approximately 3 mm) LB agar layer (1% agar) to the bottom of the Petri-dish. After incubation for 24 to 48 h at 30 °C, the isolates were observed for the appearance of the hazy zone of growth at the interface between the agar and the polystyrene surface.

2.2.7Amplification of 16S ribosomal RNA gene

To identify different bacterial isolates, amplification and sequence analysis of 16S rRNA gene were conducted. Total genomic DNA of bacterial isolate was extracted using genomic DNA extraction kit (Quiagen, USA). 16S rRNA gene of various isolates was amplified by polymerase chain reaction (PCR) using universal primer 27F1 (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1494Rc (5′-TACGGCTACCTTGTTACGAC-3′) where M designates A or C bases. PCR reaction was carried out in a 25 µl reaction mixture containing 2.5 µl of 10 X buffer (with 15 mM $MgCl_2$), 10 pmol of each forward and reverse primers, 200 µM of each dNTP, 1.5 U of Taq DNA polymerase, and 50 ng of DNA template. Volume was maintained with nuclease free water (Genetix, India). DNA samples were amplified in a thermal cycler (Bio-Rad T100, USA). The PCR conditions were as follows: initial denaturation for 3 min at 94 °C, 30 cycles each consisting of denaturation for 1 min at 94°C, primer annealing for 1 min at 54 °C and extension

at 72 °C for 5 min, and a final elongation of 5 min at 72 °C. PCR amplified products were analyzed by electrophoresis on 1% agarose gel stained with 0.5 µg ml^{-1} ethidium bromide.

2.2.8 Sequencing and taxonomical analysis

Sequencing of purified PCR product was done at Xcelris Genomics Labs Ltd (Xcelris Ahmedabad) India. The sequenced nucleotides were compared against GenBank database using the NCBI-BLAST algorithm and deposited in the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST). Taxonomic affiliation was assigned using RDP database (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) at 98% threshold of 16S rRNA gene sequence.

2.2.9 Test for plant growth promoting activity

2.2.9.1 Phosphate solubilization

Phosphate solubilization assay of the isolates was done following the method of Mehta and Nautiyal (2001) on NBRIP (National Botanical Research Institute's Phosphate) medium containing insoluble tricalcium phosphate. The rreshly grown culture of the bacterial isolate was point-inoculated on media and kept at 28 °C for 4 days. Clear zone formed around the inoculated culture was considered as positive for phosphate solubilization activity. Solubilized Phosphate was quantified according to the method of Ames et al. (1966). For this, 1.5 ml of 3 days old culture was pelleted down by centrifuging at 10,000 g for 2 min. 3.5 ml of reagent C (mentioned below) was added to the supernatant of culture, mixed properly and incubated at room temperature for 1 h. Absorption of the resultant reaction mixture was measured at 660 nm against blank. A standard curve was prepared using varying concentrations (10-100 µg/ml) of K_2HPO_4. Following reagents were prepared for the quantification of phosphate: Reagent A: 10% Ascorbic acid (stored at 4 °C), Reagent B: 42% Ammonium molybdate in 1 N H_2SO_4, Reagent C: 1 (Reagent A):6(Reagent B).

2.2.9.2 Indole-3-acetic acid (IAA) production

An ability of the isolates to produce indole acetic acid was tested following the method of Gordon and Weber (1951). Cultures were grown in Nutrient broth (Himedia, India) containing 100µg/ml tryptophan for 72 h at 30 °C and kept on shaking at 180 rev/min. Un-inoculated media was used as a control. A 1.5 ml of stationary phase culture was taken in a sterilized eppendorf

and centrifuged at 10,000 g for 2 min. In glass test tubes, 1 ml supernatant of culture was added along with IAA reagent and kept for 30 min at room temperature. Development of cherry-red color was considered as a positive test of IAA production. For quantification of IAA, an absorbance of the resultant reaction mix was measured at 530 nm. Different concentration (10-100 µg/ml) of pure IAA (Sigma, USA) was used for constructing a standard curve. The composition of IAA reagent has been mentioned in Appendix I.

2.2.9.3 Test for gibberellic acid (GA) production

Estimation of gibberellic acid was done by spectrophotometric method following the protocol of Holbrook et al. (1961). Bacterial isolates were grown in 100 ml NB medium at 28 °C for 3 days. Thereafter, the culture was centrifuged at 8,000 g for 10 min. The pH of the supernatant was adjusted to 2.5 using 1 N HCl and it was extracted with equal volume of ethyl acetate in a separating funnel. The extract was transferred to another separating funnel and retreated with an equal volume of ethyl acetate 2 to 3 times to get the maximum amount of gibberellic acid. To 1.5 ml of extract 0.2 ml of potassium ferrocyanide was added and centrifuged at 1,500 g for 10 min. An equal volume of 30% HCl was added to the supernatant and incubated for 1h at room temperature. The absorbance of the mixture was measured at 254 nm in a UV-Visible spectrophotometer (Jasco Corporation, Japan). The amount of gibberellic acid was calculated from the standard curve (10-100 µg ml^{-1}).

2.2.9.4 Test for siderophore production

Siderophore production of the isolates was assayed by chrome azurole S (CAS)-agar method described by Schwyn and Neiland (1987). The test organisms were spot inoculated on the chrome azurole agar plates and incubated at 30 °C for 5-6 days. Development of yellow-orange halo zone around the colony was considered as positive for siderophore production. For measuring the efficiency of siderophore production, the siderophore index was calculated according to the formula: $(D_s-D_c)/D_s$, where D_s and D_c refer to diameter (in mm) of colony and diameter of clear zone (in mm) respectively. The composition of chrome azurole S has been given in Appendix II.

2.2.9.5 Assay for ammonia production

Freshly grown culture of the bacterial isolates were inoculated into 10 ml peptone water in each tube and incubated for 48 h at 37 °C. After the bacterial growth, Nessler's reagent (0.5 ml) was added to each tube. Development of brown to yellow color was observed as positive test for ammonia production (Cappuchno and Sherman, 1992).

2.2.9.6 Preliminary screening for nitrogen fixation

A preliminary test for nitrogen fixation was based on an ability of the isolates to grow on growth medium lacking any organic or inorganic form of combined nitrogen. Screening for nitrogen fixation was carried out by streaking on the $JNFb^-$ medium (Composition: per liter $CaCO_3$ 1.0 g, K_2HPO_4 1.0 g, $MgSO_4.7H_2O$ 0.2 g, $FeSO_4.7H_2O$ 0.1 g, $Na_2MoO_4.2H_2O$ 0.005 g, Sucrose 5 g, pH 5.5) (Tapia-Hernández et al., 2000). Plates were incubated at 30 °C for 48-72 h and observed for the visible appearance of bacterial growth.

2.2.10 Test of antagonistic activity

Antagonistic activity of the isolates was determined by using agar well diffusion method against important plant pathogenic fungal species namely *Aspergillus flavus, Fusarium oxysporum*, *F. moniliforme,* and *F. graminearum.* Antagonistic activity against certain bacterial pathogens such as *Bacillus cereus, Erwinia carotovora, Escherichia coli*, and *Staphylococcus aureus* was also determined. Briefly, freshly grown cultures of selected fungal and bacterial species were spread on tryptic soy broth and potato dextrose agar plates, respectively. After adsorption, well size of 6 mm was made by metallic borer and filled with 1×10^8 CFU/ml of a freshly grown culture of bacterial isolates. The plates were incubated for 7 days at 28 °C for fungal species and 24 h at 37 °C for bacteria. Boiled culture of microbial species was used as a control. Antagonistic activity was determined by measuring zone of inhibition for which parameter used was <10 mm = poor (+), between 10 to 20 mm =good (++).

2.2.11 Abiotic stress tolerance test

Since, the selected bacterial isolates were to be used to test the effect on ameliorating salt and temperature stress, we tested the tolerance of different isolates to various level of salt and temperature stresses. For salt stress, 20 µl of overnight grown culture was inoculated into DF medium supplemented with different concentrations of NaCl (0.5% to 10%) and grown at 30 °C

for overnight. For the test of temperature tolerance, the isolates were grown in DF medium at different temperatures (25 °C to 50 °C). Cultures were grown for overnight and growth of cultures was determined by measuring absorbance at 600 nm in a spectrophotometer (Jasco Corporation, Japan) using un-inoculated broth as a blank. Cultures were incubated in triplicate sets.

2.3 Results

2.3.1 Isolation of ACC deaminase producing bacteria

ACC deaminase bacteria were enriched on selective media containing ACC as a sole nitrogen source. The appearance of bacterial colonies on selective agar media indicated growth of bacteria possessing ACC deaminase. Among all the selected plants, highest population of ACC deamianse bacteria was obtained from *Calligonum polygonoides* followed by *Ziziphus nummularia* and *Sorghum bicolor* (Table 2.2). The obtained population of ACC morphotypes was compared with total bacterial population obtained from rhizospheric soil of deferent plants. The geographical location and site of the collection have been summarized in Table 2.2. Based on the efficiency to utilize ACC as nitrogen source and difference in morphological features, a total of 25 bacterial isolates were selected for the test of plant growth promoting properties and basic biochemical and molecular characterization. All the isolates were further subjected to a quantitative assay for measuring the level of ACC deaminase activity. The ACC deaminase activity of different isolates ranged between 0.115 and 0.3962 µmol/mg protein/h. Highest ACC deaminase activity was observed in SBP-8 followed by SBP-9 and SBP-7 (0.3695 and 0.321 µmoles/mg protein/h) respectively (Table 2.3).

Table 2.2 Number of ACC positive bacterial isolates recovered from various plant species

Plants (local name)	**Total Bacterial population (CFU)**	**ACC positive bacterial population (CFU)**	**No. of ACC positive morphotypes**
Jowar	2.8×10^7	1.4×10^2	5
Jharber	1.8×10^4	2.1×10^2	5
Kair	3.4×10^4	1.5×10^2	2
Bajra root	1.6×10^6	1.3×10^2	1
Green grass (Yellow Indian grass)	2.4×10^4	1.0×10^2	2
Loonia root	1.4×10^3	1.2×10^2	1
Salt lake	0.7×10^3	1.0×10^2	3
Phog root	3.2×10^3	1.0×10^3	6

Table 2.3 ACC deaminase activity of all recovered isolates

Isolate	ACC deaminase activity (µmol/mg protein/h)
ZNP-1	0.172±0.011
ZNP-2	0.160±0.014
ZNP-3	0.209±0.003
ZNP-4	0.188±0.007
ZNP-5	0.135±0.010
SBP-6	0.284±0.008
SBP-7	0.321±0.011
SBP-8	0.396±0.021
SBP-9	0.362±0.004
SBP-10	0.154 ±0.092
SL-11	0.184±0.049
SL-12	0.376±0.021
CDP-13	0.172±0.007
CDP-14	0.208±0.004
A J S-15	0.192±0.016
HSW-16	0.267±0.019
SNP-17	0.229±0.016
SNP-18	0.157±0.002
PGP-19	0.115±0.014
CPC-20	0.179±0.002
CPC-21	0.134 ±0.006
CPC-22	0.182±0.006
CPC-23	0.177±0.005
CPC-24	0.165±0.002
CPC-25	0.191±0.003

±; standard deviation;

2.3.2 Biochemical characterization

All the isolates were screened for various biochemical characteristic features. One isolate was found to be gram positive, whereas twenty-four were gram negative. Results of basic biochemical tests are given in Table 2.4 which indicates that most of the isolates were catalase positive, except the SBP-9, HSW-16, SNP-17, CPC-23, CPC-24, and CPC-25. With respect to IMViC test, ZNP-1 and SBP-7 showed positive for indole, whereas others showed negative for indole and methyl-red test. Most of the isolates found to be positive for Voges-Proskauer test except the SBP-9, SBP-10, SNP-17, SNP-18, PGP-19, CPC-20, CPC-21, CPC-22, CPC-23, CPC-24, and CPC-25. Isolates SBP-7, SBP-9, SBP-10, and AJS-15 were found to be positive for urea production. Nitrate reductase test was used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase.Test of nitrate reductase was postive for the majority of the isolates except for SBP-6, SNP-17, PGP-19, CPC-22, CPC-23, and CPC-25. Among the antibiotic sensitivity test, most of the isolates were found to be sensitive for chloramphenicol and resistant to other tested antibiotics like tetracycline, kanamycin, gentamycin and vancomycin (Table 2.5).

Table 2.4 Biochemical characteristics of various ACC deaminase bacteria

Isolate	Gram staining	catalase	Indole	MR	VP	Urease	Nitrate reductase
ZNP-1	-	+	+	-	+	-	+
ZNP-2	-	+	-	-	+	-	+
ZNP-3	-	+	-	-	+	-	+
ZNP-4	-	+	-	-	+	-	+
ZNP-5	-	+	-	-	+	-	++
SBP-6	-	+	-	-	+	-	-
SBP-7	-	+	+	-	+	++	+
SBP-8	-	+	-	-	+	-	+
SBP-9	-	-	-	-	-	+	+
SBP-10	-	+	-	-	-	+	+
SL-11	-	+	-	-	+	-	++
SL-12	-	+	-	-	+	-	++
CDP-13	-	+	-	-	+	-	+
CDP-14	-	+	-	-	+	-	+
AJS-15	-	+	-	-	+	+	++
HSW-16	+	-	-	-	+	-	+
SNP-17	-	-	-	-	-	-	-
SNP-18	-	+	-	-	-	-	+
PGP-19	-	+	-	-	-	-	-
CPC-20	-	+	-	-	-	-	+
CPC-21	-	+	-	-	-	-	+
CPC-22	-	+	-	-	-	-	-
CPC-23	-	-	-	-	-	-	-
CPC-24	-	-	-	-	-	-	+
CPC-25	-	-	-	-	-	-	-

MR; methyl red, VP; voges-proskauer, -; negative, +; positive

Table 2.5 Antibiotic sensitivity test of ACC utilizing bacterial isolates

Isolates	Chloramphenicol	Tetracyclin	Kanamycin	Gentamycin	Vancomycin
ZNP-1	+	++	++	++	+++
ZNP-2	+	++	++	++	+++
ZNP-3	+	++	++	++	+++
ZNP-4	+	++	++	++	+++
ZNP-5	+	++	++	++	+++
SBP-6	+	++	++	++	+++
SBP-7	+	++	+++	+++	+++
SBP-8	+	++	+++	+++	+++
SBP-9	+	++	++	++	++
SBP-10	+	++	++	++	++
SL-11	+	++	++	++	+++
SL-12	+	++	++	++	+++
CDP-13	+	+	++	+	+++
CDP-14	+	+	++	+	+++
A J S-15	+	++	+++	++	+++
HSW-16	++	+	++	+	++
SNP 17	+	++	++	++	+++
SNP 18	+	++	++	++	+++
PGP 19	+	++	++	++	+++
CPC-20	+	++	++	++	+++
CPC-21	+	++	++	++	+++
CPC-22	++	++	++	+	+++
CPC-23	+	+++	++	+	+++
CPC-24	+	++	++	+	+++
CPC-25	+	++	++	++	+++

+; sensitive, ++; moderate resistant, +++; resistant

Bacteria can also be characterized based on their ability to utilize different carbon sources. therefore, the recovered isolates were subjected to carbohydrate utilization test for evaluating their efficacy to utilize various carbon sources. It is evident from the Table 2.6 that all the isolates were found to utilize maltose, glycerol, dextrose, sucrose, mannose, sorbose and fructose. The tested isolate except SL-11 and SL-11 were found to utilize the xylose. Negative result was observed for galactose in SBP-6, SL-11, for raffinose in ZNP-2,4, for trehalose in CPC-21,23,25, for melibiose in SL-11, SL-12, CPC-23, for L-arabinose in ZNP-2, SL-11, CPC-21, CPC-23,CPC-25 etc.

Table 2.6 Carbohydrate utilization efficacy of ACC utilizing bacterial isolates

Isolate	Xylose	Maltose	Fructose	Dextrose	Galactose	Raffinose	Trehalose	Melibiose	Sucrose	L-Arabinose	Mannose	Sorbose
ZNP-1	+	+	+	+	+	+	+	+	+	+	+	+
ZNP-2	+	+	+	+	+	-	+	+	+	-	+	+
ZNP-3	+	+	+	+	+	+	+	+	+	+	+	+
ZNP-4	+	+	+	+	+	-	+	-	+	+	+	+
ZNP-5	+	+	+	+	+	+	+	+	+	+	+	+
SBP-6	+	+	+	+	-	-	+	+	+	+	+	+
SBP-7	+	+	+	+	+	+	+	+	+	+	+	+
SBP-8	+	+	+	+	+	+	+	+	+	+	+	+
SBP-9	+	+	+	+	+	+	+	+	+	+	+	+
SBP-10	+	+	+	+	+	+	+	+	+	+	+	+
SL-11	-	+	+	+	-	+	+	-	+	-	+	+
SL-12	-	+	+	+	+	+	+	-	+	+	+	+
CDP-13	+	+	+	+	+	-	+	+	+	+	+	+
CDP-14	+	+	+	+	+	+	+	+	+	+	+	+
AJS-15	+	+	+	+	+	+	+	+	+	+	+	+
HSW-16	+	+	+	+	+	+	+	+	+	+	+	+
SNP-17	+	+	+	+	+	+	+	+	+	+	+	+
SNP-18	+	+	+	+	+	+	+	+	+	+	+	+
PGP-19	+	+	+	+	+	+	+	+	+	+	+	+
CPC-20	+	+	+	+	+	+	+	+	+	+	+	+
CPC-21	+	+	+	+	+	+	-	+	+	-	-	+
CPC-22	+	+	+	+	+	+	+	+	+	+	+	+
CPC-23	+	+	+	+	+	+	-	-	+	-	+	+
CPC-24	+	+	+	+	+	+	+	+	+	+	+	+
CPC-25	+	+	+	+	+	+	-	+	+	-	+	+

+; positive, -; negative

Isolate	Inulin	Sodium gluconate	Glycerol	Salicin	Dulcitol	Inositol	Sorbitol	Mannitol	Adonitol	Arabitol	Erythritol	Xylitol
ZNP-1	-	-	+	-	-	+	+	+	-	-	-	-
ZNP-2	-	-	+	-	-	+	+	+	-	-	-	-
ZNP-3	-	-	+	-	-	+	+	+	-	-	-	+
ZNP-4	+	+	+	+	+	+	+	+	+	+	+	+
ZNP-5	+	+	+	-	-	+	+	+	-	-	+	-
SBP-6	+	-	+	+	-	-	+	+	+	-	-	-
SBP-7	+	-	+	+	+	+	+	+	+	+	-	-
SBP-8	+	-	+	+	-	-	+	+	+	-	-	-
SBP-9	-	-	+	-	-	-	+	+	-	-	-	-
SBP-10	+	+	+	+	+	+	+	+	+	+	+	-
SL-11	-	-	+	+	-	+	+	-	-	+	+	-
SL-12	-	-	+	+	+	+	+	+	-	+	-	-
CDP-13	-	-	+	+	-	+	+	+	+	-	-	-
CDP-14	+	+	+	+	+	+	+	+	+	-	+	-
AJS-15	+	-	+	+	-	+	+	+	+	+	+	+
HSW-16	-	-	+	+	+	+	+	+	-	-	-	-
SNP-17	+	+	+	+	+	+	+	+	+	+	+	+
SNP-18	-	+	+	+	+	+	+	+	+	+	+	+
PGP-19	+	-	+	+	-	+	+	+	+	+	+	+
CPC-20	+	+	+	+	+	+	+	+	+	-	-	+
CPC-21	-	-	+	+	-	+	+	+	-	+	+	+
CPC-22	+	+	+	+	+	+	+	-	-	-	-	+
CPC-23	+	+	+	+	+	+	+	+	-	+	-	+
CPC-24	-	+	+	+	-	+	+	-	+	+	+	+
CPC-25	-	-	+	+	+	+	-	+	+	-	-	+

+; positive, -; negative

Isolate	α-Methyl-D-glucoside	Rhamnose	Cellobiose	Melezitose	α-Methyl-D-mannoside	ONPG	Esculin hydrolysis	D-Arabinose	Citrate	Malonate	Lactose
ZNP-1	-	+	+	-	+	+	-	+	-	-	-
ZNP-2	+	-	-	-	+	+	-	+	+	+	-
ZNP-3	+	-	+	-	+	+	+	+	-	+	+
ZNP-4	+	+	+	+	+	-	-	-	+	+	+
ZNP-5	+	-	+	-	+	+	+	+	-	-	+
SBP-6	+	+	+	-	+	+	+	-	-	-	+
SBP-7	-	+	+	-	+	+	+	+	+	+	+
SBP-8	-	+	+	+	+	+	+	+	+	-	+
SBP-9	-	+	+	-	+	+	+	+	-	-	+
SBP-10	+	+	+	+	+	+	+	+	+	+	+
SL-11	-	+	+	-	+	+	+	-	-	-	-
SL-12	+	+	+	+	+	+	+	-	-	-	-
CDP-13	+	+	+	+	+	+	+	+	+	+	+
CDP-14	-	+	+	+	-	+	+	+	-	-	+
AJS-15	+	+	+	+	-	+	+	+	-	-	+
HSW-16	-	+	+	-	+	-	+	+	+	-	-
SNP-17	-	+	+	+	+	+	+	+	+	+	+
SNP-18	+	+	+	+	-	+	+	+	+	+	+
PGP-19	-	+	+	+	+	+	+	+	-	+	+
CPC-20	+	+	-	+	+	-	+	+	+	+	+
CPC-21	+	+	+	+	-	+	+	+	+	-	+
CPC-22	-	+	-	+	+	-	+	+	+	-	-
CPC-23	+	+	+	+	+	-	+	+	+	+	+
CPC-24	+	+	+	+	-	+	+	+	+	+	-
CPC-25	-	-	+	+	-	+	+	+	+	-	+

+; positive, -; negative

The presence of cellulolytic and pectinolytic activities in PGPR are considered to be crucial for colonization particularly with reference to endophytic bacteria. Out of twenty five isolates, most of the isolates showed the presence of at least one of the tested hydrolytic enzymes. None of the isolate showed all cellulolytic and pectinolytic activities. Isolate ZNP-4, ZNP-5, SBP-10, CDP-14, AJS-15, HSW-16, CPC-23, and CPC-25 did not show the presence of any cellulolytic enzymes. Pectinase helps in loosening of the cellulosic cell wall of plant cell and, therefore, it helps bacteria to pave the way for endophytic colonization in plants. Out of 25 isolates, ten namely ZNP-3, ZNP-5, SBP-8, SBP-9, SL-12, CDP-14, SNP-17, SNP-18, CPC-20, CPC-24, and CPC-25 were found to be pectinase positive. Another hydrolytic enzyme chitinase which is responsible for protection against fungal infections, was found to be present in all isolates except ZNP-2, ZNP-5, SBP-7, CDP-13, CDP-14, SNP-18, CPC-20 and CPC-23. Amylase test was shown by the isolate CDP-13, and CDP-14 only whereas lipolytic activity was observed in isolate ZNP-1, ZNP-2, ZNP-3, SBP-7, SBP-8, SBP-9, CDP-13, CDP-14, AJS-15, HSW-16 and CPC-24.

Table 2.7 Test of hydrolytic enzymatic assay by all twenty five ACC utilizing bacteria

Isolates	Cellulase	Cellobio-hydrolase	β-glucosidase	Pectinase	Chitinase	Amylase	Lipase
ZNP-1	+	+	++	--	--	-	+
ZNP-2	++	++	--	--	+	-	+
ZNP-3	+	--	--	+	--	-	+
ZNP-4	--	++	--	--	--	-	-
ZNP-5	--	--	+	++	+	-	-
SBP-6	++	+	++	--	--	-	-
SBP-7	+	+	+	--	+	-	+
SBP-8	++	++	+	++	++	-	+
SBP-9	+	--	+	++	--	-	+
SBP-10	--	++	+	--	--	-	-
SL-11	+	--	--	--	_	-	-
SL-12	+	++	--	+	--	-	-
CDP-13	++	--	--	--	++	+	++
CDP-14	--	++	--	++	++	+	++
AJS-15	--	++	++	--	+	-	+
HSW-16	--	+	--	--	--	-	++
SNP 17	+	+	--	+	--	-	-
SNP 18	++	--	++	++	++	-	-
PGP 19	--	--	++	--	--	-	-
CPC-20	+	--	--	+	+	-	-
CPC-21	+	--	--	-	--	-	-
CPC-22	++	--	++	--	--	-	-
CPC-23	--	++	--	--	+	-	-
CPC-24	++	++	++	+	--	-	+
CPC-25	--	++	--	+	--	-	-

--; negative activity, +,++; positive test

2.3.3 Test of motility

Bacterial motility is one of the most important properties required by a microorganism for successful colonization on or inside the host plant. Therefore, all 25 isolates were screened for three different types of motility behaviors namely swimming, swarming and twitching. Most of the isolates showed all three types of motility. In the swimming test, inoculated bacterial cells formed the circular turbid zone, in swarming it was spreaded on the media plate, whereas in the case of twitching the bacterial inoculum was found to be attached to the media containing plastic plate surfaces. The isolate CPC-21 showed only swimming, whereas CPC-20, CPC-23, and CPC-24 lack the swarming motility. The motility images of few isolates ZNP-1, ZNP-3, SL-11, SL-12 and HSW-16 has been shown in Fig. 2.2.

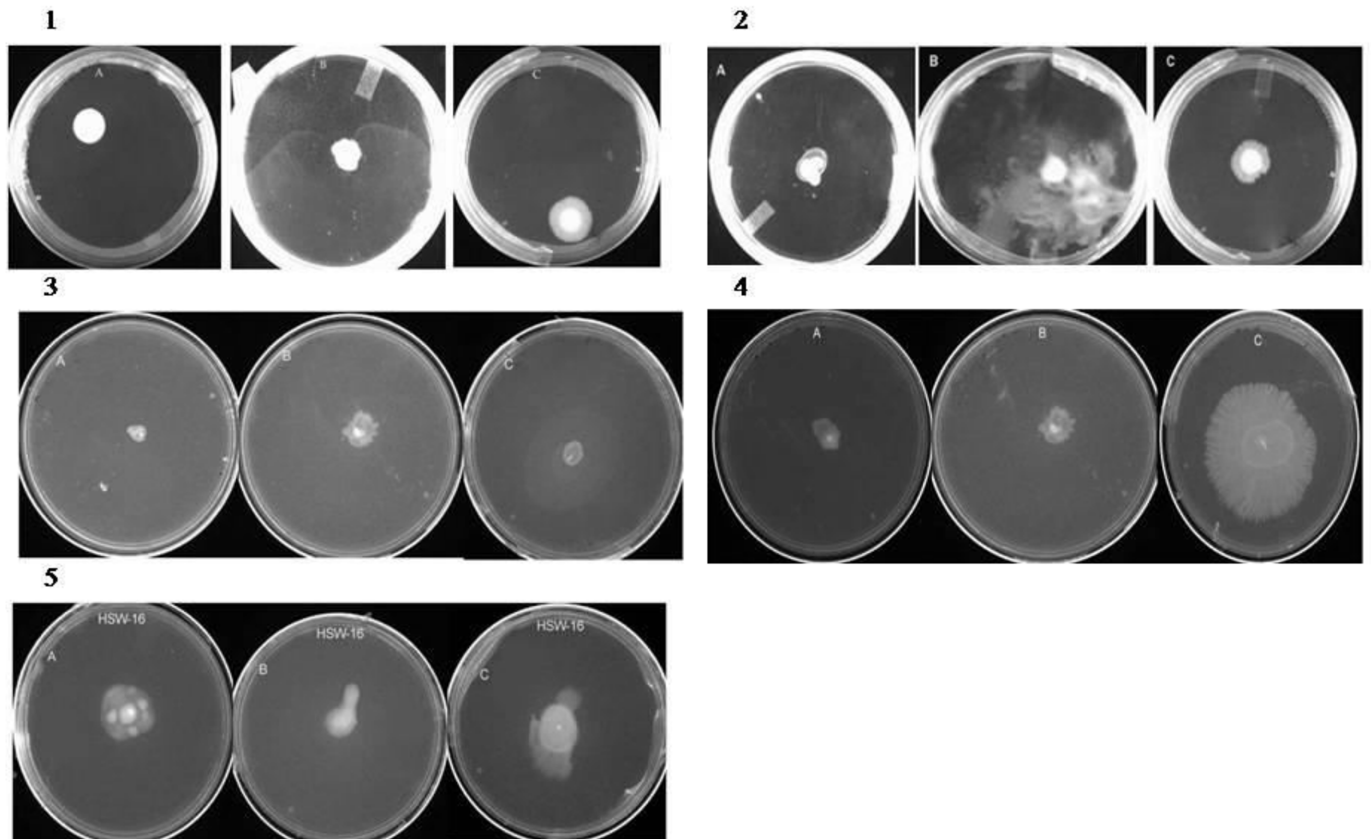


Fig. 2.2. Test of motility shown by the isolates ZNP-1 (1), ZNP-3 (2), SL-11 (3), SL-12 (4) and HSW-16. Panel A, B, C represent the swimming, swarming and twitching motility respectively.

2.3.4 Molecular identification of bacterial isolates and phylogenetic analysis

All the selected isolates were identified by analysis of the partial sequence of 16S rRNA gene. A 1.5 kb of 16S rRNA gene of each isolate was amplified by PCR and sequenced for molecular identification. The nucleotide sequence was analyzed by comparing with 16S rRNA genes available at GenBank database of National Centre for Biological Information (NCBI) using BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST) to find the closest match to type strain. Based on the sequence similarity, the test isolates were identified as the member of different genera including *Enterobacter, Serratia, Bacillus, Phylobacterium, Pantoea, Mesorhizobium, and Klebsiella.* The obtained sequence of isolates was submitted to Genbank database for the accession number. The various bacterial species isolated in the present study with their accession number are listed in Table 2.8. The majority of the bacterial species belong to phylum Proteobacteria, whereas *Bacillus* sp. belongs to Firmicutes.

Table 2.8 The closest affiliations of the representative isolate in the GenBank according to the 16S rRNA gene sequences

S. No.	Code	Bacterial species	Closest match	Sequence coverage	% Identity	Accession No.
1.	ZNP-1	*Enterobacter cloacae*	*E. cloacae* 113CL	100	100	KJ950702
2.	ZNP-2	*Enterobacter cloacae*	*E. cloacae* ST15	100	100	KJ950703
3.	ZNP-3	*Enterobacter cloacae*	*E.cloacae* PW113	100	100	KJ950704
4.	ZNP-4	*Enterobacter cloacae*	*E. cloacae* PM93	100	100	KJ950705
5.	ZNP-5	*Pantoea* sp.	*Pantoea* sp. F-92-1	97	96	KJ950706
6.	SBP-6	*Enterobacter* sp.	*Enterobacter* sp. PCX1	100	100	KJ950707
7.	SBP7	*Serratia marcescens*	*S. marcescens* A-CS1B	100	100	KJ950708
8.	SBP-8	*Klebsiella* sp.	*K. pneumoniae* HKG219	99	96	KJ950709
9.	SBP-9	*Stenotrophomonas maltophilia*	*S. maltophilia* T 25	100	99	KJ950710
10.	SBP-10	*Klebsiella pneumoniae*	*K. pneumoniae* ATCC 700603	100	100	KJ950711
11.	SL-11	*Serratia marcescens*	*S. marcescens* BHWSF4	100	100	KJ950712
12.	SL-12	*Serratia marcescens*	*S. marcescens* IHB B 7064	100	99	KJ950713
13.	CDP13	*Serratia marcescens*	*S. marcescens* U36365	100	100	KJ950714
14.	CDP-14	*Enterobacter ludwigii*	*E. ludwigii* TLAB1	100	99	KJ950715
15.	AJS15	*Enterobacter cloacae*	*E. cloacae* F21	100	98	KJ950716
16.	HSW-16	*Bacillus licheniformis*	*B. licheniformis* 08BF01CA	100	100	KJ950717
17.	SNP-17	*Mesorhizobium opportunistum*	*M. opportunistum* M809	100	99	KJ950718
18.	SNP-18	*Citrobacter freundii*	*C. freundii* LS-013	100	99	KJ950719
19.	PGP-19	*Enterobacter ludwigii*	*E. ludwigii* 144CL	100	99	KJ950720
20.	CPC-20	*Enterobacter cancerogenus*	*E. cancerogenus* PSM1	100	99	KJ950721
21.	CPC-21	*Enterobacter cloacae*	*E. cloacae* IITRCS11	100	100	KJ950722
22.	CPC-22	*Stenotrophomonas maltophilia*	*S. maltophilia* NA108	100	100	KJ950723
23.	CPC-23	*Stenotrophomonas maltophilia*	*S. maltophilia* PS14	100	100	KJ950724
24.	CPC-24	*Phyllobacterium myrsinacearum*	*P. myrsinacearum* PP R-6	100	100	KJ950725
25.	CPC-25	*Phyllobacterium myrsinacearum*	*P. myrsinacearum* STM-402	100	100	KJ950726

2.3.5 Test of plant growth promotion (PGP) activities

Isolates were screened qualitatively and/or quantitatively for their various PGP features. The results of PGP properties have been summarized in Table 2.9. All twenty five isolates were found positive for phosphate solubilization and indole acetic acid production, while only nine isolates showed positive for siderophore production. Based on the quantitative estimation, highest phosphate solubilization was observed in SBP-8 (13.71±1.15 µg/ml), followed by SL-11 (13.44±6.25), and ZNP-4 (13.35±3.05). Similarly, highest production of indole acetic acid was recorded in SBP-9 (3.158±0.12) followed by SL-11 (0.655±0.065) and SL-12 (0.599±0.073). Siderophore production was exhibited by the isolates SBP-7, SBP-8, SL-11, SL-12, CDP-13, CDP-14, SNP-17, CPC-20 and CPC-21 (Table 2.9).Test of nitrogen fixation was qualitatively evaluated by their growth on $JNFb^-$. Most of the isolates showed luxuriant growth on the $JNFb^-$, thus indicated positive for nitrogen fixation. For the test of ammonia production, it showed positive results for all the isolates except SNP-18, PGP-19, CPC-20, and CPC-25.

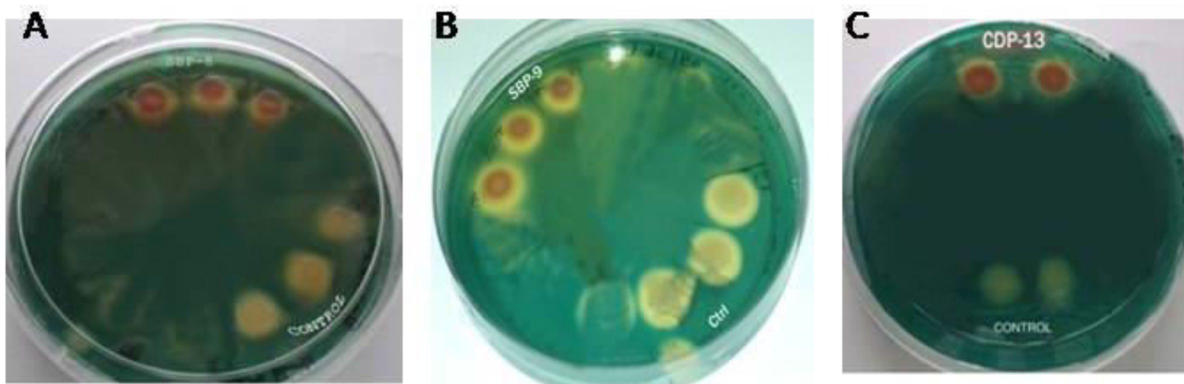


Fig. 2.3. Test of siderophore production by isolate, Panel (A) SBP-8, (B) SBP-9, and (C) CDP-13.

Table 2.9 Plant growth promoting features of bacterial isolates

Isolate	IAA production (µg/ml)	Phosphate solubilization (µg/ml)	Gibberellic acid production	Siderophore index	NH_3 production
ZNP-1	0.545±0.038	11.73±1.88	-	-	+
ZNP-2	0.259±0.005	9.84±1.01	-	-	+
ZNP-3	0.362±0.022	12.41±0.45	-	-	+
ZNP-4	0.364±0.0234	13.35±3.05	-	-	+++
ZNP-5	0.270±0.036	11.25±2.75	-	-	+++
SBP-6	0.403±0.025	09.50±0.80	+	-	++
SBP-7	0.491±0.026	13.18±1.98	-	+	++
SBP-8	0.410±0.083	13.71±1.15	+	+	++
SBP-9	3.158±0.125	10.43±2.34	-	-	++
SBP-10	0.385±0.029	10.70±1.23	-	-	++
SL-11	0.655±0.065	13.44±2.25	+	+	+++
SL-12	0.599±0.073	13.18±1.92	+	+	++
CDP-13	0.336±0.020	13.22±0.12	-	+	+++
CDP-14	0.441±0.020	10.38±1.72	-	+	+++
A J S-15	0.531±0.050	8.61±2.15	-	-	+++
HSW-16	2.894±0.025	11.04±2.44	+	-	++
SNP-17	0.365±0.034	13.11± 1.12	-	+	+
SNP-18	0.214±0.023	7.44± 0.850	-	-	-
PGP-19	0.214±0.031	9.11 ± 1.14	-	-	-
CPC-20	0.218±0.021	12.24 ± 0.97	-	+	-
CPC-21	0.326±0.035	10.74± 0.54	-	+	+
CPC-22	0.312±0.021	12.11± 1.11	-		+
CPC-23	0.367±0.024	13.12± 1.21	-	-	+
CPC-24	0.287±0.032	11.04±1.11	-	-	+
CPC-25	0.365±0.024	10.51±1.03	-	-	_

±; standard deviation, -; negative, +; positive

2.3.6 Antagonistic test

Apart from plant growth promoting tests, the recovered isolates were also screened for antagonistic behavior against the selected bacterial and fungal pathogens. It is evident from the table 2.10 that most of the isolates except ZNP-3, SBP-7, SBP-9, SBP-10, SL-12, HSW-16, SNP 18, PGP 19, CPC-23, and CPC-25 showed antagonistic activity to *Fusarium oxysporum*. Antagonistic behaviour against the *Fusarium moniliforme* was observed by the isolate ZNP-4, ZNP-5, SBP-6, SBP-7, SBP-9, SL-11, SL-12, CDP-14, AJS-15, HSW-16, SNP 18, PGP 19, CPC-21, CPC-22, CPC-23, and CPC-25. Isolates ZNP-1, ZNP-2, SBP-7, SBP-10, SL-11, HSW-16, CPC-22, and CPC-23 were found to inhibiting the mycelial growth of *Fusarium graminearum* and *Aspergillus flavus* (Table 2.10).

Among the tested bacterial isolates, ZNP-1, ZNP-2, ZNP-4, ZNP-5, SBP-6, SBP-10, SL-11, CDP-13, CDP-14, AJS-15, SNP 17, CPC-20, CPC-21, CPC-22, and CPC-24 were effective against the *Bacillus cereus*. Likewise, ZNP-1, ZNP-2, SBP-6, SBP-7, SBP-8, SBP-9, SL-12, CDP-14, AJS-15, HSW-16, SNP 17, SNP 18, CPC-23, CPC-24, and CPC-25 showed antibacterial effect against *Erwinia carotovora.* Most of the isolates except ZNP-2, SL-11, CDP-13, CDP-14, SNP 17 and CPC-20 showed the antagonistic behaviour against *Escherichia coli*. The isolates ZNP-1, ZNP-4, SBP-6, SBP-10, HSW-16, PGP 19 and CPC-23 were ineffective against *Staphylococcus aureus* (Table 2.11).

Table 2.10 Antifungal activity of isolates against tested fungal species

Isolates	FO	FM	FG	AF
ZNP-1	++	--	+	++
ZNP-2	++	--	++	++
ZNP-3	--	--	--	+
ZNP-4	+	++	++	--
ZNP-5	++	++	--	++
SBP-6	+	++	++	--
SBP-7	--	++	++	+
SBP-8	++	--	++	--
SBP-9	--	++	--	++
SBP-10	--	--	+	++
SL-11	++	++	++	++
SL-12	--	++	--	+
CDP-13	+	--	++	--
CDP-14	++	++	--	--
AJS-15	+	++	--	--
HSW-16	--	++	++	++
SNP 17	++	--	++	--
SNP 18	--	++	--	++
PGP 19	--	++	--	+
CPC-20	+	--	++	--
CPC-21	++	++	--	++
CPC-22	+	++	++	+
CPC-23	--	++	++	++
CPC-24	++	--	++	--
CPC-25	--	++	--	++

FO; *Fusarium oxysporum*, FM; *Fusarium moniliforme*, FG; *Fusarium graminearum*, AF; *Aspergillus flavus*
++; good (13-18mm), +; poor (8-12mm), --; no inhibition

Table 2.11 Antibacterial activity of isolates against tested pathogenic bacterial isolates

Isolates	BC	Er C	EC	SA
ZNP-1	++	++	++	--
ZNP-2	++	++	--	++
ZNP-3	--	--	++	+
ZNP-4	++	--	+	--
ZNP-5	++	--	++	++
SBP-6	--	++	++	--
SBP-7	++	+	+	++
SBP-8	++	+	++	++
SBP-9	--	++	+	++
SBP-10	++	--	++	--
SL-11	++	--	--	+
SL-12	+	+	++	+
CDP-13	++	--	--	++
CDP-14	--	++	--	++
A J S-15	--	++	++	++
HSW-16	--	+	++	--
SNP 17	+	+	--	++
SNP 18	--	++	++	++
PGP 19	++	--	++	--
CPC-20	--	--	--	+
CPC-21	++	--	++	+
CPC-22	++	--	++	++
CPC-23	--	++	++	--
CPC-24	--	++	++	++
CPC-25	--	++	++	++

BC; *Bacillus cereus*, Er C; *Erwinia carotovora,* EC; *Escherichia coli*, SA; *Staphylococcus aureus*
++; good (13-18mm), +; poor (8-12mm), --; no inhibition

2.3.7 Abiotic stress tolerance test

The isolates were evaluated for their ability to tolerate abiotic stressors like different salt concentrations, and temperature stress. Criteria for testing the tolerance/sensitivity were a spectrophotometric measurement of bacterial growth after incubation under various levels of abiotic stressors. From Table 2.12 it can be seen that most of the isolates showed growth at a salt concentration between 0.5% to 10%, thereafter, no growth was observed. Bacterial isolates ZNP-1, ZNP-2, ZNP-3, ZNP-4, ZNP-5, SBP-6, SBP-7, SBP-8, SBP-9, SBP-10, AJS-15, HSW-16, SNP-17, CPC-21, CPC-22, CPC-23, CPC-24, CPC-25 showed growth even at 10% NaCl in broth, whereas SL-11, SL-12, CDP-13, CDP-14, SNP-18, PGP-19, CPC-20 showed reduced growth. For temperature stress, the optimal growth was found in the range of 25 °C to 35 °C for most of the isolates. Further increase in temperature showed reduced growth for most of the isolates.

Table 2.12 Test for growth of various ACC deaminase bacteria at various temperatures

Bacterial isolates	Optical density of bacterial growth at A_{600}					
	25 °C	**30 °C**	**35 °C**	**40 °C**	**45 °C**	**50 °C**
ZNP 1	1.954±0.11	2.069±0.14	1.253±0.13	0.907±0.11	0.676±0.03	0.247±0.01
ZNP 2	1.085±0.08	2.105±0.08	1.443±0.10	0.969±0.08	0.504±0.03	0.124±0.02
ZNP 3	1.772±0.10	2.279±0.12	1.561±0.11	1.063±0.13	0.415±0.02	0.145±0.03
ZNP 4	1.839±0.12	2.181±0.06	1.407±0.04	0.685±0.05	0.474±0.04	0.142±0.02
ZNP 5	2.039±0.11	2.211±0.09	1.554±0.13	1.009±0.10	0.784±0.04	0.112±0.02
SBP 6	2.080±0.13	2.380±0.16	1.744±0.12	1.423±0.11	0.695±0.03	0.230±0.01
SBP 7	2.114±0.12	2.315±0.19	1.613±0.15	1.490±0.10	0.815±0.02	0.109±0.01
SBP 8	2.097±0.14	2.426±0.17	1.010±0.06	0.943±0.13	0.645±0.03	0.412±0.01
SBP 9	1.845±0.10	2.003±0.16	1.212±0.10	0.876±0.11	0.660±0.03	0.321±0.01
SBP 10	2.057±0.10	2.213±0.13	1.234±0.08	1.040±0.13	0.482±0.01	0.119±0.01
SL 11	1.945±0.09	2.228±0.13	1.426±0.09	1.108±0.13	0.775±0.02	0.436±0.01
SL 12	2.106±0.09	2.256±0.11	1.055±0.10	0.993±0.04	0.740±0.02	0.320±0.01
CDP 13	2.060±0.13	2.191±0.11	0.902±0.11	0.681±0.03	0.428±0.01	0.026±0.00
CDP 14	1.830±0.12	2.123±0.10	1.055±0.12	0.843±0.07	0.286±0.01	0.153±0.01
AJS 15	2.046±0.11	2.208±0.14	1.418±0.13	1.063±0.07	0.593±0.02	0.110±0.00
HSW 16	0.932±0.10	2.591±0.18	1.176±0.12	0.996±0.05	0.643±0.03	0.412±0.02
SNP 17	2.040±0.10	2.210±0.16	1.680±0.12	0.850±0.03	0.410±0.02	0.240±0.00
SNP 18	1.990±0.11	1.680±0.10	1.280±0.11	0.980±0.05	0.650±0.03	0.310±0.00
PGP 19	1.450±0.11	1.970±0.13	1.450±0.10	0.810±0.03	0.440±0.02	0.210±0.00
CPC-20	1.250±0.12	1.734±0.14	1.016±0.05	0.545±0.03	0.482±0.01	0.190±0.00
CPC-21	1.390±0.13	1.840±0.15	1.320±0.07	0.970±0.04	0.540±0.01	0.340±0.01
CPC-22	1.210±0.09	1.965±0.13	1.540±0.06	1.110±0.04	0.760±0.03	0.540±0.02
CPC-23	1.390±0.09	1.760±0.16	1.210±0.09	0.870±0.05	0.640±0.01	0.420±0.01
CPC-24	1.452±0.08	1.840±0.15	0.981±0.08	0.750±0.06	0.540±0.01	0.210±0.01
CPC-25	1.440±0.12	1.642±0.11	1.122±0.10	1.010±0.10	0.770±0.02	0.420±0.02

±; standard deviation, values are means of three replicates

Table 2.13 Test for growth of various ACC deaminase bacteria at various salt concentrations

Bacterial isolates	Optical density of bacterial growth at A_{600}								
	0.5%	1%	2%	3%	4%	5%	7%	8%	10%
ZNP 1	1.846±0.11	1.680±0.10	1.661±0.09	1.564±0.12	1.350±0.17	1.144±0.10	0.880±0.05	0.736±0.04	0.210±0.01
ZNP 2	1.989±0.14	1.785±0.10	1.546±0.10	1.370±0.11	1.250±0.12	1.045±0.09	0.836±0.08	0.512±0.03	0.150±0.02
ZNP 3	1.857±0.12	1.714±0.18	1.510±0.12	1.420±0.19	1.230±0.10	1.143±0.10	0.939±0.06	0.725±0.03	0.310±0.03
ZNP 4	1.899±0.11	1.720±0.16	1.603±0.11	1.413±0.11	1.243±0.14	0.981±0.14	0.409±0.03	0.373±0.04	0.254±0.03
ZNP 5	1.541±0.14	1.371±0.10	1.141±0.10	0.927±0.08	0.871±0.14	0.772±0.14	0.613±0.04	0.545±0.04	0.245±0.02
SBP 6	2.235±0.18	2.091±0.13	1.805±0.17	1.743±0.17	1.691±0.15	1.494±0.12	1.182±0.11	0.712±0.05	0.397±0.01
SBP 7	2.130±0.14	2.084±0.18	1.862±0.15	1.694±0.11	1.256±0.14	1.146±0.09	0.989±0.14	0.565±0.04	0.321±0.02
SBP 8	2.110±0.11	2.058±0.14	1.852±0.16	1.533±0.13	1.420±0.11	1.300±0.12	1.296±0.14	0.946±0.05	0.426±0.02
SBP 9	1.785±0.16	1.976±0.13	1.548±0.11	1.441±0.12	1.344±0.18	1.069±0.09	0.966±0.11	0.723±0.04	0.445±0.01
SBP 10	1.828±0.15	1.930±0.10	1.836±0.14	1.718±0.13	1.416±0.10	1.034±0.10	0.920±0.07	0.650±0.03	0.245±0.01
SL 11	1.773±0.10	1.845±0.16	1.411±0.13	1.361±0.05	1.213±0.09	1.109±0.10	0.870±0.10	0.047±0.10	0.039±0.00
SL 12	1.884±0.16	1.539±0.16	1.381±0.13	1.227±0.12	1.110±0.10	0.957±0.06	0.747±0.10	0.092±0.10	0.044±0.00
CDP 13	1.689±0.17	1.309±0.18	0.976±0.10	0.764±0.05	0.429±0.04	1.139±0.12	0.166±0.02	0.128±0.00	0.096±0.00
CDP 14	1.561±0.14	1.240±0.12	1.126±0.10	0.946±0.08	0.739±0.05	0.493±0.02	0.129±0.00	0.061±0.00	0.021±0.00
AJS 15	1.613±0.14	1.712±0.11	1.392±0.12	1.276±0.11	1.156±0.08	0.940±0.03	0.828±0.03	0.730±0.04	0.340±0.01
HSW 16	1.976±0.11	2.069±0.11	2.187±0.12	1.949±0.16	1.803±0.09	1.578±0.11	1.312±0.10	0.970±0.02	0.533±0.02
SNP 17	1.541±0.11	1.310±0.13	1.220±0.13	1.010±0.09	0.965±0.04	0.840±0.03	0.721±0.04	0.690±0.01	0.450±0.02
SNP 18	1.710±0.12	1.540±0.12	1.245±0.13	1.115±0.09	0.980±0.05	0.750±0.01	0.550±0.01	0.150±0.01	0.050±0.00
PGP 19	1.880±0.14	1.620±0.10	1.446±0.13	1.350±0.10	1.150±0.10	0.980±0.04	0.336±0.02	0.112±0.01	0.040±0.00
CPC-20	2.110±0.14	1.864±0.10	1.630±0.14	1.432±0.16	1.115±0.08	0.932±0.03	0.540±0.03	0.110±0.01	0.090±0.00
CPC-21	1.670±0.16	1.530±0.13	1.340±0.12	1.260±0.10	1.070±0.09	0.995±0.04	0.710±0.03	0.310±0.02	0.242±0.03
CPC-22	1.850±0.16	1.642±0.13	1.550±0.18	1.420±0.12	1.330±0.14	0.976±0.07	0.786±0.02	0.610±0.04	0.452±0.01
CPC-23	1.980±0.13	1.940±0.16	1.845±0.14	1.615±0.16	1.475±0.13	1.240±0.10	1.120±0.10	0.910±0.04	0.510±0.02
CPC-24	1.875±0.13	1.750±0.16	1.680±0.14	1.415±0.13	1.215±0.11	0.950±0.05	0.740±0.02	0.616±0.02	0.480±0.04
CPC-25	1.650±0.18	1.540±0.18	1.410±0.10	1.320±0.10	1.290±0.10	1.080±0.09	0.930±0.03	0.712±0.03	0.550±0.01

±; standard deviation, values are means of three replicates

2.4 Discussion

Although, a large number of bacterial isolates containing ACC deaminase have been isolated by different laboratories worldwide and have shown statistically significant plant growth-promoting activity (Glick et al., 2007; Onofre-Lemus et al., 2009), in-depth characterization of several ACC deaminase bacteria and their application is still lacking. Therefore, the present study was undertaken to explore the potential of ACC deaminase containing bacteria from the rhizosphere of various plants growing in the desert region of Rajasthan, which can protect host plants from abiotic stressors. Isolation and testing of bacteria for PGPR activities revealed that a large number of them showed such activities. These bacteria showed positive PGPR traits *in-vitro* such as ACC deaminase activity, production of indole acetic acid (IAA) and siderophore, solubilization of inorganic phosphate, fixation of atmospheric nitrogen and HCN production etc. The isolates were also subjected to various biochemical tests, motility, antibiotic sensitivity, and carbohydrate utilization pattern. Growth behaviors under various stress condition like temperature and salt concentration were monitored to check for survivali and their further use as inocula for plant growth promotion test under stress conditions. Antagonistic behavior was tested to check the bio-control efficacy of the isolates. The result of the present study is a valuable addition to understanding properties of PGPR associated with plants growing in the stressed condition of deserts. To the best of our knowledge, very few studies have been carried out to characterize ACC deaminase containing bacteria associated with plants of Thar desert (Sharma et al., 2013). Isolation and characterization of these bacteria were based on the assumption that plants growing in the desert may recruit microorganisms which can help them tolerate deleterious effects of stressors with reference to salt and temperature in particular.

The catabolic activity of isolated PGPR was assessed by their ability to utilize and digest 36 different carbohydrates. This test is sometimes called fermentation test, as it is used for measuring the production of acids that result from the metabolization of a small carbohydrate. Most sugars other than glucose are said to undergo "fermentation" when they are hydrolyzed or converted into glucose or both with the glucose then being metabolized. This results in the production of acids that change the color of the medium. When the organism ferments carbohydrates, acidic organic by products (Lactic acid, formic acid or acetic acid) is accumulated which turns the medium into yellow color with a reduction in the pH (acidic). In our study, most

of the isolates were found to utilize the tested carbohydrates, however sodium gluconate and xylitol was less preferred by the isolates belonging to bacterial genera like *Enterobacter*, *Serratia* and *Klebsiella*. A detailed study relevant to non-utilization of these carbon sources by such bacteria is still lacking. Very few studies have been done regarding the utilization of various carbon sources by bacterial isolates like *Stenotrophomonas, Pantoea* and *Mesorhizobium*. Thus the present study provides insight into utilization of various carbon sources by a diverse group of bacterial genera. An ability to utilize various carbon sources helps the organisms to survive under deficiency conditions of their conventional carbon sources.

Identification of the isolates was performed by partial 16S rRNA gene sequence analysis and their phylogenetic relationship. It is quite normal to find that the majority of the identified bacteria were assigned to *Proteobacteria* because it is the second largest known bacterial phylum and one of the major ones in soil. It is noteworthy that many bacteria have 5 to 10 copies of 16S rRNA. Therefore, there is the possibility of sequence divergence between different copies of the gene from the same organism. ACC deaminase activity is an efficient marker for plant-associated bacteria to promote plant growth by lowering the levels of stress ethylene in plants. Moreover, bacteria producing ACC deaminase can down-regulate the plant genes involved in ethylene-induced stress responses and defense signaling pathways and up-regulate the plant genes involved in growth and protein production (Hontzeas et al., 2004). Recently, utilization of PGPR containing ACC deaminase activity in promoting plant growth and development both under stress and normal conditions and genetic manipulation of cultivars with genes expressing this enzyme has attracted much attention among the scientists (Belimov et al., 2002; Safronova et al. 2006).

In addition to ACC deaminase, we also looked for other plant growtth promoting attributes which are critical in influencing the growth of the associated plants. In the present study, all the isolates exhibited production of IAA which plays a critical role in the development of rooting system and in turn helps in the acquisition of nutrients due to increased root surfaces and thus, contributes to overall plant growth and yield (Lalande et al., 1989). The extensive proliferation of root system also helps to cope with the adverse environmental conditions (Baset Mia et al., 2010). The production of IAA by the bacteria may be involved at different levels in plant-bacterial interactions. The endogenous plant IAA may be altered by the acquisition of IAA

that has been secreted by the soil bacteria. The acquisition of additional IAA may alter the IAA level to either optimal or suboptimal level resulting in plant growth promotion or inhibition, respectively. In addition, inoculation with IAA-producing bacteria induces the proliferation of lateral roots and root hairs. In our study, all the selected bacterial isolates were able to produce the varying level of IAA. The secretion of IAA by the rhizobacteria modifies the micro-habitats of bacteria by increasing nutrient leakage of plant cells and also enhances the nutrient availability that enables the IAA producing bacteria to colonize the rhizosphere (Mohite, 2013). Similar to our results, previous studies have shown the IAA production by *P. fluorescens* (15.38±0.537µg/ml) and *B. subtilis* as (12.67±0.325 µg/ml) respectively (Reetha et al., 2014). Similarly, *Pseudomonas thivervalensis* (STF3) and *Serratia marcesens* (STJ5) produced 13.02 ±1.36 and 19.68 ±1.34 mg/l IAA, respectively (Shahzad et al., 2013).

In the soil, a large amount of phosphorus in the range of 400-1200 mg Kg^{-1} is present, however, most of this phosphorus is present in an insoluble form and therefore not available to support the plant growth. The insoluble phosphorus is present as either an inorganic mineral such as apatite or in organic forms like phytase (Inositol hexa- and penta-phosphates) which constitute 60% of soil organic phosphorus (Khan et al., 2007). The limited bioavailability of phosphorus from the soil often limits the plant growth (Feng et al., 2004). Therefore, solubilization of phosphorus by PGPR as a consequence of the low molecular weight organic acids such as gluconic and citric acid helps in mineralization of phosphorus. These organic acids can chelate the cation bound to phosphate with their hydroxyl and carboxyl groups (Kpomblekou and Tabatabai, 1994). In our study, the highest phosphate solubilisation was shown by *Klebsiella* sp. SBP-8, followed by *Enterobacter cloacae* ZNP-4 and *Serratia marcescens* SL-11. The low phosphate solubilization was observed by *Citrobacter freundii* (SNP-18) and *Enterobacter* sp. (SBP-6). The presence of such activities increases the applicability of PGPR as a bioinoculant for facilitating plant growth.

In addition to above mentioned plant growth promoting properties, we also tested the ability of the isolate to fix atmospheric nitrogen and make nitrogen bioavailable. Biological nitrogen fixation by the microbes could be the better alternative to minimize the use of industrially manufactured fertilizers to provide the fixed form of nitrogen to the plants. The nitrogen-fixing bacteria convert the atmospheric nitrogen to ammonia with the help of

nitrogenase enzyme and found as both free living as well as symbiotically with the leguminous crops. Therefore, characterization of rhizospheric bacteria with nitrogen fixation ability from diverse plant species appears to be promising in sustainable agriculture. Based on the growth of bacteria on minimal medium lacking any bioavailable nitrogen source, all isolates obtained in this study were found to be positive for nitrogen fixation. However, merely growth on nitrogen deficient media does not confirm ability to fix atmospheric nitrogen. It can be confirmed by acetylene reduction assay and ^{15}N method in future studies. Production of ammonia by the rhizobacteria is one of the important traits which benefit the crop (Kundu, 1987). The accumulation of ammonia in the soil suppresses the proliferation of fungi and inhibits the germination of fungal spores (Martin, 1982). The isolates tested in our study showed the production of ammonia except for the *Citrobacter freundii* SNP-18, *Enterobacter ludwigii* PGP-19, *Enterobacter cancerogenus* CPC-20 and *Phyllobacterium myrsinacearum* CPC-25.

Besides plant growth promoting characteristics, rhizospheric bacteria is considered to be better alternative to chemical pesticides for reducing the growth of pathogenic organisms at laboratory or field level as it is eco-friendly and cost effective (Compant et al., 2005). The rhizospheric microorganism can inhibit the pathogens directly by competitions for iron, antagonistic activity or detoxification of virulence factors or indirectly by augmenting induced systemic resistance in plants against certain disease (Lugtenberg and Kamilova, 2009). Certain microorganisms produce small molecular weight compound known as 'siderophore' that has the capability to chelate iron from the environment and makes it available to host plants under iron limiting conditions (Saha et al., 2012). Although the siderophores as chemical entities display considerable structural variation, the majority of them are either hydroxamates or phenolates-catecholates and all exhibit a very strong affinity for Fe (III). Rhizospheric bacteria generally produce three types of siderophores namely catecholate, hydroxymate and/or phenolate type (Rajkumar et al., 2010). As the siderophore production offers a mechanism of plant growth promotion by chelation of iron with the resultant decrease in iron availability in the surrounding medium. In the present study, siderophore production was observed in *Serratia marcescens* SL-11, SL-12, CDP-13, *Enterobacter ludwigii* CDP-14, *Mesorhizobium opportunistum* SNP-17, *Enterobacter cancerogenus* CPC-20 and *Enterobacter cloacae* CPC-21 on CAS-agar plate. Previous study have shown the siderophore production by the bacterial isolates like *Enterobacter cloacae* (Glick, 2012), *Serratia marcescens* (Karthikeyan et al., 2012) and in *Mesorhizobium* sp.

(Jha et al., 2012). Our results suggest that siderophore production is not very common phenomena exhibited by PGPR, however, the isolates showing this trait may play an additional role of iron transport for its host plants.

The antagonistic behavior of the microorganism can be exploited as biocontrol agents to overcome the infestation of microbial diseases. Bacteria can also restrict the growth of pathogens by producing hydrolytic enzymes such as chitinase, β-1,3-glucanase, protease, and lipase (Ordentlich et al*.,* 1988). The tested isolates in the present study showed the most of the hydrolytic enzymes production, however none of them showed the all enzymatic activity. Presence of these hydrolytic activity has extra advantages like β-1,3-glucanase production by *Bacillus cepacia* inhibit the growth of *Rhizoctonia solani*, *Rhizoctonia rolfsii* and *Pythium ultimum* (Fridlender et al*.,* 1993). Similarly, secretion of protease and chitinase by *Enterobacter* and *Pantoea* species isolated from cotton were found to protect the plants against fungal pathogen *Fusarium oxysporum* (Li et al*.,* 2010). In addition, production of certain hydrolytic enzymes may be required by few bacteria to enter inside the plant and establish the endophytic association inside the host plants. The exception to this, *Pseudomonas aeruginosa* PM389 does not possess any hydrolytic activities however it showed the passive mode of colonization (Gupta et al., 2013). Similarly, *Herbaspirillum seropedicae* strain SmR1 is well known as endophytic bacteria because of its active mode of colonization, however, it does not possess any hydrolytic enzymatic activity.

Tolerance to various abiotic stress conditions makes the isolates to survive and metabolically functional under extreme conditions. The tested isolates in the present study showed the varying degree of tolerance to salt and temperature stressors. Ability to tolerate high salinity stress (8 to 10% NaCl) makes the isolate a good candidate for stress studies as they have the advantage of being relatively protected from the competitive, high-stress environment of the soil. Temperature optima for the growth of bacterial isolates were more or less similar to that of mesophilic bacteria. Our results pertaining to temperature requirement for growth are consistent with other previous reports, where the majority of bacterial growth falls in the range of 28-37 ºC (Kirchhof et al., 2001).

Colonization of root is the crucial step for the establishment of plant-microbe interaction. The bacterial motility plays a key role in the root colonization. Motility is closely linked with chemotaxis, the ability to orient along certain chemical gradients. Most bacteria move by the use

of flagella, a thread like locomotor appendages extending outward from the plasma membrane and cell wall. Some bacteria exhibit a gliding motility by which they crawl over surfaces by waves of contraction produced within the cytoplasm. Gliding is defined as the movement of a non-flagellated cell in the direction of its long axis on a surface. Colonization by microorganisms with exudation of specific metabolic products induces the flagellar motility that directs their colonization on plant roots. It is the most important aspect of the successful colonization events (Lugtenberg et al., 2001). Followed by chemotaxis, these bacteria attach and spread on the plant surface and the interior on plant tissues. In addition, secretion of root exudates influences the expression of the certain gene and this alternation in gene expression observed to be decisive factor for colonization (Tadra-Sfeir et al., 2011). Therefore, we looked for different types of motility which are required for effective colonization on or in the plants. In our study, most of the bacterial isolate showed presence of all tested motility, however, these motilities are responsible for different purposes. The role of twitching (mediated by type IV pili) is responsible for colonization inside the plants (Burrows, 2012). Besides, flagellar and twitching motility of *Pseudomonas sp.* helps its movement towards rhizosphere in response to root exudates and play a major role in biofilm development (O'Toole and Kolter, 1998). After chemotaxis, many PGP bacteria need hydrolytic enzymes which can exert controlled degradation of plant cell wall and middle lamella to enter the plant tissue for endophytic colonization. The cell walls of the plants contain cellulose, whereas the middle lamella between cell walls contains mainly pectin. The presence of cellulase and pectinase activity may confer an advantage for intercellular ingress and spreading of isolates into the host cell by loosening the outer barrier (Hallmann et al., 1997). The presence of hydrolytic enzymes in the tested strains suggests the colonizing ability of the isolated PGPR used in the present study.

In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil. Unfortunately, the interaction between associative PGPR and plants can be unstable. To achieve the maximum growth promoting interaction between PGPR and plant seedlings, it is important to discover how the rhizobacteria are exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms. The good results obtained *in vitro* can not always be dependably reproduced under field conditions. Further

evaluation of the isolates exhibiting multiple plant growth promoting (PGP) traits on soil–plant system is needed to uncover their efficacy as effective PGPR.

CHAPTER III

Physiological characterization of ACC deaminase activity and plant growth promoting test under abiotic stress conditions

3.1 Introduction

Soil salinity is one of the most important abiotic factors adversely affecting the soil microbial activities and crop productivity. Reports suggest that at least 20% agricultural land worldwide is affected by salt stress (Pitman et al., 2002). There is an estimation of about 50% loss of arability globally by middle of the twenty-first century due to increased salinization of agricultural land (Wang et al., 2003). In plants, one of the effects of salt stress is an increase in the pool of 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene, which results in accumulation of ethylene beyond threshold level termed as 'stress ethylene'. Stress ethylene inhibit plant root and shoot growth (Penrose and Glick, 2003), suppresses leaf expansion (Peterson et al., 1991), alters photosynthesis and photosynthetic components (Koyro, 2006) and promote epinasty (Abeles et al., 1992). In addition to salt stress, other stressors like temperature stress also lead to significant rise in the level of endogenous 'stress ethylene' (Morgan and Drew, 1997; Stearns and Glick, 2003).

Association of plant growth promoting bacteria (PGPB) equipped with ACC deaminase activity can have tremendous effect on mitigating plant growth inhibition resulted from stress ethylene. The use of plant growth promoting bacteria with ACC deaminase activity for enhancing the plant growth and yield is a promising biotechnological approach to the agriculture sector in the near future. The enzyme ACC deaminase catalyzes the cleavage of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of stress ethylene to α-ketobutyrate (KB) and ammonia. Breakdown products of ACC serve as a source of nitrogen and energy for associated bacteria (Glick, 2007). Thus, by minimizing the generation of stress ethylene these bacteria help the plants to cope with stress conditions. The bacterial uptake of rhizospheric ACC decreases the root ACC concentrations and ethylene generation, therefore maintain the dynamic equilibrium of ACC concentration among root, rhizosphere and bacterium (Glick et al., 1998; Penrose et al., 2001). Different bacterial genera exhibit different levels of enzyme activity under various environmental conditions. However, organisms with ACC deaminase activity of approximately >20 nmol α-ketobutyrate/mg protein/h are sufficient to alleviate the stress response (Penrose and Glick, 2003). Isolation of ACC deaminase producing bacteria and their beneficial effect on plant growth under various environmental stressors have been reported by several research groups (Ahmad et al., 2011; Cheng et al., 2007; Shaharoona et al., 2007).

PGPR can improve plant growth through one or more mechanisms directly through providing nitrogen, phosphate, iron nutrition and production of phytohormones or indirectly through protecting from pathogenic and insect pests by antagonistic mechanism or generating induced systemic resistance in host plants. In addition, certain PGPR can also protect plants from abiotic stressors by induced systemic tolerance (IST) which enables plants to tolerate or attenuate the deleterious effect of abiotic stressors (Yang et al., 2009). PGPB isolated from saline habitats could be adapted to tolerate the saline conditions and hence, increase plant resistance to salt stress. As described in the introduction section, plant-associated bacteria can ameliorate different abiotic stressors including salt and temperature by restricting the level of 'stress ethylene' by producing ACC deaminase. Mayak et al. (2004) reported that the salt-tolerant ACC deaminase bacteria help plants to overcome stress effects. Hence, the presence of ACC deaminase activity could be one of the primary mechanisms by which bacteria support the plant growth under salt stress (Saleem et al., 2007).

Recently, use of ACC deaminase possessing bacteria has become a promising option for plant growth promotion and alleviation of plant stress caused by salinity (Jha et al., 2012; Karthikeyan et al., 2012; Rashid et al., 2012) and other stressors. For instance, ACC deamianse bacteria *Achromobacter piechaudii* ARV8 was found to be efficient in growth promotion of tomato under salt stress (Mayak et al., 2004). Increase in seedling growth, biomass and reduction of stress ethylene (57%) in red pepper was observed after inoculation of ACC deaminase bacteria (Siddikee et al., 2011). Similarly *Pseudomonas fluorescens* strain TDK1 with ACC deamianse activity enhanced salt resistance in groundnut plants as compared to other *Pseudomonas* strains lacking ACC deaminase activity (Saravanakumar and Samiyappan, 2007). Thus, salt tolerant ACC deaminase containing bacteria could be advantageous in a saline environment to deliver beneficial effects on plants. The influence of high temperatures on growth and development of wheat and other crops is well documented (Porter and Gawith, 1999; Wheeler et al., 2000). At the molecular level, high temperatures adversely affect cell metabolism and cause changes in the pattern of protein synthesis (Larkindale et al., 2005). A recent review by Barlow et al. (2015) on the effect of temperature extremes in wheat (*Triticum aestivum* L.) revealed that excessive heat caused reduction in grain number and reduced duration of the grain- filling period. Besides, very few studies have been done on deciphering the role of ACC deaminase producing bacteria for improving the plant growth under temperature stress.

Therefore, understanding the mechanism of development of salt tolerant plants either by genetic engineering or use of plant growth promoting bacteria are essential for solving the problems of productivity in salty regions (Dimpka et al., 2009). However, little information is available on the effectiveness of ACC deaminase bacteria on the growth of wheat plant, one of the most economically important crop plant, facing the salinity stress (Nadeem et al., 2006). Wheat along with rice, represent the most important staple food crops, as they provide more calories and proteins in the diet than any other crop. Annually, total wheat production in India is 80.2 million tons (http://www.agricoop.nic.in) which corresponds to about 12% of total world production (http://dacnet.nic.in). Like other crops, germination of wheat seed and seedling growth are severely affected by salt stress (Hampson and Simpson, 1990; Nasri et al., 2015). Wheat growing under field conditions is exposed to various abiotic stressors including high salt, and high temperature at various developmental stages. Therefore, the present work was aimed to evaluate the selected ACC deaminase bacteria for their plant growth stimulating effect on the wheat plant under salt and temperature stress conditions. In addition, the ACC deaminase activity of selected isolates was characterized under the various physiological conditions like different salt, temperature, pH, varying the concentration of ACC, at different time intervals and in different concentration of carbon and nitrogen sources.

3.2 Material and Methods

3.2.1 Selection of bacterial isolates

Based on the results of ACC deamianse activity and other plant growth promoting tests, twelve bacterial isolates namely ZNP-1, ZNP-2, ZNP-3, ZNP-4, SBP-6, SBP-7, SBP-8, SBP-9, SBP-10, SL-11, SL-12, and HSW-16 were selected for detail characterization of ACC deaminase activity under various physiological conditions such as different salt, pH, temperature, substrate ACC concentrations, incubation period, and various carbon and nitrogen sources (Table 3.1).

Table 3.1 The selected isolates used for physiogical characterization of ACC deaminase activity and plant growth promoting test

S.No	Code	Bacterial species
1.	ZNP-1	*Enterobacter cloacae*
2.	ZNP-2	*Enterobacter cloacae*
3.	ZNP-3	*Enterobacter cloacae*
4.	ZNP-4	*Enterobacter cloacae*
5.	SBP-6	*Enterobacter* sp.
6.	SBP-7	*Serratia marcescens*
7.	SBP-8	*Klebsiella* sp.
8.	SBP-9	*Enterobacter cloacae*
9.	SBP-10	*Klebsiella pneumoniae*
10.	SL-11	*Serratia marcescens*
11.	SL-12	*Serratia marcescens*
12.	HSW-16	*Bacillus licheniformis*

3.2.2 Test of ACC deaminase activity under different physiological conditions

Bacterial ACCD activity can be affected by different physiological and environmental factors. Therefore, ACCD activity in selected isolates was investigated under various physiological conditions namely salt, temperature, pH, substrate (ACC), different C and N sources, and at different time intervals. The bacterial isolates were grown in DF media and ACC deaminase enzyme activity was measured as described in Chaper I, section 2.2.4. Suitable changes in growth condition were made for different variables. Based on preliminary observations, various concentration of NaCl (2 to 8%) was added in DF medium for estimating ACC deaminase under salt stress condition, whereas the different concentration of ACC (1 to 5 mM) was used to estimate optimum substrate concentration for ACC deaminase enzyme activity. For imposing different pH conditions, pH of the culture media was adjusted with 2 N HCl and 1 M NaOH to attain pH 5.0 to 10.0 using the pH meter (Eutech, pH 1100, South Korea). Similarly, bacteria were grown at different temperatures ranging between 25 °C and 40 °C in an incubator. For the time course assay, ACC deaminase enzyme activity was tested at different time intervals after adding an optimal concentration of ACC in the medium. Cultures of equal OD (OD_{600}) were used for ACC deaminase assay. To determine the influence of various carbon sources on ACC

deaminase activity, bacterial isolates were grown in DF medium supplemented separately with sucrose, dextrose, lactose, and xylose at the concentration of 0.2%. After growth, the enzyme activity was assayed. Similarly, determination of ACC deaminase activity under different nitrogen sources was carried out by growing the isolates in a medium supplemented with different nitrogen sources such as ammonium chloride (NH_4Cl), ammonium sulfate $(NH_4)_2SO_4$, ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) at the concentration of 0.2%

3.2.3 Amplification, sequencing, and analysis of *AcdS* gene

AcdS gene, a functional gene encoding ACC deaminase was amplified in the tested bacterial isolates using a universal pair of primers. PCR was also carried out with universal primers designed by Duan et al. (2009). The sequence of primers used for *AcdS* amplification has been shown in Table 3.2. 50 µl of PCR mix contained 50–200 ng DNA, 1× PCR buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.5% DMSO, 20 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate (Fermentas), 1 µg BSA (Promega) and 1 U Taq DNA polymerase (Invitrogen). PCR was performed under the following conditions: initial denaturation was performed for 3 min at 95 °C followed by 30–35 cycles consisting of denaturation for 30 s at 95 °C, primer annealing for 1 min at 46 °C (DegACCf/DegACCr), 51°C (AccF/AccR), 56 °C (Uni F/UniR) or 53 °C (F1937f/F1939r) and elongation for 1 min at 72 °C followed by a final extension for 5 min at 72 °C.

After getting failure with universal primers, PCR was performed with Duan et al. (2009) in a final volume of 50 µl reaction mix containing 50 ng genomic DNA (template), 20 pmole each of forward and reverse primers, 200 µM of each d-NTPs (Genei), 1X Taq polymerase buffer with 1.5 mM $MgCl_2$, and 2.5 U Taq DNA polymerase (Genei). PCR was carried out in a thermal cycler (T100, BioRad, USA). The reaction conditions of PCR included an initial denaturation step at 94 °C for 3 min, 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and primer extension at 72 °C for 3 min, followed by a final extension at 72 °C for 5 min. Amplified prouduct was confirmed by agarose gel electrophoresis and purified using PCR purfication kit (Qiagen, USA) as per the instructions of the manufacturer. The purified PCR product was sequenced at Xceleris Genomics Labs Ltd (Xcelris, Ahmedabad). The sequence of *AcdS* amplicons was analyzed using BLAST algorithm available at http://www.ncbi.nlm.nih.gov/BLAST. The sequence of *AcdS* of test isolates was aligned with

that of related bacterial species by ClustalW using MEGA 6.0 software and a Neighbor-Joining (NJ) tree with bootstrap value 500 was generated using the software (Tamura et al., 2013).

Table 3.2 List of primers used in PCR amplification

Primers	Sequences (5'-3')	Reference
AccF	5'-ATGAATCTGAATCGTTTTGAAC-3'	Farajzadeh et al., 2010
AccR	5'-TCAGCCGTTGCGGAACAG-3'.	
UniF	5'-AGAGTTTGATCCTGGCTCAG-3'	Ramadoss et al., 2013
UniR	5'-AAGGAGGTGATCCAGCCGCA-3'.	
DegACCf	5'-GGBGGVAAYAARMYVMGSAAGCTYGA-3'	Glick et al., 2005
DegACCr	5'-TTDCCHKYRTANACBGGRTC-3'	
F1937f	5'-MGVAAGCTCGAATAYMTBRT-3'	Blaha et al., 2006
F1939r	5'-GARGCRTCGAYVCCRATCAC-3'	
F	5'-GGCAAGGTCGACATCTATGC-3	Duan et al., 2009
R	5'-GGCTTGCCATTCAGCTATG-3'	

3.2.4 Effect of ACC deaminase containing bacteria on plant growth under abiotic stress conditions

3.2.4.1 Selection of plant and bacterial inoculum preparation

For the experimental studies, wheat plant (*Triticum aestivum* C309) was used. This variety is commonly used for making the breads and chapati. It is a drough resistant variety commonly grown in the arid regions with the soil of mild salinity. For the plant growth promoting test inoculums of selected isolate was prepared. The selected bacterial cultures were grown in the Luria-Bertani (LB) broth medium and optical density (OD) was maintained to 0.15 using 0.03 M $MgSO_4$ in a UV-Visible spectrophotometer (JASCO Corporation, Japan).

3.2.4.2 Physiochemical characteristics of soil

Soil sample used for pot study was analyzed for its various physico-chemical properties at National Horticulture Research and Development Foundation (NHRDF, Nashik, India). pH and electrical conductivity of soil were analyzed by digital pH and EC meter on an 1:2.5 ratio of soil and water suspension. Estimation of organic carbon was done by the method of Walkley and Black (1934) using 1 N potassium dichromate for titration and 0.5 N ferrous ammonium sulfate for back titration. Available soil phosphorus (Olsen P) was determined by chlorostannus-reduced

molybdophosphoric blue color method after extraction with 0.5 M sodium bi-carbonate as described by Olsen et al. (1954). Available nitrogen and potassium and other micronutrients (Fe, Cu, Zn and Mn) were estimated by the method of Jackson (1967).

Table 3.3 Physico-chemical properties of soil used for pot study

Parameter	**Value**
pH	7.20±0.02
EC	0.158±0.01ds m^{-1}
Olsen P	32.8 ±1.4 mg kg^{-1}
Total N	63 ±2.1 mg kg^{-1}
Total K	126.7 ±4.2 mg kg^{-1}
Zn	0.213 ±0.001 mg kg^{-1}
Cu	0.131 ±0.001 mg kg^{-1}
Fe	2.76 ±0.03 mg kg^{-1}
Mn	0.963 ±0.02 mg kg^{-1}

±; standard deviation, each value is mean of three replicates

3.2.4.3 Seed sterilization and bacterial treatments

The selected bacterial isolates were tested for their effect on the growth of wheat plants (*Triticum aestivum* C-309) under salt and temperature stresses in a controlled environment. Preparation of bacterial inoculum and seed treatment were performed according to Penrose and Glick (2003) with slight modifications. Briefly, freshly grown culture (30 ml) was harvested and suspended in 0.5 ml sterile 0.03 M $MgSO_4$ and diluted in the same solution to adjust absorbance to 0.15 at 600 nm. Wheat seeds were surface sterilized by treating with 70% ethanol for 2 min followed by three times washing with sterilized water. Then, the seeds were treated to 1.0% sodium hypochlorite (NaOCl) solution for 3 min followed by three consecutive washes using sterile water to remove all traces of sodium hypochlorite. Each Petri-dish containing around 15-20 seeds was incubated at room temperature with appropriate treatment: sterile 0.03 M $MgSO_4$ control and bacterial suspension in sterile 0.03 M $MgSO_4$ (OD of 0.15 at 600 nm) as treated one.

Bacterized seeds were air dried for one hour under aseptic conditions. Following the incubation period, five seeds were sown in each plastic pots filled with sterile soil in triplicates in a growth chamber with 16:8 photo-period at 28±2 °C with a humidity of 70% and 140 µmol $m^{-2}s^{-1}$ of light intensity. Hoagland media was applied to soil as a nutrient solution on alternate days (Hoagland and Boyer, 1936) and it's detail composition has been given in Appendix III.

3.2.4.4 Plant growth test under stress conditions

For imposing salt stress, plants were watered with 150 mM, 175 mM, and 200 mM NaCl every alternate day after germination of seeds. Pots were arranged in completely randomized block design with three replicates in each treatment in the controlled condition of plant growth chamber. For temperature stress, bacterized seeds were grown at different temperatures (25 °C, 30 °C, and 35 °C) maintained in the plant growth chamber (Labtech, S. Korea). Plants were harvested after 15 days of germination and plant growth was measured based on various parameters such as percent germination, root and shoot length, the fresh and dry weight of five randomly selected seedlings and chlorophyll content. For measurement of photosynthetic pigments chlorophyll a/b, fresh leaf samples (1 g) were homogenized in 80% acetone, and pigments were extracted and quantified as per the method of Duxbury and Yentch (1956). The absorbance was measured at 480, 510, and 663 nm in a UV–Vis spectrophotometer (Jasco Corporation, Japan). Amount of chlorophyll was estimated using following formula: Chl a = $12.7A_{663} - 2.59A_{645}$; Chl b = $22.9A_{645} - 4.67A_{663}$

3.2.4.5 Statistical analysis

All the experiments were conducted in triplicates and results were expressed as mean ± standard deviation (SD). Data was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test (DMRT) at $p<0.05$.

3.3 Results

3.3.1 Physiological characterization of ACC deaminase activity

ACC deaminase activity of selected bacterial isolates varied under different physiological conditions. It is evident from Table 3.4 that ACC deaminase activity increased with the increase in salt concentrations form 2% to 6% for most of the isoltes. However, there was no activity in 8% NaCl. The isolates showed the basal level of activity in the range of 120 to 231 nmol/mg protein/h at 2% NaCl concentration, whereas it increased with increase in NaCl concentration from 2% to 6%, in the range of 160 to 416 nmol/mg protein/h. With respect to increasing in salinity, the highest increase in enzyme activity was observed for SBP-8 (80%), followed by SL-11 (78%) at 6% NaCl concentration.

Table 3.4 ACCD activity of various isolates under different NaCl concentrations

		ACC deaminase activity*			
S. No.	**Isolates**	**2% NaCl**	**4% NaCl**	**6% NaCl**	**8% NaCl**
1.	ZNP-1	125.1±13.5	135.6 ± 10.0	167.4±8.7	-
2.	ZNP-2	145.0±14.0	183.5±8.9	220.7±21.0	-
3.	ZNP-3	132.7±13.5	176.5±7.9	246.4±23.6	-
4.	ZNP-4	122.3±9.1	156.2±10	190.4±11.7	-
5.	SBP-6	147.0±17.4	187.0±21.4	279.2±4.4	-
6.	SBP-7	196.1±14.5	227.7±11.8	284.0±11.6	-
7.	SBP-8	231.0±10.4	330.5±13.5	416.0±11.7	-
8.	SBP-9	121.0±11.9	149.5±5.8	170.1±13.8	-
9.	SL-11	146.3±13.2	188.9±18.0	200.5±20.6	-
10.	SL-12	212.3±11.0	320±14.5	391.0±10.5	-
11.	SBP-10	169.5±9.7	196.1±14.3	217.4±13.4	-
12.	HSW-16	155.9±5.8	183.5±15.6	213.3±9.1	-

* values are in n mol α-ketobutyrate/mg/protein/h. Each value represents mean±SD of triplicates.

Testing the enzyme activity at various temperatures revealed that most of the isolates showed their maximum enzyme activity at 30 °C. ACC deaminase activity decreased at a higher temperature than 30 °C in all the tested isolates (Fig. 3.1).

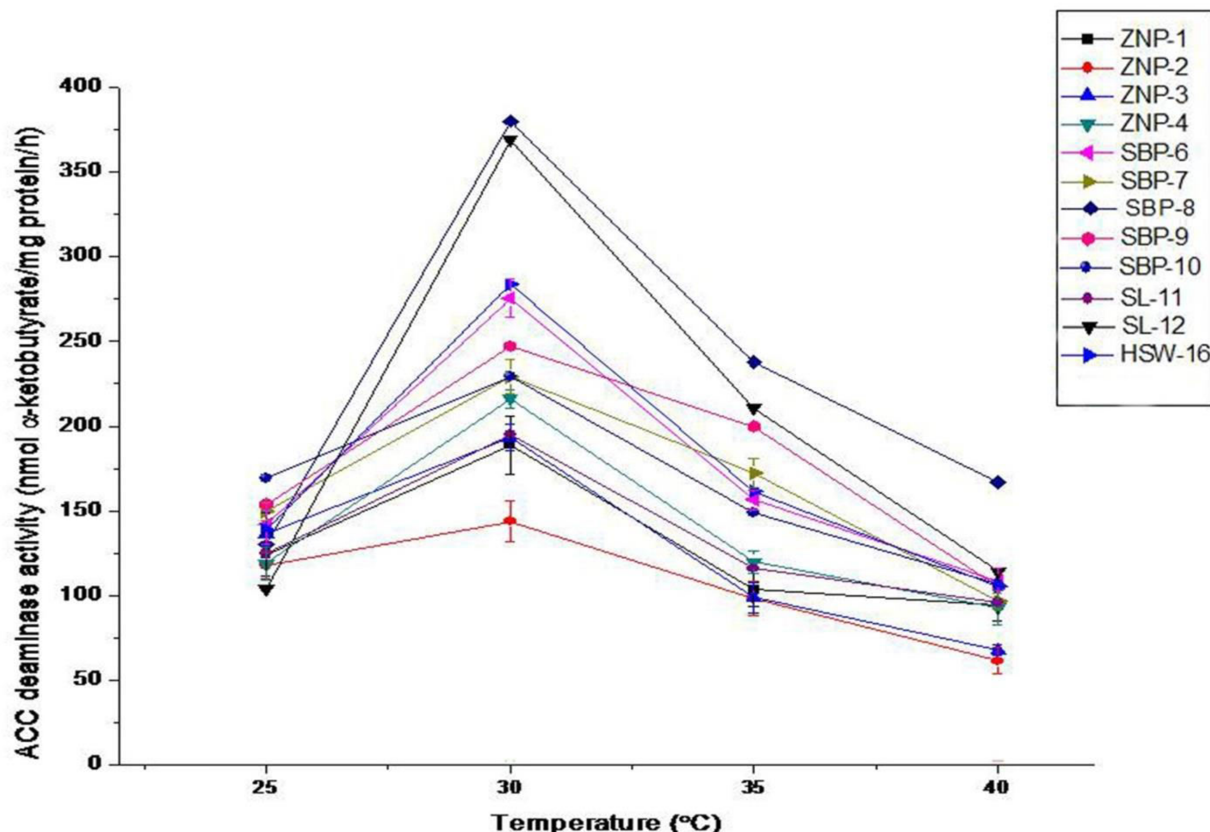


Fig. 3.1. ACC deaminase activity of selected isolates at different temperature (25 to 40 ºC) concentration. Each value represents mean±standard deviation of triplicate sets

Availability and concentration of substrate is also one of the important determinants for optimum activity of an enzyme. Therefore, ACC deaminase activity was estimated in bacteria grown with different concentration of ACC. ACC deaminase activity increased in all the tested isolates with an increase in the concentration of ACC with optimal activity at 3 mM ACC, which followed the decrease in activity with a further increase in concentration (Fig. 3.2). Among all the isolates, the highest activity was noted for the isolate SBP-8 with 396.20±8.0 nmol/mg protein/h, followed by 370±2.3 nmol/mg protein/h for SL-12. Most significant effect of substrate concentration was observed in SL-12 which showed 130% increase in ACC deaminase activity from 1 to 3 mM. Enzyme activity was also assessed under different incubation periods from 4 to 48 h following ACC induction. Some of the isolates namely ZNP-1, ZNP-3, ZNP-4, SBP-9,

SBP-10, SL-11, and HSW-16 showed higher enzyme activity after 24 h of ACC induction. Further incubation, decreased the enzyme activity in these isolates. However, the isolates ZNP-2, SBP-6, SBP-7, SBP-8, and SL-12 showed higher enzyme activity at 48 h of incubation (Table 3.5).

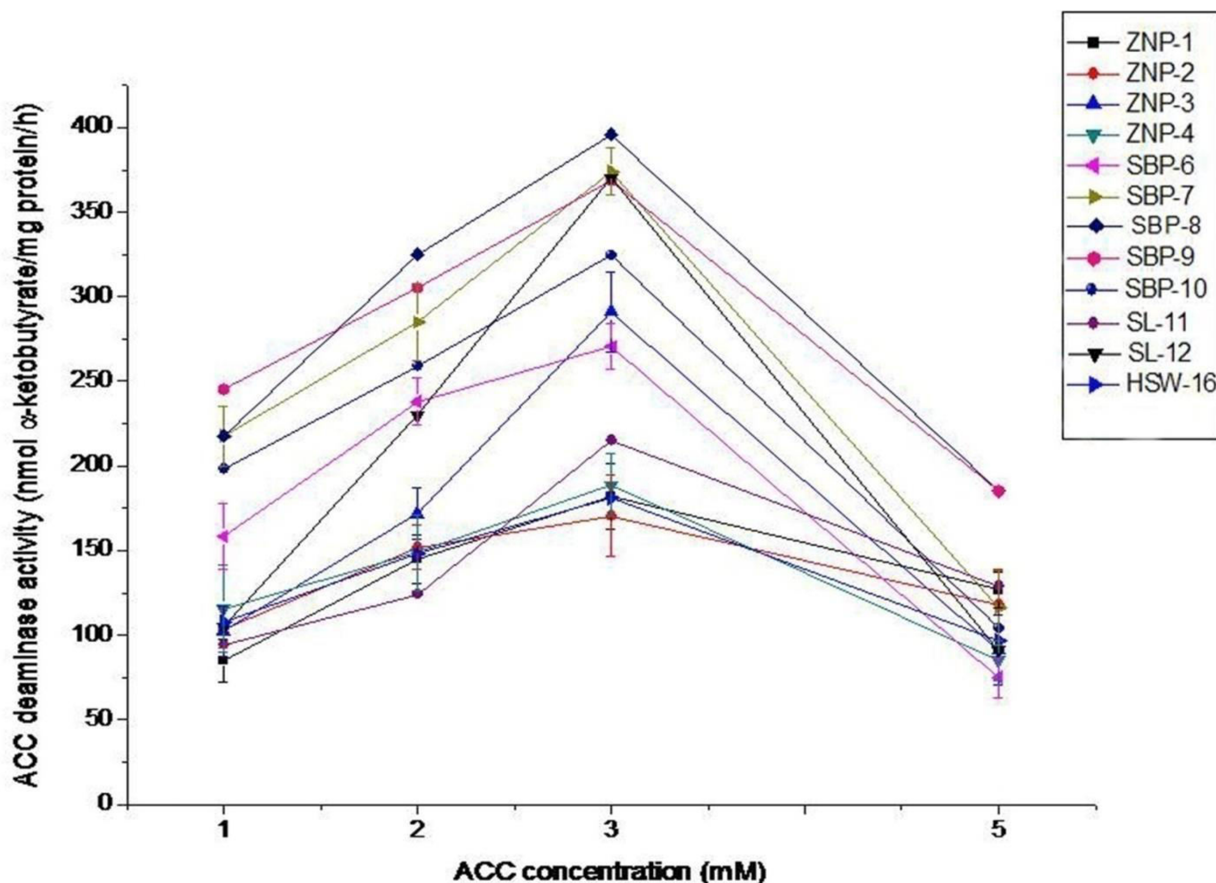


Fig. 3.2 ACC deaminase activity of selected isolates at different ACC (1 to 5 mM) concentration. Each value represents mean±standard deviation of triplicate sets.

Similar to the other conditions, ACC deaminase activity was also tested under varying pH (5.0 to 9.0), which showed that ACC deaminase activity was lower at acidic pH wheras it was increased at slightly alkaline pH. The optimum pH for ACC deaminase activity was 8.0 for all the tested isolates. Highest effect of pH on ACC deaminase activity was observed in SBP-8 where ACCD increased by 99.49% over its counterpart grown in pH 5.0 with enzyme activity of

391.0±8.9 nmol/mg protein/h. On further increase in pH (9.0), enzyme activity decreased upto 234%. Isolate ZNP-4 showed the lowest enzyme activity 152±3.7. nmol/mg protein/h (Fig. 3.3).

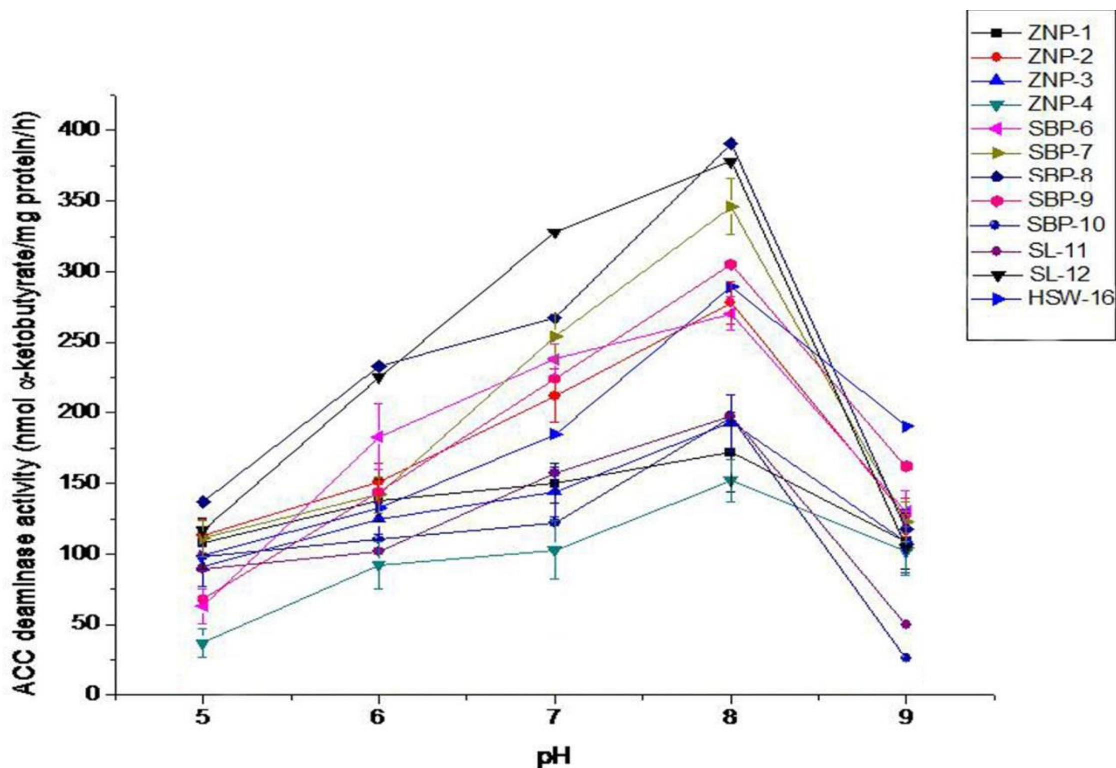


Fig. 3.3 ACC deaminase activity of selected isolates at different pH (5.0 to 9.0) concentration. Each value represents mean±standard deviation of triplicate sets.

Table 3.5 ACCD value of selected isolates at different time intervals

	ACC deaminase activity*				
Isolates	**4 h**	**8 h**	**12 h**	**24 h**	**48 h**
ZNP-1	40.6±6.4	96.7±12.7	124.8±16.3	180.9±18.4	125.8±14.8
ZNP-2	33.6±7.5	82.7±16.3	110.7±21.7	196.9±20.4	274.8±17.4
ZNP-3	54.6±9.3	110.7±11.9	145.8±26.2	194.9±23.4	104.8±10.7
ZNP-4	87.5±8.4	102.0±17.2	124.5±19.6	162.5±22.5	115.9±19.5
SBP-6	82.7±13.2	154.9±23.4	136.2±11.6	225.3±18.4	281.5±20.9
SBP-7	90.7±15.4	13.70±21.3	198.3±25.3	287.0±20.2	341.5±24.4
SBP-8	54.6±10.4	166.9±16.3	221.1±11.4	271.3±18.4	393.0±19.4
SBP-9	104.9±24.2	120.8±21.6	212.0±18.5	301.3±29.4	245.4±14.6
SL 11	96.9±12.7	105.8±23.1	121.5±14.5	185.5±17.3	105.9±11.7
SL12	47.6±12.5	103.8 ±26.3	207.9±28	290.4±16.2	372.4±21.3
SBP-10	56.8±11.4	88.7±13.2	124.8±18.9	192.1±23.3	145.8±19.2
HSW-16	87.0±10.5	112.0±16.1	143.8±22.4	285.5±21.5	135.9±22.2

*values are in nmol α-ketobutyrate/mg protein/h. Each value represents mean±standard deviation of triplicate sets.

Further, understanding the composition and influence of root exudates on microbial activity, we tested ACC deaminase activity in all the selected isolates grown in the presence of various carbon and nitrogen sources. The optimum enzyme activity varied in different isolates under the influence of various carbon sources. The isolates ZNP-1, ZNP-2, and SBP-6 showed the higher activity in the range of 150 to 325 nmol/mg protein/h in dextrose amended medium, whereas ZNP-3, ZNP-4 SBP-7, SBP-8, SBP-10, SL-11, SL-12, and HSW-16 showed maximum ACC deaminase activity in lactose. On the other hand, highest activity in SBP-9 was observed in sucrose supplemented medium (Table 3.6). Similar to the results of carbon sources, ACC deaminase activity was also affected by different nitrogen sources (Table 3.7). It revealed that ammonium chloride was found to be most favored nitrogen source for most of the isolates. The highest ACC deaminase activity was observed by the isolate SBP-8 with 412±1.8 nmol/mg protein/h. On the contrary, isolate SBP-6 showed the higher enzyme activity 338±1.5 nmol/mg protein/h in the potassium nitrate supplemented medium.

Table 3.6 ACC deamianse activity of selected isolates in presence of different carbon sources

		ACC deaminase activity*			
S. No.	**Isolates**	**Sucrose**	**Dextrose**	**Lactose**	**Xylose**
1.	ZNP 1	158.6±20.5	169.2±19.8	126.9±10.9	72.1±9.8
2.	ZNP 2	147.4±13.6	156.1±21.3	122.6±21.4	69.3±12.9
3.	ZNP 3	168.2±17.2	146.2±17.6	165.4±12.2	56.8±10.9
4.	ZNP 4	153.0±23.9	135.5±15.4	143.3±18.3	65.5±9.1
5.	SBP 6	223.0±13.5	325.7±22.5	297.8±13.5	50.4±6.5
6.	SBP 7	239.6±19.4	292.9±14.7	317.1±23.7	192.5±13.4
7.	SBP 8	288.2±18.2	312.2±24.9	327.3±31.4	131.1±12.3
8.	SBP 9	309.9±29.7	276.7±18.4	300.8±28.5	91.7±14.3
9.	SBP 10	123.2±13.8	105.0±14.8	122.5±14.5	82.3±9.8
10.	SL 11	138.8±22.6	95.8±7.3	122.9±12.1	61.7±11.5
11.	SL 12	120.6±19.8	104.2±9.6	123.4±18.4	82.0±8.3
12.	HSW 16	137.3±17.6	110.7±19.3	144.7±16.7	78.5±9.8

* values are in µmol α-ketobutyrate/mg protein/h. Each value represents mean±standard deviation of triplicate sets.

Table 3.7 ACC deamianse activity of selected isolates in presence of various nitrogen sources

		ACC deaminase activity*			
S. No.	**Isolates**	**KNO_3**	**NH_4Cl**	**NH_4NO_3**	**$(NH4)_2SO4$**
1.	ZNP-1	142.9± 11.9	213.0±23.6	176.8 ±29.3	146.0 ±19.5
2.	ZNP-2	143.6±17.3	188.7±25.2	168.0±24.7	151.8±18.4
3.	ZNP-3	142.3± 21.4	198.7 ±10.6	177.4 ±21.4	167.3± 11.8
4.	ZNP-4	154.6±22.7	197.5±18.4	167.0±15.9	138.5±17.6
5.	SBP-6	338.0±25.7	293.3± 24.3	215.6±13.9	173.4±18.8
6.	SBP-7	135.0±18.4	285.0±15.8	113.5±16.6	176.0±14.4
7.	SBP-8	116.5±12.6	412.0±38.7	387.0±27.5	325.9±13.6
8.	SBP-9	286.7±15.8	346.6±31.5	305.8±25.7	125.6±19.9
9.	SBP-10	114.5±19.5	224.5±23.8	134.6±27.3	128.0±25.7
10.	SL-11	125.8±18.3	223.1±24.5	153.4±22.5	144.6±18.4
11.	SL-12	125.4±16.8	185.5±19.2	145.6±15.9	138.5±24.7
12.	HSW-16	118.7±16.9	285.8±25.3	168.8±16.7	157.8±23.4

*values are in n mol α-ketobutyrate/mg protein/h. Each value represents mean±standard deviation of triplicate set.

Molecular characterization of *AcdS* gene and phylogenetic analysis

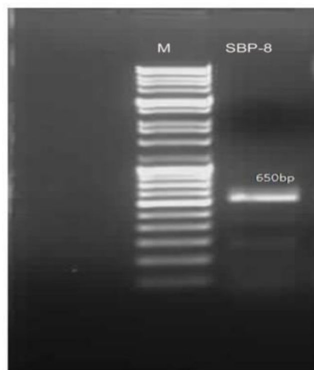
Amplification of *AcdS* gene was performed for all the isolates, however, the successful amplicon was obtained only in the isolate ZNP-2, SBP-8, and HSW-16 (Duan et al., 2009 primers). It is evident from Fig. 3.4 A that a 650 bp amplicon specific to *AcdS* was obtained from genomic DNA of *Klebsiella* sp. SBP-8. On performing BLAST analysis, identification of given amplified fragment as *AcdS* was confirmed with 99% sequence similarity with *AcdS* gene of *Klebsiella pneumoniae* AcdSPB 2. The obtained sequence of isolate SBP-8 was submitted to NCBI GenBank database under the accession number KM501058. Phylogenetic analysis revealed that *AcdS* sequence of SBP-8 is closely related to *Klebsiella* AcdSPB and other strains belonging to genera *Klebsiella, Pseudomonas, Bacillus,* and *Serratia* sp. It also showed identity with one of the most characterized ACCD bacteria *Pseudomonas putida* UW4 (Fig. 3.4 B).

For the isolate HSW-16, 800 bp amplicon was obtained by PCR from genomic DNA of isolate HSW-16 (Fig. 3.5 A). Sequence analysis of amplicon confirmed its identity where it

showed the similarity of 98% identity with *AcdS* sequence of *Bacillus cereus* AcdSPB4. The obtained sequence was submitted under the accession number KM501059. Gene sequence alignment and phylogenetic analysis of *AcdS* gene obtained from HSW-16 and other bacterial species revealed that *AcdS* sequence of HSW-16 is closely related to *B. cereus* AcdSPB4, *B. subtillis,* other members belonging to other genera including *Klebsiella, Pseudomonas, Burkholderia, Achromobacter,* and *Serratia* sp. and *Pseudomonas putida* UW4 (Fig. 3.5 B). Phylogenetic analysis showed that sequence of *AcdS* of genus *Bacillus* clustered together.

A 600 bp amplicon was obtained by PCR from genomic DNA of isolate ZNP-2 using *AcdS* gene specific primers (Fig. 3.6 A). Sequence analysis of amplicon confirmed its identity where it showed the similarity of 93% identity with *AcdS* sequence of other *Enterobacter cloaceae.* The obtained sequence was submitted under the accession number KM501057. Gene sequence alignment and phylogenetic analysis of *AcdS* gene obtained from ZNP-2 and other related bacterial species revealed that *AcdS* sequence of ZNP-2 is closely related *Enterobacter cloaceae* and other gram negative bacteria (Fig. 3.6 B).

A



B

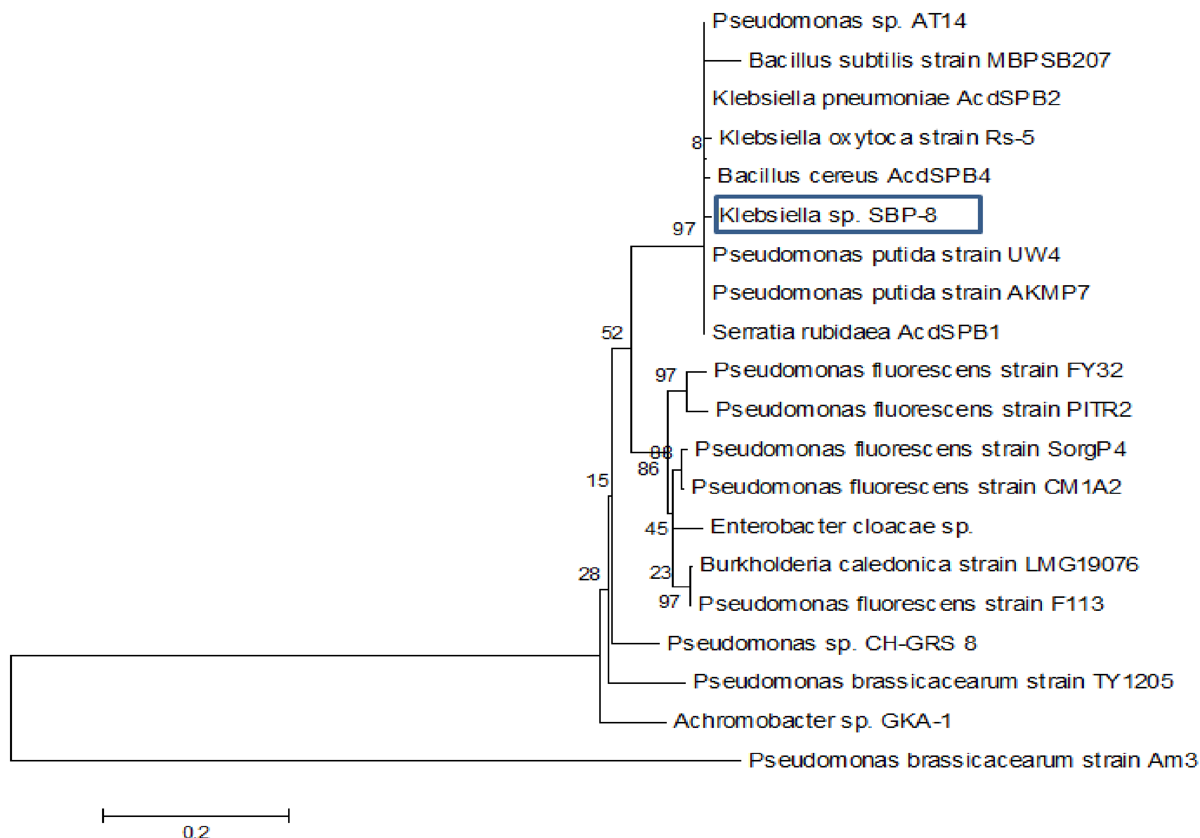
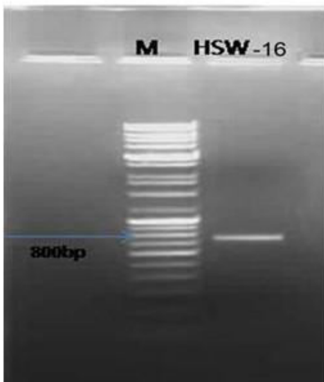


Fig. 3.4. Amplification and analysis of *AcdS* gene of *Klebsiella* sp. SBP-8. Panel 'A' shows amplicon of *AcdS* (650 bp) of test isolate in Lane 2 and DNA ladder mix (SM0331) in Lane 1. Panel 'B' represents dendrogram based on *AcdS* sequence of test isolate and other species which showed similar *AcdS* sequence. Other sequences of *AcdS* used for analysis were retrieved from NCBI Genbank data base. Neighbor-joining method was performed using the software packages Mega version 6.0, at the bootstrap value of (n = 500).

A



B

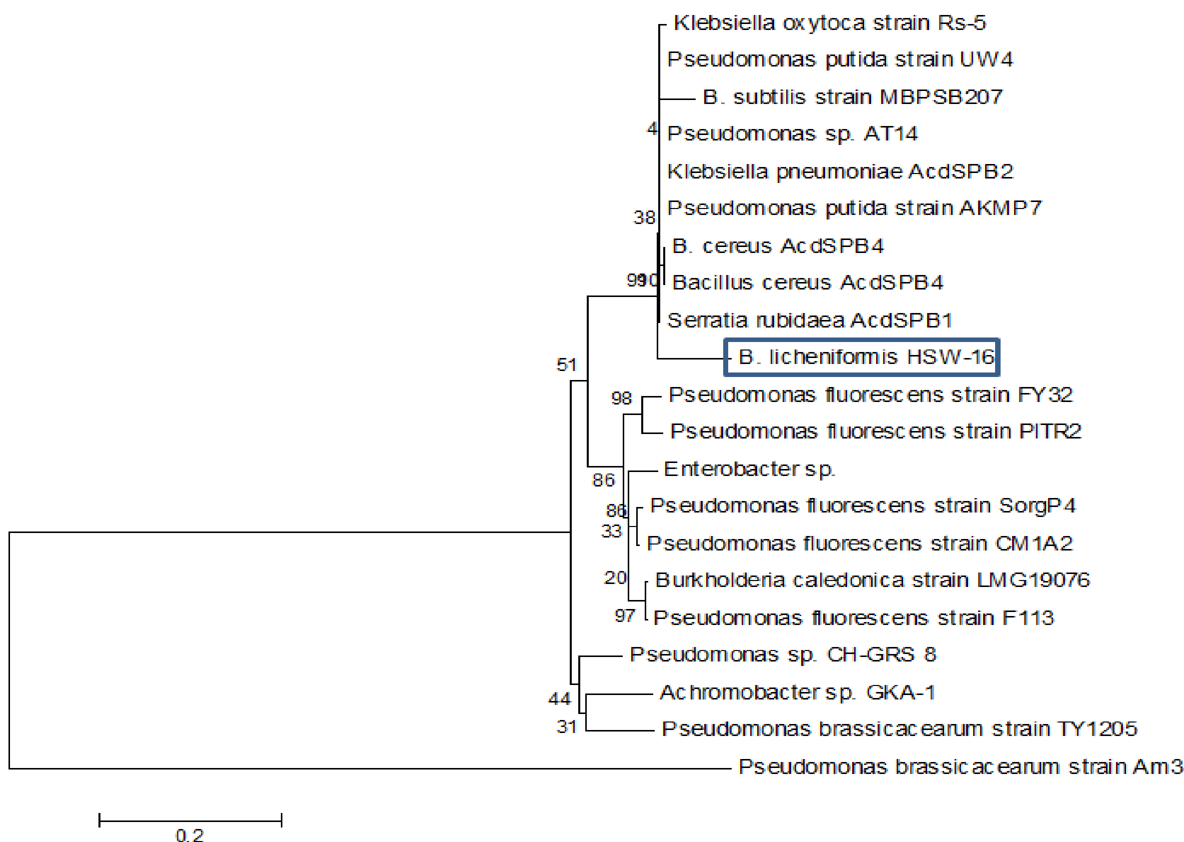
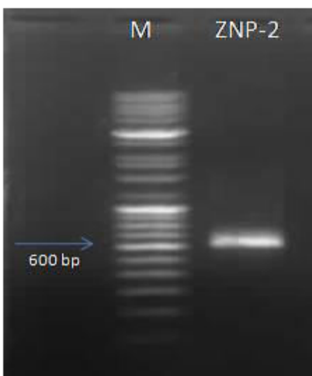


Fig. 3.5. Amplification and analysis of *AcdS* gene of *Bacillus lichniformis* HSW-16. Panel 'A' shows amplicon of *AcdS* (800 bp) in Lane 2 and DNA ladder mix (SM0331) in Lane 1. Panel 'B' represents dendrogram based on *AcdS* sequence of test isolate and other species which showed similar *AcdS* sequence. Other sequences of *AcdS* used for analysis were retrieved from NCBI Genbank data base. Neighbor-joining method was performed using the software packages Mega version 6.0, at the bootstrap value of (n = 500).

A



B

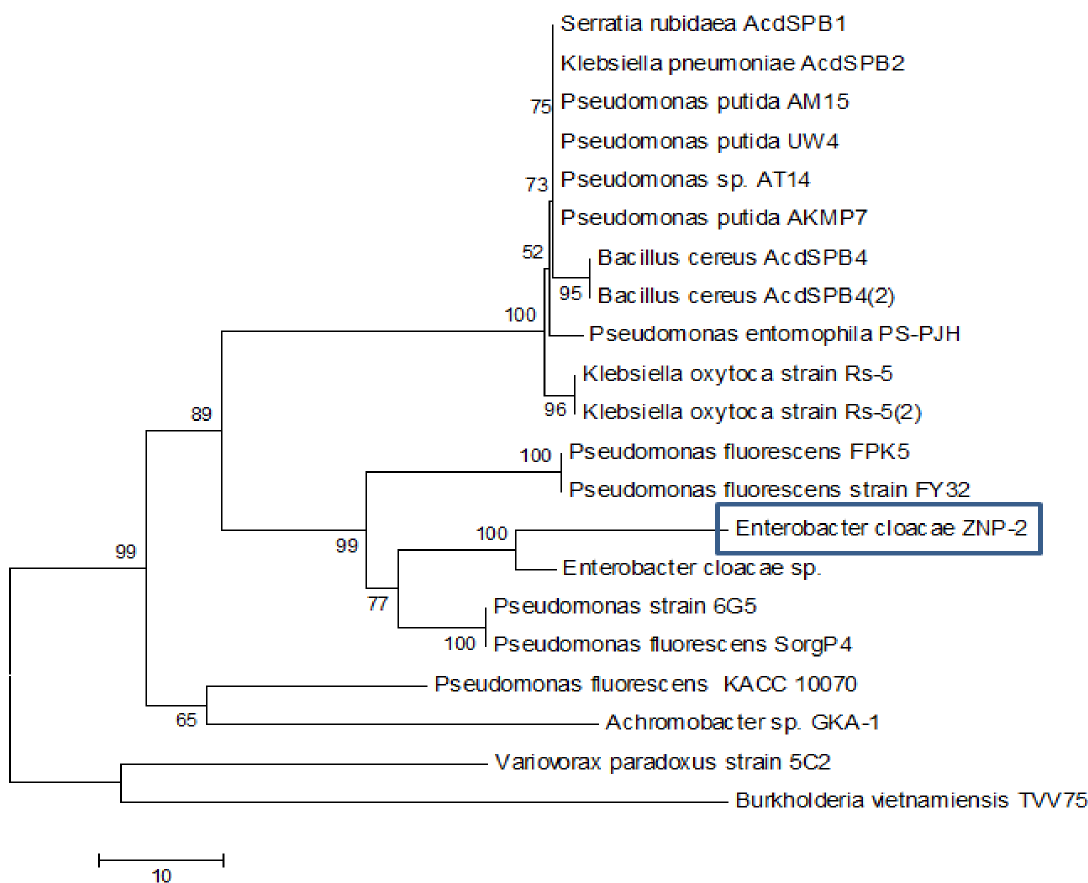


Fig. 3.6. Amplification and analysis of *AcdS* gene of *Enterobacter cloacae* ZNP-2. Panel 'A' shows amplicon of *AcdS* (600 bp) in Lane 2 and DNA ladder mix (SM0331) in Lane 1. Panel 'B' represents dendrogram based on *AcdS* sequence of ZNP-2 and other closely related species. After retrieving the other sequences from NCBI Genbank data base, Neighbor-joining method was performed using the software packages Mega version 6.0, at the bootstrap value of (n = 500).

Plant growth promotion test under salt stress conditions

All the isolates were tested for pot study with and without salt stress to evaluate their effect on the growth of wheat seedlings under stress conditions. Except few, inoculation with all the isolates significantly increased the growth of wheat plants in terms of various growth parameters tested. It is evident from Table 3.8 A that highest significant ($P<0.05$) growth in shoot length was observed for SBP-8 (19.60%) followed by HSW-16 (15%) at 0 mM NaCl, as compared to uninoculated control. SBP-8 was found to be the best isolate which increases the root length (25.25%), fresh weight (31.36%), dry weight (32.9%), and chlorophyll a (72%) at 0 mM NaCl. Similarly, increases in root length (23.30%), fresh weight (22.88%), dry weight (21.09%), and Chlorophyll a (62.46%) was observed following inoculation of HSW-16. Among the tested isolates, ZNP-1, ZNP-2, SBP-9, SBP-10, and SL-11 were found to increase significantly the fresh weight and chlorophyll content. Following application of NaCl stress of 150 mM, the reduction in shoot length (10.5%), root length (8.2%), fresh weight (7.4%), dry weight (8.7%), chlorophyll a (4.8%) and chlorophyll b (36%) was observed in control plants. At 150 mM NaCl, SBP-8 inoculation significantly increased the shoot length (20.8%), fresh weigh (18.8%), chlorophyll a (70.64%) and chlorophyll b (31.39%) as compared to control plants treated with respective salt stress (Table 3.8 B). There was no any significant increase in root length was observed for any of the tested isolates. Few of the bacterial isolates namely ZNP-4, SBP-6, SBP-7, SBP-10 and SL-11 could not show an increase in chlorophyll b content at 150 mM NaCl.

Furthermore, at 175 mM NaCl stress, the test isolate SBP-8 showed the significant ($p<0.05$) increase in shoot length (19.70%), root length (19%), fresh weight (20.4%), and dry weight (19.5%) as compared to uninoculated plants treated with respective salt stress. The isolates were further tested for their growth promotion effect at a higher salinity of 200 mM NaCl. However, treatment with 200 mM NaCl, decrease in various growth parameters of wheat was observed as shoot length (53.7%), root length (31%), fresh weight (49.4%), dry weight (48%), chlorophyll a (210%) and chlorophyll b (92%) in uninoculated control plants. It is evident from Table 3.8 C that SBP-8 was found to be a most efficient strain for promoting the growth among the other test strains. SBP-8 inoculation significantly increases the shoot length (47.42%), root length (36%), fresh weight (25%), and dry weight (43.7%) as compared to corresponding control plants. At 200 mM NaCl, SBP-8 inoculation remarkably increases the phtotosynthetic

pigments of chlorophyll a (168%) and chlorophyll b (59%) as compared to control plants treated with respective salt stress. Among the other tested isolates, HSW-16 inoculation was found to be second best isolates for promoting the wheat growth at 200 mM NaCl stress. HSW-16 inoculation significantly increase the shoot length (45%), root length (31.5%), fresh weight (21%), dry weight (37.5%), chlorophyll a (159%) and chlorophyll b (43%) as compared to control plants treated with 200 mM NaCl (Table 3.8 D).

Table 3.8 A Plant growth promotion test following inoculation of selected isolates under stress conditions

Control: 0 mM NaCl

S. No.	Isolates	Shoot length (cm)	Root length (cm)	Fresh wt.	Dry wt.	Chl a (mg g^{-1}FW)	Chl b (mg g^{-1}FW)
	Control	26.57± 1.59	20.08±2.11	2.36±0.34	0.237±0.028	3.25±0.56	1.88±0.32
1.	ZNP-1	29.10±2.70	23.90±2.87	2.88±0.031**	0.280±0.014**	4.82±0.21**	1.93±0.43
2.	ZNP-2	28.98±2.20	23.86±1.82	2.71±0.035**	0.272±0.019	4.78±0.29**	1.63±0.32
3.	ZNP-3	28.76±2.89	24.05±2.50**	2.68±0.050	0.258±0.011	4.58±0.34**	1.47±0.26
4.	ZNP-4	28.21±2.43	24.09±2.01**	2.74±0.025	0.271±0.010	4.76±0.49**	1.68±0.30
5.	SBP-6	29.07±2.42	24.16±1.87**	2.63±0.037	0.267±0.016	4.32±0.33**	1.30±0.29
6.	SBP-7	28.58±1.78	23.51±2.01	2.67±0.033	0.275±0.020	4.10±0.18**	1.18±0.23
7.	SBP-8	31.78±1.76**	25.15±1.35**	3.10±0.035**	0.315±0.014**	5.59±0.44**	2.10±0.30
8.	SBP-9	28.13±2.85	23.74±2.34	2.84±0.029**	0.250±0.017	4.45±0.31**	1.85±0.38
9.	SBP-10	29.35±2.28	23.86±2.02	2.81±0.023**	0.247±0.011	4.39±0.56**	1.32±0.47
10.	SL-11	28.59±2.63	24.12±2.24**	2.78±0.041	0.278±0.014**	4.35±0.31**	1.76±0.22
11.	SL-12	29.02±2.36	23.77±2.36	2.81±0.017**	0.253±0.019	4.20±0.39**	1.27±0.19
12.	HSW-16	30.50±2.10**	24.76±1.90**	2.90±0.023**	0.287±0.015**	5.28±0.41**	1.83±0.25

All the experiments were conducted in triplicates and results were expressed as mean±standard deviation (SD) (n=15). Data was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test at $p<0.05$(**). wt.: weight; Chl: chlorophyll, cm: centimeter.

Table 3.8 B Plant growth promotion test following inoculation of selected isolates under stress conditions

Stres: 150 mM NaCl

S. No.	Isolates	Shoot length (cm)	Root length (cm)	Fresh wt.	Dry wt.	Chl a ($mg\ g^{-1}FW$)	Chl b ($mg\ g^{-1}FW$)
	Control	24.05± 2.45	19.89±2.69	2.20±0.99	0.218±0.012	3.10±0.15	1.37±0.45
1.	ZNP-1	27.78±2.70**	22.09±2.87	2.50±0.230**	0.232±0.014**	4.10±0.17**	1.61±0.27**
2.	ZNP-2	28.18±2.20**	21.16±1.82	2.32±0.095**	0.238±0.019**	4.57±0.19**	1.79±0.22**
3.	ZNP-3	28.06±2.89**	20.55±2.50	2.58±0.070**	0.243±0.011**	4.10±0.17**	1.67±0.16**
4.	ZNP-4	28.21±2.43**	20.31±2.01	2.14±0.025**	0.237±0.011**	4.45±0.31**	1.52±0.10
5.	SBP-6	28.10±1.15**	21.05±1.87	2.22±0.085**	0.230±0.011**	3.87±0.17**	1.70±0.22
6.	SBP-7	28.18±1.78**	21.00±2.01	2.17±0.150**	0.242±0.027**	4.90±0.38**	1.57±0.34
7.	SBP-8	29.06±1.03**	22.56±1.30	2.59±1.05**	0.267±0.021**	5.29±0.22**	1.80±0.22**
8.	SBP-9	26.13±2.85**	21.04±2.34	2.14±0.055**	0.251±0.007**	4.76±0.36**	1.61±0.26**
9.	SBP-10	27.70±2.28**	20.86±2.02	2.26±0.145	0.237±0.071**	4.25±0.32**	1.55±0.29
10.	SL-11	26.59±2.63**	20.52±2.24	2.30±0.041**	0.230±0.014**	4.09±0.35**	1.60±0.20
11.	SL-12	26.02±2.36**	20.17±2.36	2.31±0.094**	0.250±0.009**	4.65±0.39**	1.76±0.19**
12.	HSW-16	27.24±2.12**	21.12±2.20	2.43±0.19**	0.24±0.025**	5.18±0.31**	1.62±0.18**

All the experiments were conducted in triplicates and results were expressed as mean±standard deviation (SD) (n=15). Data was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test at $p<0.05$ (**). wt.: weight; Chl: chlorophyll, cm: centimeter.

Table 3.8 C Plant growth promotion test following inoculation of selected isolates under stress conditions

Stress: 175 mM NaCl

S. No.	Isolates	Shoot length (cm)	Root length (cm)	Fresh wt.	Drt wt.	Chl a (mg g^{-1}FW)	Chl b (mg g^{-1}FW)
	Control	22.40±2.72	18.06±1.71	1.81±0.48	0.184±0.013	2.37±0.48	1.09±0.22
1.	ZNP-1	26.32±1.90**	19.41±1.68	1.97±0.21**	0.212±0.022**	3.80±0.24**	1.34±0.19**
2.	ZNP-2	26.37±2.65**	19.49±1.62	1.92±1.35**	0.208±0.024**	3.90±0.26**	1.38±0.15**
3.	ZNP-3	26.12±2.57**	19.38±1.53	1..98±0.07**	0.203±0.011**	3.72±0.38**	1.20±0.11
4.	ZNP-4	25.95±2.01**	19.72±1.71	2.10±0.07**	0.208±0.027**	3.90±0.26**	1.26±0.26**
5.	SBP-6	25.93±1.95**	19.65±1.80	2.10±0.05**	0.218±0.022**	3.31±0.19**	1.29±0.18**
6.	SBP-7	26.09±1.67**	20.78±1.53**	1.82±0.06	0.207±0.015**	3.46±0.33**	1.31±0.25**
7.	SBP-8	26.82±1.48**	21.47±2.11**	2.17±1.09**	0.220±0.013**	4.48±0.18**	1.52±0.15**
8.	SBP-9	25.82±2.10	18.89±1.87	2.02±0.143**	0.219±0.022**	3.87±0.35**	1.31±0.31**
9.	SBP-10	25.70±1.29	19.17±1.44**	2.05±0.089**	0.190±0.019	3.59±0.24**	1.43±0.17**
10.	SL-11	24.75±1.91	19.02±2.06	1.87±1.64	0.194±0.022	3.26±0.28**	1.18±0.19
11.	SL-12	25.01±1.77	18.69±1.88	1.91±1.65**	0.202±0.021**	3.50±0.25**	1.29±0.24**
12.	HSW-16	25.10±1.90	19.43±1.37	1.95±0.699**	0.196±0.0170	4.27±0.20**	1.24±0.15

All the experiments were conducted in triplicates and results were expressed as mean ± standard deviation (SD) (n=15). Data was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test at p<0.05(**). wt.: weight; Chl: chlorophyll, cm: centimeter.

Table 3.8 D Plant growth promotion test following inoculation of selected isolates under stress conditions

Stress: 200 mM NaCl

S. No.	Isolates	Shoot length (cm)	Root length (cm)	Fresh wt.	Dry wt.	Chl a (mg g^{-1}FW)	Chl b (mg g^{-1}FW)
	Control	17.29± 2.15	15.34± 1.20	1.59±0.57	0.160±0.04	1.08±0.20	0.98±0.17
1.	ZNP-1	24.09±1.63**	19.21± 1.96**	1.88±0.24**	0.176±0.03**	1.66±0.30**	1.30±0.23**
2.	ZNP-2	23.85±1.97**	19.00± 1.59**	1.79±0.13**	0.182±0.19**	1.98±0.28**	1.27±0.13**
3.	ZNP-3	24.10±1.93**	19.95± 1.77**	1.76±0.21**	0.23±0.019**	1.76±0.42**	1.24±0.09**
4.	ZNP-4	22.07±1.08**	19.88± 1.10**	1.92±0.070**	0.21±0.028**	2.26±0.29**	1.20±0.28**
5.	SBP-6	23.02±1.70**	18.77±1.41**	1.81±0.058**	0.21±0.022**	1.61±0.21**	1.29±0.21**
6.	SBP-7	24.30±1.40**	18.75±1.50**	1.87±0.065**	0.21±0.016**	2.31±0.30**	1.23±0.23**
7.	SBP-8	25.49±2.02**	20.87± 2.37**	1.98±0.75**	0.230±0.021**	2.90±0.31**	1.56±0.13**
8.	SBP-9	23.68±1.28**	19.76±1.55**	1.83±0.143**	0.22±0.022**	2.35±0.33**	1.19±0.29
9.	SBP-10	23.45±1.54**	18.91±1.19**	1.78±0.089*	0.19±0.02**	2.70±0.23**	1.27±0.26**
10	SL-11	24.09±1.95**	18.79±1.90**	1.91±0.132**	0.17±0.05**	2.81±0.42**	1.25±0.28**
11.	SL-12	25.10±1.86**	19.64±1.10**	1.89±0.15**	0.21±0.021**	1.90±0.40**	1.32±0.24**
12.	HSW-16	24.83± 1.90**	20.18± 1.36**	1.88±0.050**	0.186±0.017*	2.81±0.30**	1.41±0.21**

All the experiments were conducted in triplicates and results were expressed as mean±standard deviation (SD) (n=15). Data was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test at $p<0.05$(**). wt.: weight; Chl: chlorophyll, cm: centimeter.

Plant growth promotion test under temperature stress conditions

All the twelve selected isolates were tested for their effects on wheat growth under temperature stress also. Following application of isolates, we observed the higher growth, biomass and photosynthetic pigments in SBP-8 inoculated plants as compared to other tested isolates. As seen from Table 3.9, the highest significant ($P<0.05$) increase in shoot length (29.52%) was observed by SBP-8, followed by HSW-16 (25%), as compared to control plants. Considering the other parameters, the highest increase in root length (29.32%), fresh weight (59.17%), dry weight (39.8%), chlorophyll a (62.5%) and chlorophyll b (25%) was recorded in SBP-8 inoculated plants. The isolates ZNP-1, ZNP-2 and ZNP-3 were not effective for significantly increasing the root length, whereas the isolate SBP-10 and SL-11 did not show any significant effect on the fresh weight. Following the application of temperature stress of 30 ºC, the reduction in shoot length (18.6%), root length (14.4%), fresh weight (16%), dry weight (15%), chlorophyll a (61%) and chllorophyll b (63%) was recorded. However, among the other tested isolates, SBP-8 inoculation significantly increased the shoot length (26.46%), root length (27.13%), fresh weight (28.19%), dry weight (24.40%), chlorophyll a (47.14%) and chlorophyll b (60%) as compared to respective control plants treated with temperature stress of 30 ºC (Table 3.9 B). Isolates SBP-9 and ZNP-1 were found to be least effective for increasing the root growth. Further increase in temperature stress of 35 ºC, the decrease in various growth parameters of wheat plants was observed in uninoculated control plants. At 35 ºC, the decrease in shoot length (69.7%), root length (59%), fresh weight (28%), and weight (33%) was noted. A remarkable decrease in photosynthetic pigments chlorophyll a (250%) and chlorophyll b (128%) was recorded at 35 ºC in uninoculated control plants. The test isolate SBP-8 inoculation significantly increased the shoot length (44%), root length (23.96%), fresh weight (21%), dry weight (38%), chlorophyll a (94%) and chlorophyll b (55.8%) as compared to respective plants treated with temperature stress. Among the tested isolates, SL-12 was found to be ineffective for increasing the dry weight at 35 ºC of temperature stress as compared to other isolates (Table 3.9 C).

Table 3.9 A Plant growth promotion test following inoculation of selected isolates under temperature stress; 25 ^{0}C

S. No.	Isolates	Shoot length (cm)	Root length (cm)	Fresh wt.	Dry wt.	Chl a (mg g^{-1}FW)	Chl b (mg g^{-1}FW)
	Control	25.87±1.31	18.72±2.10	2.18±0.40	0.240±0.032	3.40±0.40	1.55±0.70
1.	ZNP-1	27.68±2.08*	20.58±2.10	2.88±0.031**	0.280±0.019**	4.70±0.26**	1.39±0.15
2.	ZNP-2	28.54±2.22**	19.87±1.91	2.71±0.035**	0.275±0.014**	4.31±0.25**	1.27±0.13
3.	ZNP-3	29.08±3.05**	19.75±2.19	2.68±0.050**	0.288±0.032**	4.10±0.17**	1.21±0.33
4.	ZNP-4	28.89±2.51*	20.08±1.38**	2.92±0.15**	0.291±0.023**	4.34±0.19**	1.26±0.38
5.	SBP-6	27.90±1.90**	21.10±1.65**	3.04±0.39**	0.293±0.013**	4.88±0.34**	1.56±0.23
6.	SBP-7	27.57±1.20**	20.18±2.20**	2.80±0.31**	0.287±0.018**	4.50±0.24**	1.38±0.34
7.	SBP-8	30.45±2.31**	22.90±2.35**	3.47±0.27**	0.335±0.022**	5.04±0.21**	1.90±0.33
8.	SBP-9	28.71±1.60**	21.87±1.57**	2.61±0.26**	0.271±0.031	4.28±0.27**	1.26±0.40
9.	SBP-10	29.30±1.41**	21.48±1.90**	2.50±0.19	0.289±0.016**	4.57±0.32**	1.21±0.29
10	SL-11	27.90±0.95**	21.29±2.49**	2.49±0.21	0.278±0.027**	4.07±0.28**	1.18±0.27
11.	SL-12	28.37±1.49**	19.56±1.28**	2.65±0.23**	0.253±0.033	4.49±0.32**	1.35±0.24
12.	HSW-16	29.51±1.30**	22.25±1.36**	3.18±0.31**	0.310±0.024**	4.91±0.22**	1.83±0.30

All the experiments were conducted in triplicates and results were expressed as mean±standard deviation (SD) (n=15). Data was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test at $p<0.05$(**). wt.: weight; Chl: chlorophyll, cm: centimeter.

Table 3.9 B Plant growth promotion test following inoculation of selected isolates under temperature stress; 30 ^{0}C

S. No.	Isolates	Shoot length (cm)	Root length (cm)	Fresh wt.	Dry wt.	Chl a (mg g^{-1}FW)	Chl b (mg g^{-1}FW)
	Control	21.80±2.53	16.36±1.88	1.88±0.05	0.209±0.027	2.10±1.08	0.95±0.42
1.	ZNP-1	26.83±2.80**	18.36±2.55	2.28±0.120**	0.228±0.0104*	2.76±0.34**	1.26±0.20**
2.	ZNP-2	26.20±2.006**	19.27±1.386**	2.23±0.680**	0.244±0.0132*	2..95±0.40**	1.21±0.23**
3.	ZNP-3	27.00±3.143**	19.10±2.842**	2.30±0.094**	0.241±0.018*	2.89±0.29**	1.29±0.27**
4.	ZNP-4	26.01±2.73**	20.29±1.77**	2.35±0.126**	0.251±0.024**	2.81±0.36**	1.38±0.25**
5.	SBP-6	27.13±1.31**	20.85±2.92**	2.22±0.130**	0.238±0.008*	2.59±0.31**	1.26±0.19**
6.	SBP-7	26.89±1.47**	20.49±1.82**	2.26±0.14**	0.227±0.029*	2.97±0.43**	1.29±0.24**
7.	SBP-8	27.57±2.07**	20.45±1.75**	2.41±0.080**	0.260±0.016**	3.09±0.65**	1.52±0.15**
8.	SBP-9	27.24±1.33**	18.56±1.99	2.25±0.14**	0.253±0.004**	2.87±0.33**	1.37±0.27**
9.	SBP-10	26.25±1.20**	19.59±0.982**	2.12±0.22	0.232±0.058**	2.67±0.21**	1.41±0.29**
10	SL-11	25.83±0.95**	19.37±0.900**	2.16±0.27	0.237±0.011**	2.92±0.29**	1.33±0.31**
11.	SL-12	26.15±0.713**	19.88±0.794**	2.33±0.076**	0.242±0.004**	2.83±0.26**	1.40±0.34**
12.	HSW-16	26.01±1.07**	20.80±1.41**	2.29±0.170**	0.251±0.011**	2.70±0.34**	1.39±0.25**

Table 3.9 C Plant growth promotion test following inoculation of selected isolates under temperature stress; 35 ^{0}C

S. No.	Isolates	Shoot length (cm)	Root length (cm)	Fresh wt.	Dry wt.	Chl a (mg g^{-1}FW)	Chl b (mg g^{-1}FW)
	Control	15.25±1.30	11.73±1.58	1.79±0.09	0.18±0.007	0.95±0.74	0.68±0.39
1.	ZNP-1	25.43±1.53**	17.48±1.58**	2.59±0.212**	0.230±0.020**	1.58±0.44**	0.88±0.26**
2.	ZNP-2	25.90±1.30**	18.76±1.49**	2.45±0.050**	0.226±0.003**	1.61±0.26**	0.85±0.30**
3.	ZNP-3	26.07±1.03**	18.06±0.86**	2.56±0.049**	0.229±0.009**	1.59±0.34**	0.98±0.33**
4.	ZNP-4	26.15±1.46**	17.72±0.94**	2.61±0.025**	0.250±0.028**	1.70±0.37**	0.91±0.39**
5.	SBP-6	27.06±0.83**	17.86±0.70**	2.50±0.035**	0.241±0.009**	1.63±0.31**	0.87±0.34**
6.	SBP-7	27.60±0.95**	18.88±0.70**	2.63±0.064**	0.245±0.006**	1.69±0.39**	0.97±0.32**
7.	SBP-8	27.78±0.66**	19.50±0.70**	2.65±0.092**	0.259±0.018**	1.85±0.54**	1.06±0.81**
8.	SBP-9	27.19±1.01**	16.26±0.61**	2.60±0.020**	0.25±0.001**	1.81±0.41**	0.91±0.20**
9.	SBP-10	24.79±0.95**	17.59±1.13**	2.39±0.115**	0.219±0.02**	1.80±0.43**	0.95±0.24**
10	SL-11	23.29±1.34**	18.31±0.84**	2.29±0.046**	0.22±0.006**	1.67±0.29**	0.84±0.23**
11.	SL-12	22.46±1.74**	18.37±0.84**	2.43±0.051**	0.20±0.010	1.55±0.32**	0.97±0.27**
12.	HSW-16	24.92±1.13**	17.43±0.90	2.39±0.085**	0.227±0.015**	1.75±0.40**	1.02 ± 0.35**

2.4 Discussion

The present study demonstrates the effectiveness of plant growth promoting activity of rhizospheric bacteria possessing the ACC deaminase activity in improving plant growth under abiotic (salt & temperature) stress conditions. The selected isolates were isolated from the rhizospheric soil of various plants growing in the desert region of Rajasthan, which is exposed to varying degree of salt and temperature stress. Before conducting the effect of selected PGPR isolates on the growth of the wheat plant, all given bacterial isolates were characterized on physiological and molecular level to estimate the potential of PGPR to sustain in stress conditions and influence plant growth. Present report elucidates the detail characterization of ACC deaminase activity under various physiological conditions in bacterial isolates like *Enterobacter, Klebsiella, Serratia,* and *Bacillus* sp. Similarly, the present study is the first attempt to evaluate the performance of such broad range of bacterial isolates to improve the plant growth under abiotic stressors like salinity and temperature.

The properties of ACC deaminase activity and some additional mechanisms of plant growth promoting bacteria (PGPB) to ameliorate stresses in host plants are referred as 'induced systemic tolerance' (Yang et al., 2009). Since, many PGPR having ACC deaminase activity are known to promote plant growth under biotic and abiotic stress conditions, selection of the isolates in the present study was primarily based on high ACC deaminase activity and other plant growth promoting properties. As the microbial activity is severely affected by its surrounding environnment and different physiological factors, it was thought to estimate the performance of different bacterial isolates in terms of ACC deaminase activity under varying conditions including temperature, salt, pH, substrate concentration etc. From the results obtained, it was interesting to note that growth and enzyme activity for most of the isolates were highest at 6% salt concentration. This increase in ACC deaminase activity might be due to increase in *AcdS* gene expression. The increase in *AcdS* gene expression of *Enterobacter* sp. and *Chryseobacterium* sp. was observed on increasing the salt stress from 0-4% NaCl (Tittabutr et al., 2013), however the specific mechanism of gene expression with an increase in salinity is still unknown. Furthermore, the temperature of 30 ºC was found to be optimum for ACC deaminase activity in the tested isolates. In the other tested physiological parameters, the higher enzyme activity was recorded at 3 mM of ACC concentration, pH (8.0) and 48 h of the incubation period. Results of ACC deaminase activity of isolates with relation to the concentration of substrate

(ACC), pH and incubation period tallied with previous reports (Jha et al., 2012). The presence of ACC deaminase activity under extreme environmental conditions might be useful in promoting the plant growth at unusual environmental sites or conditions and thus that allow host plants to survive under adverse conditions (Glick, 2005).

Further, ACC deaminase activity was characterized at the molecular level by amplifying *AcdS*, a gene encoding for ACCD in bacteria. Amplification of *AcdS* gene in bacteria is a proof for the presence of gene responsible for enzymatic activity. Previous studies have also shown successful amplification of *AcdS* gene in a variety of bacteria (Jha et al., 2012; Shrivastava and Kumar, 2013). More recently, ACC deaminase has been found in wide range of gram positive/negative bacteria, rhizobia and fungi (Singh et al., 2015). Putative ACC deaminase gene has also been reported in several species including *R.leguminosarum bv.trifoli* (Itoh et al., 1996) and *Mesorhizobium loti* MAFF303099 (Kaneko et al., 2002). The expression level of ACC deaminase varies from one organism to another and the adjacent environmental conditions. In the present study, the successful *AcdS* gene amplicon was observed in the three isolates only. In some of the isolates, PCR yielded non-specific bands, while in other did not yield any PCR amplicon. This is not surprising, as previous studies also found nonspecific bands (Blaha et al., 2006; Shat et al., 1998). This could be due to non-conservative nature of ACC deaminase gene and also variations in the short DNA sequences of the priming sites (Blaha et al., 2006). Recent study by Nascimento et al. (2014) has suggested that ACC deaminasehas evolved mainly through vertical transfer with the occasional horizontal transfer. However, the similarity of *AcdS* sequences with diverse species suggests that prevalence of *AcdS* gene may not be directly related to the phylogeny.

To exploit the ACC deaminase containing PGPR, their role in stimulating the growth of host plant must be assessed in both laboratory and field level. In addition, the ability of the PGPR to cross-infect other plants than the plants from where it was originally isolated is also of utmost importance so that they can be used widely for ameliorating abiotic stressors in other important plants. Therefore, we tested the effect of selected PGPR to promote the growth of wheat, a staple food crop under standard conditions. Bacterial inoculation under laboratory conditions significantly enhanced plant growth for different parameters tested. The significant decrease in shoot/root lengths, fresh/dry weight was observed in un-inoculated plants under salt stress, whereas bacterial treatment increased their growth significantly. It is likely that this

growth behavior might be due to bacterial ACC deaminase activity. From the growth promotion results, *Klebsiella* sp. SBP-8 emerged as the best isolate in terms of protecting plants from the deleterious effects of salt and temperature stress. Our results are in agreement with the previous report for salt tolerance in various plants induced by PGPR (Mayak et al., 2004; Saravankumar and Samiyappan, 2007; Zahir et al., 2009). Inoculation with ACC deaminase bacterium *Pseudomonas putida* UW4 has been shown to enhance the growth in various physiological parameters of canola plants under the inhibitory level of salt stress (Cheng et al., 2007). Our results are in congruence with other studies where ACC deaminase bacteria were observed to stimulate plant growth under salt stress conditions (Kalra et al., 2014; Jha et al., 2011). The higher increase in chlorophyll contents indirectly indicates the improvement in photosynthetic activity in bacteria- inoculated plants. Increase in chlorophyll content and photosynthetic activity in response to PGPR has been demonstrated in some of the earlier studies (Nadeem et al., 2006; Han and Lee, 2005). More importantly, the isolates were found to be effective in decreasing plant growth inhibition due to salt and temperature (abiotic) stressors. The effectiveness of these isolates opens up the possibility to evaluate the performance at field scale and even with different crop plants. In this study, we have shown that PGPR with ACC deaminase activity has tremendous potential to counteract the abiotic stressors. The pot experiments suggest that inoculation of bacterial isolates protects the plants against adverse effects of salt and temperature stress. In brief, the observed results in this study indicates that use of such multifarious PGPR hold a great potential to be used as biofertilizer in saline soil.

CHAPTER IV

Evaluation of *Klebsiella* sp. SBP-8 on plant growth under abiotic stress condition: a multifunctional study

4.1Introduction

Plant growth promoting rhizobacteria have received great attention in the last few years due to the commercial considerations involved because the use of agrochemicals to enhance plant growth and combat plant stress (abiotic and biotic) is environmentally and economically not benign, and also cause a reduction in the beneficial organism population (Glick and Bashan, 1997). These rhizobacteria reside in the vicinity of the roots of plants and majority of them do not show any disease symptoms. Some of the PGPR also colonize the plant interior and behave as endophytes. They obtain their nutrients from plant and at the same time enhance plant growth, provide stress relief, and help ward off potential pathogens. Therefore, it is assumed that such mutualistic interactions between host plants and associated microorganisms must have emerged as a result of the clear positive selection exerted on these associations.

Plant rhizosphere houses a plethora of microorganisms some of which establish an intimate relationship with plants and influence their growth and physiology. Hence, it becomes vital to analyze how plants differentiate between an endophyte and a pathogen. In the present study, *Klebsiella* sp. SBP-8 was found to be an important plant growth promoting rhizobacteria due to its ability to protect plants from abiotic stress conditions and its ability to colonize plants efficiently. It has several plant growth promoting traits such as ACC deaminase activity, indole acetic acid production, N_2-fixation, phosphate solubilization and siderophore production (Chapter II), and confers induced systemic tolerance (Chapter III). Apart from above-mentioned properties, *Klebsiella* sp. SBP-8 also showed strong antagonistic activity against various phytopathogens, which indicated its ability to confer induced systemic resistance. Therefore, it can be exploited as a promising bioinoculant to be used as a biocontrol agent as well.

It is established that PGPR possessing 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity could reduce the stress ethylene level through the hydrolytic cleavage of the ethylene biosynthesis precursor ACC (Glick et al., 1998; Madhaiyan et al., 2006) and maintain the ethylene level sufficient for biological processes. In addition to the production of stress ethylene in plants under salt stress, several biochemical processes like protein synthesis and depletion of photosynthetic pigments are severely affected by increased salinity (Parida and Das, 2005). An elevated level of salinity also causes restriction of water uptake and toxicity of Na^+. Increased accumulation of Na^+ in salt rich agricultural land promotes

senescence of older leaves (Munns and Tester, 2008). High salinity induces both ionic and osmotic stress on plants by enhancing the generation of reactive oxygen species (ROS), which finally cause the deleterious oxidative damage (Gill and Tuteja 2010; Munns and Tester, 2008). In addition, higher accumulation of salt ions reduces the K^+ uptake by roots of higher plants (Maathuis et al., 2014). A number of experimental studies have demonstrated that salinity negatively affected the Ca^{2+} absorption and their translocation in plants (Liu et al., 2014; Zhang and Shi, 2013). Amelioration of salt stress using physical and chemical methods is not practically feasible. Therefore, a PGPR having ability to support plant growth under salt stress will have immense agricultural importance. In recent times, the potential of PGPR to alleviate agricultural loss arising due to salinity is being explored by several researchers. Some of the PGPR can enhance plant growth under stressful conditions w.r.t. salt stress through multiple mechanisms other than by decreasing the level of stress ethylene in plants.

The plants have developed several physiological and biochemical mechanisms to combat salt and other stress conditions. These mechanisms include osmotic adjustment by secretion of compatible solutes 'osmolytes' (Shabala and Shabala, 2011). Osmolytes mediated protective mechanism is still not fully understood, however, it generally favors the osmotic adjustment (Colmer et al., 1995). Moreover, to mitigate the salinity-induced oxidative damages, plant produce antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) which protect plants from oxidative stresses. The PGPR can ameliorate salt stress by abetting host plants in employing one or more of above-mentioned mechanisms to the greater extent. Therefore, exploration and selection of efficient PGPR can be successfully used to manage salt stress in plants growing in saline soil.

Therefore, present work made an effort to investigate abilities of *Klebsiella* sp. SBP-8 to diminish deleterious effects resulted from salinity of soil and influence plant growth in sustainable manner and to characterize its ability to colonize wheat plants. In addition, the *AcdS*$^-$ mutant of selected isolate (SBP-8) was constructed with transposon-based mutagenesis to evaluate role of ACCD in improving plant growth under salt stress.

4.2 Material and Methods

4.2.1 Bacterial culture and growth conditions

The bacterium *Klebsiella* sp. SBP-8 isolated from the rhizospheric soil of *Sorghum bicolor* was used in this study. The growth condition and detail characterization of plant growth

promoting properties has been reported earlier (Singh et al., 2015, Chapter II, Section no. 2.2). The bacterium was maintained on the DF-agar medium supplemented with 3 mM ACC (Sigma-Aldrich, USA).

4.2.2 Preparation of FAME samples and GC-MS analysis

To test the effect of salt stress on composition of fatty acid in bacterial cell membrane, fatty acid contents were analyzed using GC-MS. For this, the isolate was grown for 24 h in DF minimal medium (with 3 mM ACC) amended with different concentrations of salt (0 to 200 mM). The bacterial cultures were harvested and the cell pellets were sonicated in lysis buffer containing Tris–HCl (50 mM, pH 7.6), dithiothreitol (1.l mM), 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysozyme (0.2%). The resulting suspension was extracted with 2:1 ratio of chloroform and methanol and centrifuged at 5,000 g for 10 min. The supernatant was diluted ten times with the above solvent system and kept on a shaker at 160 rpm for 3 h to separate the phases. The lower organic phase was concentrated on a rotary evaporator, re-dissolved in toluene, and converted to fatty acid methyl ester (FAME) by trans-esterification reaction following the standard protocol (Chriestie, 2003). The concentrated samples (l µl) were used for intracellular product profiling using gas chromatography-mass spectroscopy (GC-MS) on a Shimadzu QP-2010 Plus instrument equipped with a split/splitless injector and capillary column DB-5 MS (0.25 µm film thickness, 0.25 mm id, 30 m length) under following conditions: splitless mode, initial oven temperature 50 °C held for 2 min, injector temperature 250 °C and detector temperature 230 °C. The temperature was increased from 50 to 250 °C at a rate of 10 °C per min and was held for 3 min on reaching 250 °C. The conditions applied for MS analysis were: scan mode, start time 400 min, end time 3199 min, scan speed 1250 amu sec^{-1}, event time 05 s, start m/z 4000 and end m/z 65000 (Mishra et al., 2015).

4.2.3 Plant growth and experimental design

Surface sterilization of wheat seeds (*Triticum aestivum* var. C309), preparation of bacterial inoculum, soil sterilization, and seed treatment were performed as per the protocol mentioned in chapter III, section no 3.2.4.3). Plants were grown for 15 days after germination under controlled condition in a plant growth chamber (Labtech, South Korea) set with 140 µmol $m^{-2}s^{-1}$ of light for 16:8 h light/dark period at 24±2 °C with humidity of 70%. Hoagland medium supplemented with salt (150 mM, 175 mM, 200 mM) was used for providing the nutrient as well as imposing the salt treatments at every alternate day. Each treatment was

taken in triplicate, and pots were arranged in completely randomized block design. The treated plants were used for estimation of ionic content, biochemical analysis to estimate the osmolyte content, antioxidative activity and for colonization studies.

4.2.4 Estimation of ionic content (Na^+, K^+, Ca^{2+}) under salt stress

For estimation of Na^+, K^+, and Ca^{2+}, shoots were washed with autoclaved Milli-Q water five to six times and oven dried at 70 °C for 48 h. Subsequently, 1g of shoot tissue was ground in liquid N_2 and digested in a mixture of 30% H_2O_2, 65% HNO_3, and deionized water in a ratio of 1:1:1 at 120 °C for 2 h to a final volume of 12 ml in a microwave digester. The final volume was adjusted to 20 ml with deionized water. Ions namely Na^+, K^+, and Ca^{2+} were estimated by an atomic absorption spectrophotometer (AAS 2380, Perkin Elmer, USA) at National Horticultural Research and Development foundation (Nashik, India). For accuracy, each sample was analyzed in triplicates.

4.2.5 Biochemical Analysis

To understand physiological changes and adaptation mechanisms of plants under salt stress conditions, bacterium-treated samples were analyzed for various ions, osmolytes, and chemical protectants following standard protocols described below.

4.2.5.1 Proline content

Proline content in the leaves was determined by following the standard protocol with minor modifications (Bates et al., 1973). A 0.5 g of fresh leaves was homogenized in 3 ml of 5% (w/v) sulfosalicylic acid and centrifuged at 8,500 g for 10 min. To 500 µl supernatant, sterile water was added to make volume of 1 ml and 2 volume of 2% ninhydrin was added to the reaction mix. The mix was gently vortexed and boiled for 30 min at 100 °C. After cooling, an equal volume of toluene was added to the mixture and upper aqueous phase was used for taking absorbance at 520 nm in a spectrophotometer (Jasco Corporation, Japan). The proline content was calculated by comparing with a standard curve using L-proline as standard (Sigma-Aldrich, USA).

4.2.5.2 Estimation of total soluble sugar (TSS) and protein content

Total soluble sugar was estimated by anthrone reagent (Irigoyen et al., 1992). Alcoholic leaf extract (0.1 ml) was prepared by homogenizing 0.5 g leaf in 3 ml of 80% ethanol. 3 ml of freshly prepared anthrone reagent was added to the reaction mix and placed in a boiling water bath for 10 min. The absorbance of the resultant sample was measured at 620 nm along with

reaction mixture containing 20-400 µg ml^{-1} of glucose as a standard for making calibration curve for quantification of soluble sugar synthesized in plants. For the estimation of total protein content, 0.5 g plant tissue was homogenized in extraction buffer containing 50 mM Tris-HCl (pH 8.3), 1 mM EDTA, 3 mM DTT, 0.08% ascorbic acid, and 1 mM PMSF. The total protein was quantified by Bradford method (Bradford, 1976) keeping BSA as a standard.

4.2.5.3 Estimation of auxin

An alcoholic extract of shoot tissue, as prepared above was used for measuring the auxin content (Andreae and Ysselstein, 1959). One ml alcoholic extract was mixed with 2 ml of Salkowsky reagent in the dark and incubated for 20 min. The absorbance was measured at 535 nm and compared with the standard curve of IAA (0 to 10 µg ml^{-1}).

4.2.5.4 Malondialdehyde (MDA) content

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content produced by thiobarbituric acid reaction following method of Hodges et al. (1999) with minor modification. Briefly, 1 ml of the alcoholic extract prepared from 0.5 g of leaves was mixed with 1ml of 0.5% thiobarbituric acid containing 20% trichloroacetic acid. The mixture was heated up to 90 °C for 30 min in a water bath. After cooling to room temperature, the sample was centrifuged at 5,000 g for 5 min and absorbance was measured at three wavelengths i.e. 400, 532 and 600 nm. After subtracting the non-specific absorbance, the MDA concentration was determined by its molar extinction coefficient (155 $nm^{-1}cm^{-1}$) and the results were expressed as mmol MDA g^{-1} fresh weight (FW).

4.2.6 Antioxidant enzyme activities

4.2.6.1 Superoxide dismutase (SOD) activity

The activity of SOD was determined in control and bacterium-inoculated plants by the photoreduction of nitroblue tetrazolium (NBT). The reaction mixture contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 13 mM methionine, 75 mM NBT, 2 mM riboflavin, and 100 ml of enzyme extract. Riboflavin was added as the last component, and the reaction was started by placing the tubes under two 15 W fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Absorption of the reaction products were measured at 560 nm. The volume of the supernatant corresponding to 50% inhibition of the reaction was assigned a value of one enzyme unit and activity was expressed as unit mg^{-1} protein.

4.2.6.2 Catalase assay

To measure catalase activity, 0.5 g of leaf material was homogenized with 50 mM phosphate buffer (pH 7.0) and the homogenate was centrifuged at 8,000 g for 20 min at 4 °C. Twenty ml of enzyme extract was added to 3 ml of hydrogen peroxide phosphate buffer (pH 7.0). The time required for the decrease in the absorbance at 240 nm from 0.45 to 0.40 was noted. Enzyme solution containing hydrogen peroxide-free phosphate buffer was used as a control.

4.2.6.3 Peroxidase (POD) activity

For the peroxidase (POD) activity, assay mixture consisting of 0.1 M phosphate buffer, 0.1 mM pyrogallol, and 5 mM H_2O_2 with enzyme extracts (100 µl) was incubated for 5 min at 25 °C. The reaction was stopped by adding 1.0 ml of 2.5 N H_2SO_4. The absorbance of indigo color formed was read at 420 nm against blank containing water in place of enzyme extract.

4.2.7 Colonization study

An ability of *Klebsiella* sp. SBP-8 to colonize wheat plants was examined by flurorescent microscopy of plant surface, ERIC (Enterogenic repetitive integenic consensus)-PCR-based fingerprinting, and counting of colony forming unit (CFU) of bacteria isolated from SBP-8-treated plants as described below.

4.2.7.1 Fluorescence microscopy

From 15 days old plants treated with *Klebsiella* sp. SBP-8, 1-2 cm of root segments were cut using sterile scalpel and used for staining with 0.1% acridine orange for 2-3 min. The stained roots were thoroughly washed three times with sterile distilled water and placed on a glass slide with a cover slip on top of it and viewed under epi-fluorescence microscope (Olympus-CKX41, Olympus, Japan) at intensity between 450 and 490 nm using 100 X objective lens and 10 X eyepiece lens.

4.2.7.2 ERIC-PCR

For confirming identity of colonized bacteria, ERIC-PCR was performed to amplify DNA fragments containing enterobacterial repetitive intergenic consensus (ERIC) sequence. At 15th day of plant growth, total DNA was extracted from plant root using plant genomic DNA extraction kit (Himedia, India). For ERIC-PCR, primers 1R F' (5'-ATGTAAGCTCCTGGGGATTCAC-3') and 2R (5'-AAGTAAGTGACTGGGGTGAGCG-3') were used. PCR reaction was performed in a 50 µl reaction volume containing 50 ng (3µl) of template, 125 µM each dNTPs (Genei, India), 1.5 mM $MgCl_2$, 20 pmol of each primer and

1.5 U of Taq DNA polymerase in a DNA thermal cycler (T100, Bio-Rad, USA). The cycling condition was: initial denaturation for 5 min at 94 °C, 30 cycles each consisting of denaturation for 1 min at 94 °C, primer annealing for 1 min at 52 °C and extension at 72 °C for 5 min and a final extension of 5 min at 72 °C. Analysis of amplified products was done on 1.5% agarose gel containing 0.5μg ml^{-1} ethidium bromide using gel documentation system (Bio-Rad, USA).

4.2.7.3 Colony forming units (CFU) counting

To estimate the colonization efficiency of the test isolate, bacterial number was counted in terms of CFU from experimental plants. The plants were surface sterilized and cut into small sections of 0.3×0.4 cm. The sections were vigorously macerated in sterilized 1X PBS buffer, plated onto nutrient agar (NA) media and incubated for 2-4 days at 30 °C. The colonies were counted using plate dilution method and represented as CFU/g fresh tissue.

4.2.8 Generations of *AcdS*$^-$ mutant

To confirm the role of ACC deaminase in amelioration of stress, null mutant of *AcdS* (*AcdS*$^-$) was generated using transposon-based mutagenesis as per the method described below.

4.2.8.1 Bacterial growth and materials

Bacterial isolate *Klebsiella* sp. SBP-8 was initially revived from –80 °C glycerol stock on Luria-Bertani (LB) agar plates. The antibiotic sensitivity of the isolate was confirmed by growing it on LB containing trimethoprim (100 μg/ml). After transposon mutagenesis, the bacterium was grown and maintained on trimethoprim (Tm) containing LB-medium.

4.2.8.2 Preparation of competent cells

Freshly grown single isolated colony was inoculated into 25 ml of LB media and incubated at 37 °C. Overnight cultures (250 µl) were transferred to 250 ml of no salt LB media with/without 30 µg/ml of trimethoprim at 37 °C under aerobic conditions until turbidity at 600 nm of 0.4-0.5 was reached. Afterward, cultures (250 ml) were cooled on ice immediately and incubated for the next 45 min. Cultures were transferred to 250 ml pre-cooled centrifuge bottles and centrifuged at 5,000 g for 10 min at 4 °C. Cell pellets were re-suspended (with gentle shaking) in 250, 200 and 100 ml of volume consecutively with 10% glycerol and then centrifuged again at 5,000 g for 10 min at 4 °C. The cell pellet was re-suspended in the remaining glycerol into the tube after decanting and 50 µl aliquots of high density cells were stored in pre-cooled 1.5 ml micro-centrifuge tubes at -80 °C for future applications.

4.2.8.3 Transposon mutagenesis

Transposon mutagenesis of selected isolate was performed using EZ::TNTM <DHFR-1> Transposome TM Kit (Epicentre, USA) following instructions of manufacturer with slight modifications. A mixture of competent cells, transposon and restriction inhibitor reagent to increase transposon insertion efficiency was prepared and incubated for 15 min on ice. The mix was transferred into an electroporation cuvette (BioRad, USA), and cells were electroporated at 25 ℃ in an electroporator (BioRad, USA) set at 1250 volts. After electroporation, the cells were immediately transferred on ice and then cultured in 1 ml of SOC medium at 30 °C for 1 h to let them grow and recover. Cultured cells (100 µl) were spread on Muller-Hinton (M-H) agar plates containing Tm (30 µg/ml). Plates were incubated at 30 °C overnight. Materials and reagents to conduct transposon mutagenesis for this study were prepared the day before to perform the experiment. Bacterial colonies growing on Muller Hinton-Tm agar plates were selected for further confirmation of ACC utilization by growing on the DF-ACC plate. The bacterial colony unable to grow on DF-ACC agar plate was further selected for enzymatic assay and plant growth test against wild type.

4.2.8.4 ACCD enzyme assay of wild type and mutant strain

To confirm the mutation in the *AcdS* gene of isolate SBP-8 (*Klebsiella* sp), ACC deaminase assay was performed for the wild and selected mutant colony by following the protocol as discussed in Chapter II, section 2.2.4.2. For the assay, the ACC deaminase mutant (*AcdS*$^-$) of *Pseudomonas putida* UW4 was taken as a negative control, while the wild type *Pseudomonas putida* UW4 (a gift from Bernard R Glick, Waterloo university) was used as positive control.

4.2.8.5 Effect of *Klebsiella* sp. SBP-8 *AcdS*$^-$ mutant on plant growth

A test of plant growth promotion was performed to evaluate the *AcdS* gene mutation on the various physiological parameters of wheat (*Triticum aestivum*) plant. Preparation of bacterial inoculum of wild type and mutant was performed as mentioned in Chapter III, section 3.4.3 By using the bacterial inocula of wild type (SBP-8 *AcdS*$^+$) and mutant (SBP-8 *AcdS*$^-$), plant growth promoting test was performed as per mentioned in section III, section 3.2.4.4.

4.2.8.6 Statistical analysis

All the experiments were conducted in triplicates and results were expressed as mean ± standard deviation (SD). Data was analyzed by analysis of variance (ANOVA) and subsequently by Duncan's multiple range test at $p<0.05$.

4.3 Results

GC-MS-based FAME analysis was done to find out changes in fatty acid profile of the test isolate under various concentration (0, 175 mM, 200 mM) of NaCl stress (Fig. 4A-C). At 0 mM NaCl, the hydrocarbons like Pentacosane (0.35%), Tetracosane (1.64%), Triacontane (3.52%), Nonacosane (6.59%), Docosane (9.36%), Tetracontane (10.93%), n-Tetratriacontane (10.51%), Hexatriacontane (9.17%), n-Tetratetracontane (6.63%), Tetrapentacontane (2.65%) were observed (Table 4.1). As the NaCl increased from 0 mM to 175 mM, the extract composition changed having higher level of long chain alkanes like Pentacosane (0.56%), Tetracosane (1.78%), Triacontane (4.62%), Docosane (12.95%), n-Tetratriacontane (12.52 %), Hexatriacontane (15.27%), n-Tetracontane (8.82%), and Tetrapentacontane (3.82%) (Table 4.2). The presence of fatty alcohols has been summarized in Table 4.2. At 200 mM NaCl, the various hydrocarbons with higher percentages like Eicosane (0.20%), Hexadecane (0.42%), Pentacosane (1.54%), Tetracosane (3.74%), Triacontane (6.99%), Tetracontane (3.74%), n-Tetratriacontane (11.05%), Hexatriacontane (10.76 %), Tetrapentacontane (1.57%) were observed (Table 4.3). Thus, the GC-MS analysis of cell extract confirmed the presence of long chain hydrocarbon and fatty alcohol in bacterial cells grown at higher salt concentration. The GC-MS chromatograph of fatty acid methyl esters produced in SBP-8 under various NaCl concentrations has been given in Appendix IV.

Table 4.1 GC-MS analysis of FAME composition of *Klebsiella* sp. SBP-8 (0 mM NaCl)

S.No.	Run Time	Formula	Compound	% Area	Relative Molecular mass	Match quality %
1.	14.764	$C_{18}H_{38}O$	1-Octadecanol	0.20	270	87
2.	19.745	$C_{15}H_{32}O$	n-Pentadecanol	0.16	228	84
3.	24.448	$C_{25}H_{52}$	Pentacosane	0.35	352	96
4.	26.583	$C_{24}H_{50}$	Tetracosane	1.64	338	95
5.	28.632	$C_{30}H_{62}$	Triacontane	3.52	422	96
6.	30.598	$C_{29}H_{60}$	Nonacosane	6.59	408	95
7.	32.494	$C_{22}H_{46}$	Docosane	9.36	310	95
8.	34.319	$C_{40}H_{82}$	Tetracontane	10.93	562	95
9.	36.078	$C_{34}H_{70}$	n-Tetratriacontane	10.51	478	94
10.	37.782	$C_{36}H_{74}$	Hexatriacontane	9.17	506	94
11.	39.424	$C_{44}H_{90}$	n-Tetratetracontane	6.63	618	94
12.	45.311	$C_{54}H_{110}$	Tetrapentacontane	2.65	758	91

Table 4.2 GC-MS analysis of FAME composition of *Klebsiella* sp. SBP-8 (175 mM NaCl)

S.No.	Run Time	Formula	Compound	% Area	Relative Molecular mass	Match quality %
1.	4.915	$C_{13}H_{28}O$	1-Tridecanol	0.06	200	91
2.	5.053	$C_{17}H_{36}$	Heptadecane	0.05	240	89
3.	9.529	$C_{20}H_{40}$	9-Eicosene	0.14	280	93
4.	14.759	C21H44O	1-Heneicosanol	0.17	312	93
5.	19.742	$C_{19}H_{40}O$	1-Nonadecanol	0.20	284	94
6.	22.221	$C_{16}H_{34}$	Hexadecane	0.11	226	89
7.	24.325	$C_{27}H_{56}O$	1-Heptacosanol	0.16	396	94
8.	24.448	$C_{25}H_{52}$	Pentacosane	0.36	352	96
9.	26.580	$C_{24}H_{50}$	Tetracosane	1.62	338	95
10.	28.634	$C_{30}H_{62}$	Triacontane	4.62	422	95
11.	30.611	$C_{40}H_{82}$	Tetracontane	9.28	562	96
12.	32.513	$C_{22}H_{46}$	Docosane	12.95	310	95
13.	34.340	$C_{36}H_{74}$	n-Hexatriacontane	15.27	506	95
14.	37.796	$C_{34}H_{70}$	n-Tetratriacontane	12.52	478	94
15.	39.434	$C_{44}H_{90}$	n-Tetratetracontane	8.82	618	94
16.	45.003	$C_{54}H_{110}$	Tetrapentacontane	3.80	758	91

Table 4.3 GC-MS analysis of FAME composition of *Klebsiella* sp. SBP-8 (200 mM NaCl)

S.No.	Run Time	Formula	Compound	% Area	Relative Molecular mass	Match quality %
1.	4.916	$C_{13}H_{28}O$	1-Tridecanol	0.09	200	95
2.	9.524	$C_{20}H_{40}$	9-Eicosene	0.17	280	96
3.	14.757	$C_{21}H_{44}O$	1-Heneicosanol	0.22	312	96
4.	19.749	$C_{19}H_{40}O$	1-Nonadecanol	0.22	284	96
5.	19.895	$C_{20}H_{42}$	Eicosane	0.20	282	96
6.	22.228	$C_{21}H_{44}$	Hexadecane	0.42	226	95
7.	24.331	$C_{27}H_{56}O$	Heptacosanol	0.17	396	96
8.	24.476	$C_{25}H_{52}$	Pentacosane	1.54	352	96
9.	26.661	$C_{24}H_{50}$	Tetracosane	3.74	338	95
10.	28.758	$C_{30}H_{62}$	Triacontane	6.99	422	95
11.	30.777	$C_{40}H_{82}$	Tetracontane	9.70	562	96
12.	32.683	$C_{34}H_{70}$	n-Tetratriacontane	11.05	478	95
13.	34.098	$C_{20}H_{42}O$	1-Eicosanol	0.11	298	92
14.	34.511	$C_{36}H_{74}$	n-Hexatriacontane	10.76	506	95
15.	45.354	$C_{54}H_{110}$	Tetrapentacontane	1.57	758	91

4.3.2 Plant growth under NaCl stress, and ionic and biochemical analysis of plants

The effect of bacterial isolate *Klebsiella* sp. SBP-8 inoculation on wheat growth under control (0 mM NaCl) and in varying salinity condition (150, 175, 200 mM NaCl) has been described in Chapter III section no 3.2.4.4. Here, we are analyzing the change in ionic and biochemical constituents of the wheat plant following bacterial inoculation under salinity stress.

4.3.3 Analysis of ionic elements

Treatment with a high concentration of NaCl significantly increased Na^+ accumulation, and decreased K^+ and Ca^{2+} in wheat shoots of control plants. Highest Na^+ and less K^+ content were observed in uninoculated plants at 200 mM NaCl than the other concentrations used. However, inoculation with isolate SBP-8 decreased the level of Na^+ and increased K^+ concentration in NaCl-treated plants. As compared to uninoculated plants, bacterial inoculation inhibited the accumulation of Na^+ content by 65% and increased the K^+ by 84.21% at 200 mM NaCl (Table 4.4). In contrast, Na^+ content was unaffected in inoculated plants in non-saline (0 mM) soil. Moreover, in inoculated plants, there was an increase in K^+ content with 23.21% and 18.35% at 175 mM and 150 mM NaCl, respectively. Increased K^+ uptake by bacterial inoculation demonstrated a clear effect on potassium transport in plants. There was no significant change in Ca^{2+} in un-inoculated plants under salt stress. However, with the increase in salinity, slight increase in Ca^{2+} content was observed in inoculated plants. Higher Ca^{2+} content (27.27%) was observed at 200 mM NaCl as compared to un-inoculated plants with same salt stress treatments.

Table 4.4 Influence of *Klebsiella* sp. SBP-8 on Na^+, K^+ and Ca^{2+} ion concentrations in shoot tissue under control and salt stress

Treatments	Na^+(mg g^{-1} DW)	K^+(mg g^{-1} DW)	Ca^{2+}(mg g^{-1} DW)
Control	1.01±0.07	1.34±0.07	1.27±0.14
Control+150 mM NaCl	1.36±0.09	1.09±0.12	1.20±0.22
Control+175 mM NaCl	1.58±0.11	1.12±0.31	1.17±0.17
Control+200 mM NaCl	1.98±0.10	0.95±0.28	1.10±0.19
SBP-8 only	1.09±0.06	1.42±0.30	1.30±0.09
SBP-8+150 mM NaCl	1.11±0.15*	1.29±0.21*	1.27±0.11
SBP-8+175 mM NaCl	1.18±0.19*	1.58±0.08*	1.31±0.26
SBP-8+200 mM NaCl	1.20±0.23*	1.75±0.17*	1.40±0.20*

Values are the means of three replicates±SD. Single asterisk (*) represent significant difference from respective control based on Duncan's multiple range test ($p < 0.05$). DW: dry weight.

4.3.4 Biochemical analysis

4.3.4.1 Proline and total soluble sugar

SBP-8 inoculation significantly increased the proline content in the range of 23 to 38% as compared to uninoculated control under tested NaCl stress. The highest significant increase was observed with 38% ($p<0.05$) at 200 mM NaCl stress followed by 33.96% and 29.86 % at 175 mM and 150 mM NaCl stress respectively (Fig. 4.1 A). Simultaneously, TSS was also measured on inoculation of the test organism which could play a role in counteracting the negative effects of salt. SBP-8 inoculation significantly ($p<0.05$) increased the level of TSS in the range of 19 to 57% as compared to uninoculated control plants. The level of TSS increased with increase in NaCl concentration being highest with 57% at 200 mM in bacterium-treated plants (Fig. 4.1B).

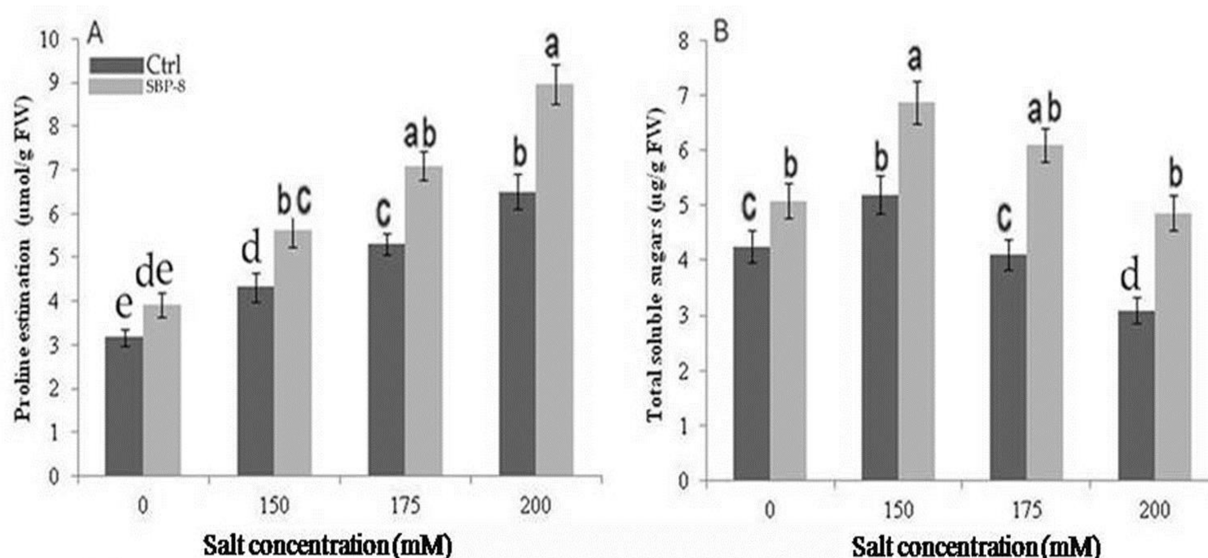


Fig. 4.1. Effect of *Klebsiella* sp. SBP-8 inoculation on proline (Panel A), and total soluble sugar (Panel B), in wheat plants grown under different salinity conditions (0 mM, 150 mM, 175 mM, 200 mM NaCl). Values are mean of three replicates±SD (n=3). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test ($p<0.05$). FW: fresh weight.

4.3.4.2 Malondialdehyde analysis

Increasing concentration of NaCl from 150 to 200 mM increased the MDA content of 26 to 46% in the uninoculated plants. However, SBP-8 inoculation significantly ($p<0.05$) decreased the MDA content in salt-treated plants. The significant decrease of 18.65%, 26.06%, 32.83%

and 46.25% was observed at 0, 150, 175 and 200 mM NaCl stress in bacterized plant as compared to respective uninoculated control plants (Fig. 4.2 C).

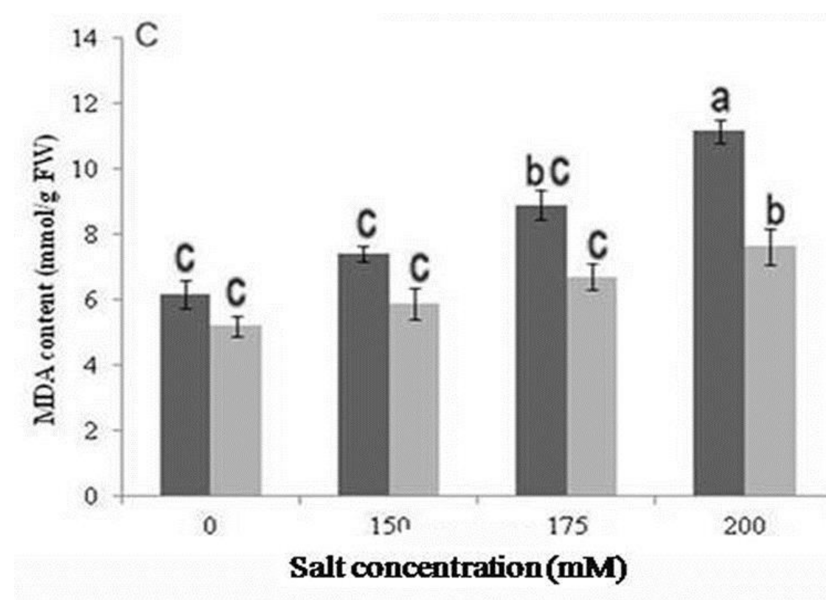


Fig. 4.2. Effect of *Klebsiella* sp. SBP-8 inoculation on malondialdehyde content under different salinity conditions. Values are mean of five replicates±SD (n=3). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test ($p<0.05$). FW: fresh weight.

4.3.4.3 Auxin and total protein content

The amount of auxin produced in host plants under gradient NaCl concentration was measured which demonstrated that the inoculation with *Klebsiella* sp. SBP-8 increased auxin content by 49% ($p<0.05$) in plants treated with 200 mM salt concentration as compared to respective control. However, enhanced auxin content of 19.63% in plants was observed even at 0 mM NaCl stress in bacterium inoculated plants (Fig. 4.3 D). Further, an increase in total protein content was also observed in bacterized plants under various salt stress conditions. The highest significant ($p<0.05$) protein content of 50.40% was observed at 200 mM NaCl stress, followed by 28.45% and 21.76% at 175 and 150 mM NaCl stress as compared to respective control plants (Fig. 4.3 E).

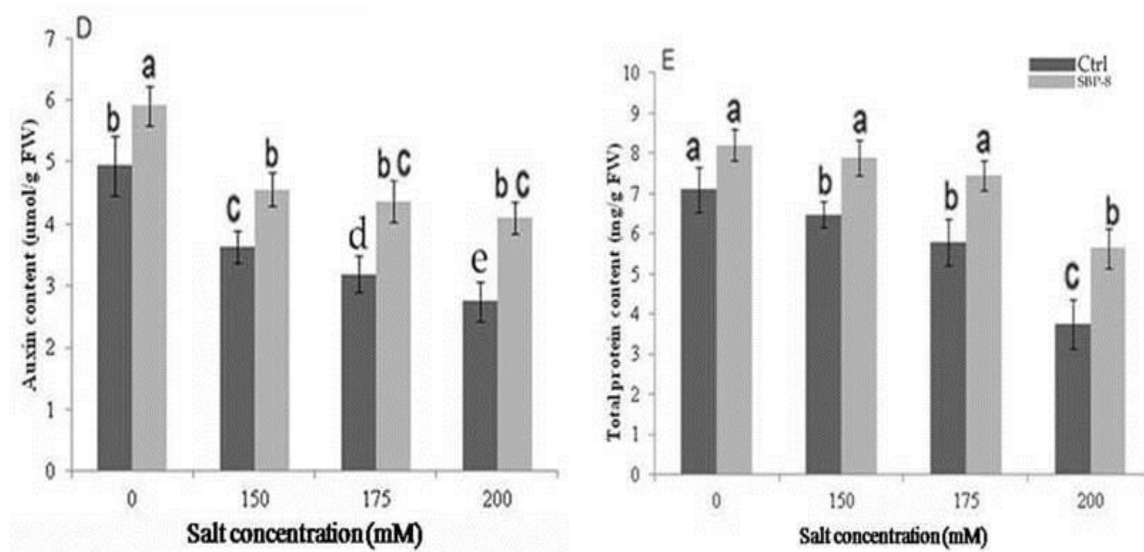


Fig. 4.3. Effect of *Klebsiella* sp. SBP-8 inoculation on auxin, and total protein content under different 0 mM, 150 mM, 175 mM, 200 mM NaCl salinity conditions. Values are mean of three replicates±SD (n=3). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test ($p<0.05$). FW: fresh weight.

4.3.5 Antioxidative activities

Inoculation with *Klebsiella* sp. SBP-8 caused a significant increase in SOD, CAT, and POX activities in the wheat plant under NaCl stress. As shown in Fig. 4.4 A, SOD activity increased in bacterium-treated plants with respect to increasing salinity stress. The highest activity was observed at 200 mM NaCl with 40.65% ($p<0.05$) increase followed by 33.76% and 25.75% at 175 mM and 150 mM NaCl stress as compared to the uninoculated plants grown with respective salt stress. Besides stimulating the activity of SOD, bacterial inoculation also increased the CAT and POX activity. The increase in CAT activity was recorded in the range of 24% to 46% in bacterium-treated plants. As compared to uninoculated control plants, its activity significantly increased with 46.60%, 34.52% and 24.43% ($p<0.05$) at 200, 175, and 150 mM salt stress respectively (Fig. 4.4 B). Similar to the results of other antioxidants, the POX activity also increased in bacterium-treated plants as compared to uninoculated plants. The increase of 38.09% ($p<0.05$) was observed at 200 mM NaCl, followed by 35% and 27.67% at 175 and 150 mM NaCl stress (Fig. 4.4 C).

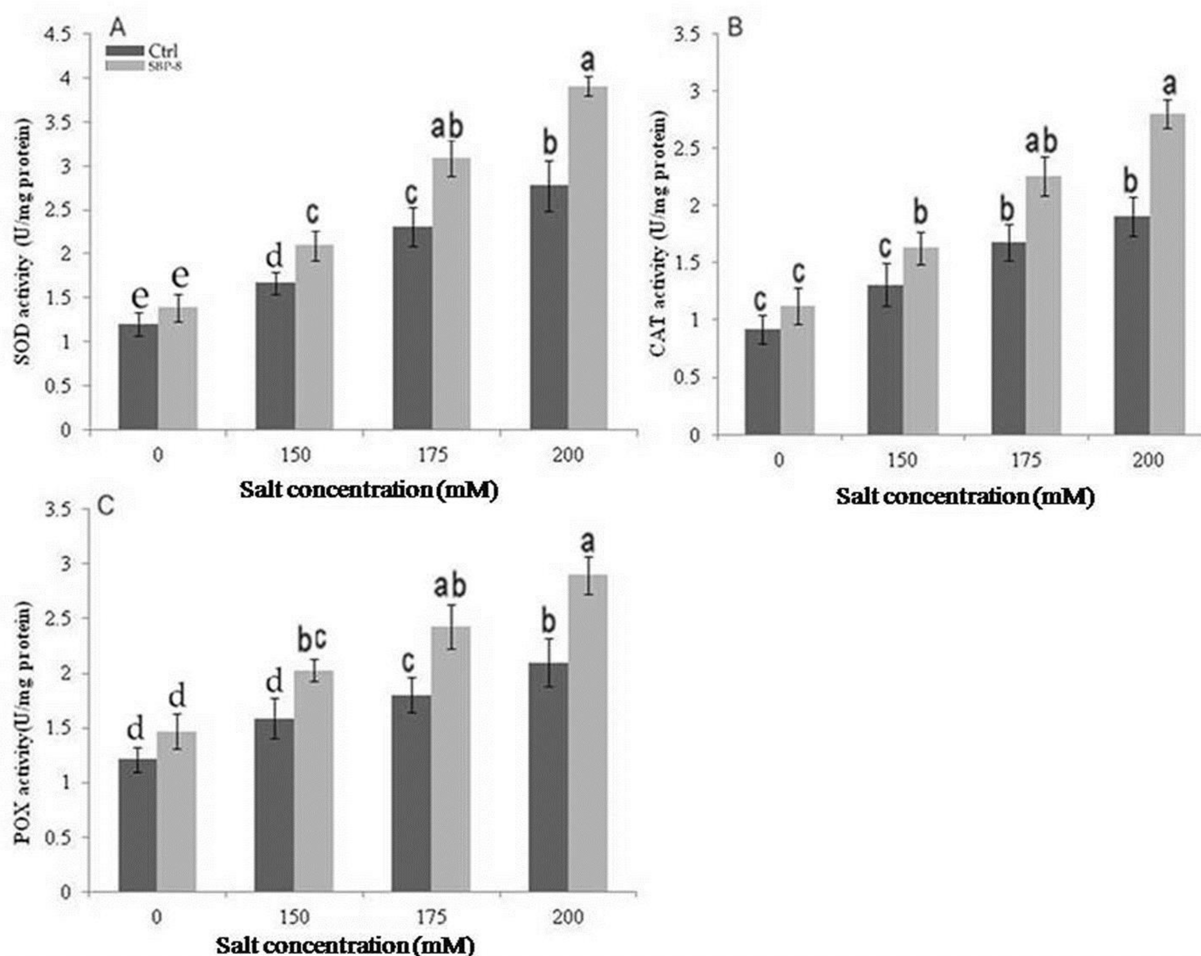
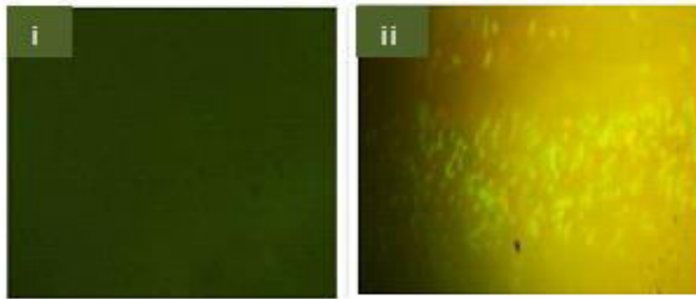


Fig. 4.4. *Klebsiella* sp. SBP-8 inoculation on anti-oxidative enzymatic activities in treated and control plants under salt stress conditions; (A) SOD activity (B) CAT activity (C) POX activity. Values are mean of three replicates±SD (n=3). Different letters as compared to respective control represents the significant difference according to Duncan multiple range test ($p<0.05$).

4.3.6 Colonization

The colonization ability of the isolate was determined by CFU counting, microscopy and ERIC–PCR of inoculated bacteria with respect to control after the experimental period. On the 15th day after seed germination, associative bacteria were detected in a range of 1.8×10^{3} CFU/g of the root. Colonization of inoculated bacteria was also evident from the micrograph (Fig. 4.5A) which indicated a large number of fluorescent cells on staining with acridine orange. No bacterial cells were observed on the root surface of uninoculated plants. To confirm an identity of the colonized bacteria, genomic DNA of treated plants was subjected to ERIC–PCR-based finger printing. ERIC–PCR profile obtained from total genomic DNA of treated plants was identical to that of pure culture, thereby confirming the identity of colonized bacteria (Fig. 4.5 B).

A



B

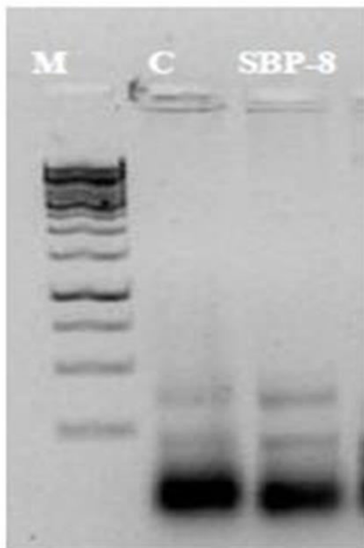


Fig. 4.5. The efficiency of colonization of *Klebsiella* sp. SBP-8 was assessed 15 days after germination of bacterized wheat plants; (A; i, ii) visualization of acridine orange-stained wheat plant roots showing bacterial cells using fluorescence microscopy (B) ERIC-PCR profile of bacterium colonized in wheat plants and confirmation of its identity using pure culture (Lane M: DNA ladder SM0311, Lane C: control DNA, Lane SBP-8: DNA of *Klebsiella* sp. isolated from inoculated plant).

4.3.7 Plant growth parameters of *AcdS*$^+$/*AcdS*$^-$ strain of *Klebsiella* sp. SBP-8

After transposon-mutagenesis, bacterial colonies growing on culture medium containing trimithoprim were selected and replica plated on minimal agar medium possessing 3 mM ACC. Bacterial colonies which did not showed growth on ACC containing selective medium were further subjected to ACC deaminase assay. As compared to wild type, mutant strain (denoted as *AcdS*$^-$) did not show the ACC deaminase production. We also checked the other PGP traits in *AcdS*$^-$ SBP-8 strain which were almost found to be unaffected. After confirmation of mutation, we tested the plant growth promotory effect of mutant and wild type strain under salt stress. Mutation in the *AcdS* gene significantly ($p<0.05$) affected the various growth parameters of wheat plant when compared to the wild type at 200 mM NaCl stress. As seen from Table 4.5, shoot length and root length was significantly reduced by 28.55% ($p<0.05$) and 24.18% ($p<0.05$) respectively. Evaluation of biomass in terms of fresh weight/dry weight revealed that mutation in the *AcdS* gene significantly reduced the fresh weight of 34.24% ($p<0.05$) and dry weight of 38.5% ($p<0.05$) as compared to wild type (Fig. 4.6).

Table 4.5 Plant growth test of wild (SBP-8, *AcdS*$^+$) and mutant strain (SBP-8, *AcdS*$^-$) at 200 mM salt stress

Isolate	Shoot length (cm)	Root length (cm)	Fresh wt.	Dry wt.
SBP-8 (*AcdS*$^+$)	27.51±2.40	21.37±1.10	2.94±0.32	0.320±0.071
SBP-8 (*AcdS*$^-$)	21.40±1.80**	17.21±2.40**	2.19±0.19**	0.231±0.03**

wt=weight; double asterisk (**) represent the significant difference as compared to respective control plants.

A

B

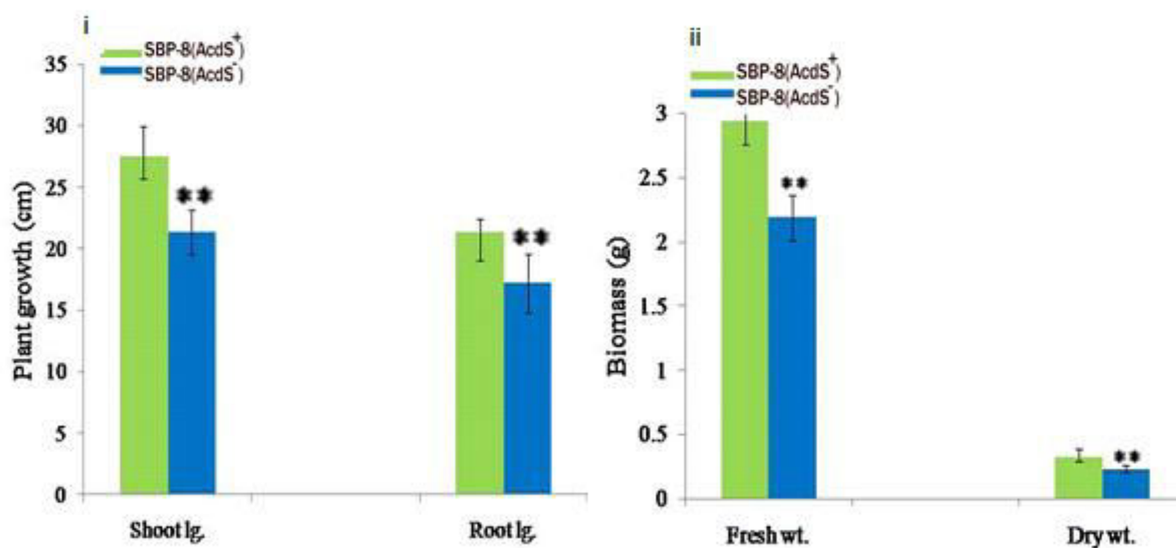


Fig. 4.6. Evaluation of plant growth promotion test following inoculation of (A) *Klebsiella* sp. SBP-8 (*AcdS*$^+$) and SBP-8 (*AcdS*$^-$) at 200 mM NaCl stress (B; i) plant growth in terms of the shoot and root length (B; ii) biomass measurement in terms of fresh weight and dry weight.

4.4 Discussion

Based on the results obtained for ACC deaminase and other plant growth promoting properties, *Klebsiella* sp. SBP-8 was selected for detailed characterization in terms of their ability to ameliorate abiotic stressors in wheat plants. In our previous report, we demonstrated that ACC deaminase containing *Klebsiella* sp. SBP-8 isolated from the rhizosphere of *Sorghum bicolor* promotes the growth of wheat plants grown at a different concentration of NaCl (Singh et al., 2015). Similar to our result, the previous study showed that PGPR *Klebsiella* Br1 and *Klebsiella pneumoniae* Fr1 improves the growth and nitrogen content of maize plant under green house conditions (Kuan et al., 2016). The present study is the first report elucidating the change in the fatty acid composition of *Klebsiella* sp. under varying salinity stress. The study is an effort to elucidate the mechanism of plant growth promotion with various perspectives like microbial-mediated changes in various osmolytes and antioxidant activities.

One of the most important criteria for the selection of an efficient PGPR to be used in high saline soil is its ability to tolerate high salt concentration. Therefore, use of the halophilic/halotolerant bacteria has achieved special attention for improving the plant growth under salt stress (Mishra et al., 2015). For gaining a maximum advantage of plant growth promoting properties of PGPR under salt stress condition, the test isolate must be able to tolerate and function under given stress. As bacterial cells are directly exposed to outside environment, their cell membrane is a primary target for damages caused by environmental stressors. Under such conditions, a bacterium can alter the fatty acid composition of their membrane lipids to maintain membrane integrity and fluidity and adapt to given abiotic stress conditions like salt (Patrignani et al., 2008). Assuming similar mechanism, we checked for a fatty acid profile in the test isolate under salt stress before it can be applied to study its role in inducing physiological changes in plants grown under salt stress. The NaCl stress resulted in multiple changes in the fatty acid composition of SBP-8, helping the membrane to maintain its integrity, fluidity, and function for growth of the bacteria under salt (NaCl) stress. In response to changing condition, bacteria can modify the structure of their lipid acryl chain by changing the ratio of saturation to unsaturation, *cis*- to *trans* unsaturation, branched to unbranched structure, type of branching and length of the acyl chain (Denich et al., 2003; Grogan et al., 1997). The modifications of fatty acids content could be a part of defense or/and repair mechanism aimed at reducing the damage caused by stress (Aghaleh and Nikman, 2009). The GC-MS profile of cell extract at different salt concentration was

comprised of hydrocarbons, fatty alcohols with carbon chain length varying in the range of C_{13} - C_{54}. FAME analysis indicated the adoption of bacterium *Klebsiella* sp. SBP-8 to tolerate salt stress through the synthesis of fatty alcohols and hydrocarbons with chain length ranging from C9-C20. It is supported by earlier study which demonstrated that accumulation of long chain alkanes and fatty alcohol helps in maintaining the cellular membrane as iso-osmotic barrier crucial for bacterial cell survival under salt stress condition (Torok et al., 1997). Therefore, the present study provided strong evidence that in response to changing environment, bacteria accumulate the different compounds of varying composition which helps for its survival. To the best of our knowledge, the present study is the first study reporting the effect of abiotic stress like salinity on fatty acid composition in PGPR *Klebsiella* sp.

The presence of salt severely affects plant growth and productivity by several mechanisms. As the salt level increases in the soil, Na^+ exerts ionic competence diminishing the ability of ion uptake by plants. Exclusion of Na^+ and influx of K^+ are the plant's strategies for mitigating salt-induced stress (Shabala and Cuin, 2008) which can be aided by certain PGP bacteria. The present study showed that bacterial inoculation significantly decreased the Na^+ and increased K^+ content favoring amenable K^+/Na^+ ratio in the plants. This adds an additional feature of the isolate SBP-8 in reducing the effect of salt stress in host plants. In consonance with our results, increase in K^+ content was observed in tomato plants inoculated with ACCD bacteria under salt stress (Mayak et al., 2004). Few other studies have reported the role of PGPR-induced regulation of Na^+ import by the high-affinity K^+ transporter 1 (HKT1) for protection from the effect of salt stress in *Arabidopsis*. Volatile organic compounds (VOC) of *Bacillus subtilis* GB03 confer salt tolerance by down- and up-regulating HKT1 in roots and shoots respectively, and result in low Na^+ accumulation throughout the plant in comparison to control (Yang et al., 2009). The differential regulation of HKT1 gene expression reduced the aggregation of Na^+ and increased accumulation of K^+ in shoot and roots of *Bacillus subtilis* GB-03-inoculated *Arabidopsis* seedlings under salt stress (Zhang et al., 2008). Hence, these PGPR act as an important promoters for HKT1 gene expression (Alizadeh et al., 2011). HKT transporter was found in many plant species, however the regulatory mechanism is still not known (Horie et al., 2008). Moreover, exopolysaccharides (EPSs) secreting PGPR have the additional advantage of inhibiting the toxic effect of Na^+ on the plants by restricting the flow of Na^+ in roots through binding of Na^+ to surface polysaccharides and make it less available to plants (Ashraf et al., 2004; Geddie

and Sutherland, 1993). However, we have not looked for expression of HKT1 transporter and exopolysaccharide which can be studied in future studies. Our results suggested that understanding the mechanism of development of salt tolerant plants either by genetic engineering or use of plant growth promoting bacteria are essential for solving the problem of productivity in salty regions (Dimpka et al., 2009).

The accumulations of osmolytes maintain optimum turgor pressure and stabilize the various macromolecular structures against the salinity-induced physiological drought stress. Proline, one of the major osmolytes is responsible for osmotic adjustment and also protects the sub-cellular organelles in stressed plants (Yamaguchi-Shinozaki, 2006). Proline provides the turgor pressure for cell expansion under stress condition and scavenges the free radicals and stabilizes DNA, proteins and membranes etc. The higher accumulation of proline in wheat plants inoculated with bacterium SBP-8 than the uninoculated counterpart indicated that SBP-8 enhanced tolerance to salinity stress in plants. The increase in proline content might be attributed to up-regulation of the biosynthetic pathway of proline to keep the proline in higher concentration for maintaining the water status of cell and membrane protection against salt stress (Han and Lee, 2005; Upadhyay et al., 2011). Increase in proline content following inoculation of PGPR *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* has been observed in rice variety GJ-17 under a different level of salinity (Hanson and Nelson, 1978). However effect of *Klebsiella* sp. on proline content in the wheat plant was not studied. In addition, an increase in TSS content suggested that bacterium inoculation helps in the hydrolysis of starch content, and therefore, more sugar was made available for osmotic adjustment to minimize the effects of salt stress (Tattini et al., 2009). Further, increase in salinity-induced oxygen radicals that react with unsaturated fatty acids induce the peroxidation of membrane lipids or intracellular organelles in the plants and results into increase in leaf MDA content (Zimmermann and Zentgraf, 2005). Therefore, we determined the leaf MDA content in presence and absence of bacterium in wheat plants. Another recent study also observed that the application of ACC deaminase bacteria *Arthrobacter protophormiae* alleviates the MDA content and reduces the membrane damage through enhanced stress tolerance in maize (Barnawal et al., 2014).

PGPR can facilitate the plant growth directly by influencing the production of phytohormone like auxin. Our result is in agreement with the previous finding where PGPR *Pseudomonas* sp. increased the auxin content in the wheat plant under salt stress (Hasnain and Sabri, 1996). Lack of IAA production in SBP-8 rules out the possibility that the auxin

was contributed by the bacterium itself. Moreover, the increase in protein content was also observed following inoculation of selected isolate. The increase in protein content could be responsible for preventing the denaturation and decomposition of the cellular molecules under stress conditions (Campbell and Close, 1997).

Plant contain a complex antioxidative defense system like superoxide-dismutase, catalase, and peroxidase to protect the cellular membranes and organelles from detrimental effects ofsalinity stress. These antioxidant enzymes are capable of detoxifying ROS-induced cellular damages and protect crop plants under oxidative stress conditions (Unyayar et al., 2006). The antioxidant enzymes of the cell have the ability to protect the membranes and DNA by removing the free radicals produced during abiotic stress conditions (Gururani et al., 2012). Kohler et al. (2009) suggested that induction of antioxidative enzymes following *Pseudomonas mendocina* inoculation increases the salt tolerance of lettuce and, therefore serve as an effective tool to mitigate the salinity stress. The bacterial SOD plays a key role in its survival in the rhizosphere zone by facilitating the removal of stress-induced free radicals (Wang et al., 2007). In general, the survival of bacteria could be attributed to contribution in alleviating abiotic stress and promoting plant growth (Dimkpa et al., 2009b). Catalase decomposes hydrogen peroxide to water and molecular oxygen and thus protects the plant cells with an energy efficient mechanism to remove the hydrogen peroxide (Scandalios et al., 1997). Similarly, hydrogen peroxide can be removed also by peroxidases and are involved in various processes like cell growth and tolerance to environmental stressors (Quiroga et al., 2000). Thus, PGPR can ameliorate salt stress employing one or more of above-mentioned mechanisms. Our observation of present study demonstrates that bacteria with multifarious PGPR activity and able to survive under stress condition can protect the wheat plant without employing any genetic manipulation of the plant.

Our test isolate showed several mechanisms that can be helpful in ameliorating salt stress in the host plant. As our primary aim was to characterize ACC deaminase activity-based alleviation of abiotic stresses, we generated null mutant of ACCD using transposon-based mutagenesis to ascertain that plant growth is stimulated due to ACC deaminase activity of the bacterium. Transposon mutagenesis is one of the most efficient strategies for creating random mutations in the genomic DNA. Transposon can be introduced into the cells through plasmids called suicide vectors, phages, and also by using the EZ::TN5<DHFR-1> Tnp Transposome Kit, cat # EZI912D manufactured by Epicentre Biotechnologies, via electroporation. The Tnp transposase (476 amino acids) catalyzes transposition by cutting the

ends of the transposon, letting the transposon hop into the target DNA. The use of this kit, allows more stable mutations because the DNA sequence that jumps into the bacterial genomic DNA does not contain the Tnp enzyme; hence it cannot jump more than once. One of the main advantages of using the transposon-mutagenesis is that it is easy to create a complete library of mutants. The transposome used in this work is a stable complex of the EZ::TN transposase and the EZ::TN <DHFR> transposon. The transposon contains the dihydrofolate reductase (dhfr) gene, which confers trimethoprim resistance (Madigan et al., 1996) and is flanked by hyperactive 19 bp mosaic end (ME) EZ::TN transposase recognition sequences (Goryshin et al., 2000). Moreover, it is one of the best characterized transposon, therefore, Tn*5* is ideal for creating random mutants with high frequencies of insertion due to its ability to interact with non-specific DNA. Additionally, Tn*5* may be used in almost any gram-negative bacterium. Use of transposons to transform various bacteria has typically led to stable single random insertions in the bacterial genome (Goryshin et al., 2000). The generated *AcdS*$^{-}$ mutant of *Klebsiella* sp. SBP-8 did not show the ACC deaminase activity. Further, comparing the plant growth test using wild and mutated strain demonstrated that *AcdS*$^{-}$ strain was unable to improve the plant growth compared to wild type under stress conditions.

The close association of plant and microbe resulting from successful colonization provides an optimum advantage for both of the partners. Therefore, we tested the ability of bacteria to colonize the plant surface. The test isolate showed all forms of motility which are required for chemotactic responses and colonization on plant surface (Lugtenberg et al., 2001). Further, colonization efficiency of the isolate was examined by fluorescence microscopy and ERIC–PCR. Bacterial cells were visualized in inoculated plant roots using fluorescent dye acridine orange which binds to bacterial nucleic acids. This dye has been frequently used for tracking microbial colonization using fluorescent and confocal laser scanning microscope (Morris et al., 1997). In addition to the visual confirmation, colonization was also confirmed using repetitive (ERIC) DNA-based finger printing approach (Li et al., 2009). Typing and confirmation of DNA strains using ERIC–PCR have been demonstrated in previous reports too (Syrmis et al., 2004; Singh et al., 2015). It is likely that SBP-8 alleviated the salt stress to wheat plant by co-ordination of antioxidative machinery, and improvement in osmolyte contents. Therefore, the present study suggests that bacterium inoculated wheat plants were able to cope better with salt stress than uninoculated control and can serve as a promising bioinoculant for enhancing the growth of wheat like cereal crops under saline

stress. Therefore, exploration and selection of efficient PGPR can be successfully used to manage salt stress in plants growing in saline soil.

Chapter V

Proteomic analysis of wheat plant following inoculation of *Klebsiella* sp. SBP-8 under salt stress

Introduction

Soil salinity is a major problem in the agriculture sector that inhibits the crop growth and productivity. Plant's response to salinity is a complex phenomenon which involves activation or modification of processes occurring at physiological, biochemical, and molecular levels (Greenway and Munns, 1980). Various injuries induced by salt stress are controlled by a chain of gene expression and several proteomic changes. A detailed identification and analyses of changes occurring at the protein level in a salt stressed-crop, therefore, seems to be a rational approach for understanding the molecular mechanism of response to salt. Coping with salt stress involves complicated mechanisms that include developmental, morphological, physiological, and biochemical strategies (Taji et al., 2004). Further, salt stress-regulation genes are expressed in abundance, which leads to the changes in total protein profile that help plants to adapt to salt accumulation (Parker et al., 2006).

Despite the fact that the generation of the salt-resistant genetically modified plants (GMP) are promising (Sergeeva et al., 2006; Zhang and Blumwald, 2001; Zhang et al., 2001), they are not very popular among users firstly, due to ethical issues and secondly, due to low public acceptance. Thus, GMPs have not been widely applied at the field level. Use of plant growth promoting rhizobacteria (PGPR) with 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity have been reported to improve plant growth at high salt concentrations (Mayak et al., 2004a; Cheng et al., 2007). Advantages of ACC deaminase producing bacteria in conferring induced systemic tolerance in plants have been described in previous chapters of the thesis.

Wheat (*Triticum aestivum* L.), the second most important food crop, has high nutritional value and is rich in protein, minerals, calcium, iron, riboflavin and vitamin-A, etc. (Šramková et al., 2009). The proteins in wheat seeds can be divided into albumins, globulins, gliadins, and glutenins that are important enzymes in plant growth (Wiesner et al., 1980). Few previous studies have been conducted on wheat to reveal the molecular mechanism of germination (Ahmad et al., 1998), determination of the influence of the external environment (Tucaliuc et al., 2008), and specific protein changes (de Gara et al., 1997). However, little is known about the physiological and molecular adaptive mechanism of wheat plant tolerance to salt in response to PGPR inoculation. Investigation of proteomic profile of wheat plant would give us insights into the molecular mechanism of salt tolerance.

The use of proteomic tools and technologies has facilitated the application of proteomics in the characterization of plant–environmental interactions and should expand our understanding of these processes in the future (Glick et al., 2011). The proteomes of plants in response to environmental stimuli have been reviewed previously that illustrated plant proteins are involved in various aspects of plant–bacterial interactions, including plant resistance to pathogenic bacteria, and symbiotic relations for nutrient availability (Jorrin-Novo et al., 2009). To date, little work has been done on the combinational effects of plant growth-promoting rhizobacteria and environmental stresses, and corresponding effects on plant proteomes. Thus, the present work was aimed to examine the effect of beneficial PGPR on plant proteomic profile under high salinity stress. Here, a proteomic approach was conducted using QTOF with column chromatography to explore the alteration in protein expression of wheat plant treated with *Klebsiella* sp. SBP-8 under salt stressors. This approach could be promising to provide new insights into the molecular adaption of plants toward abiotic stressors using a more functional approach.

Material and methods

Plant growth and bacterial treatment

The ACC deaminase-containing bacterium *Klebsiella* sp. SBP-8 was selected for the study based on its plant growth promoting properties under salt-stress conditions (described in the previous chapter). Wheat plants (*Triticum aestivum* C-309) was grown and treated with the test isolate (SBP-8) as described previously with minor modification (Singh et al., 2015). Briefly, plant seeds were surface sterilized by treating with 70% ethanol for 2 min followed by three times washing with sterilized water. The seeds were exposed to 1.0% sodium hypochlorite (NaOCl) solution for 3 min followed by three consecutive washes using sterile water to remove all traces of bleach solution. The sterilized seeds were treated at room temperature for 1 h with bacterial suspensions (10^8cfu mL^{-1}) of *Klebsiella* sp. SBP-8 (Penrose and Glick, 2003). Sterile 0.03 M $MgSO_4$ solution-treated seeds were used as a negative control. For the preparation of bacterial inoculum, the bacterial isolate SBP-8 was grown in 250 ml Luria-Bertani (LB) medium at 30 °C for 18 h with continuous shaking. The bacterial cells were harvested by centrifugation at 8000 g for 10 min at 4 °C and re-suspended in 25 ml sterilized 0.03 M $MgSO_4$ on ice (Farajzadeh et al., 2010). The absorbance of cell suspension was measured at 600 nm, and the turbidity of bacterial suspension was adjusted to a concentration of approximately 10^8cfu ml^{-1} using sterilized 0.03 M MgSO4. The sterilized seeds were placed on Petri-dishes containing moistened whatman filter paper

with distilled water (10-15 seeds on each plate) for germination. Following germination, five seedlings (3 days old) were sown in each plastic pot filled with sterile soil (400 g). The plants were grown for 15 days after germination under controlled conditions in a plant growth chamber (Labtech, South Korea) set with 140 µmol $m^{-2}s^{-1}$ of light for 16:8 h light/dark period at 24±2 °C with 70% humidity The plants were watered with Hoagland medium (pH 6.8) containing NaCl (200 mM) to providing the nutrients as well as imposing the salt stress. Each treatment was taken in triplicate, and pots were arranged in completely randomized block design way. The set of plants like control plants treated with and without salt stress (Treatment T-1), control and bacterial inoculated plants (Treatment T-2), and bacteria-inoculated plants with and without salt stress (Treatment T-3) were used for protein comparison. After the experimental period (15 days after seed germination), wheat plants were thoroughly washed with distilled water to remove any adhered soil particles and stored in liquid nitrogen until use.

Protein extraction

Proteins were extracted using a TCA-Acetone precipitation method with some modifications. The frozen wheat plants (1 g) were finely powdered in liquid nitrogen using a pestle and mortar and suspended in 1 ml of extraction buffer [sucrose 0.9 M, Tris-HCl 0.5 M, ethylenediaminetetraacetic acid 5 mM, KCl 0.1 M, and dithiothreitol (DTT) 1% w/v], and vortexed into a thick paste. The suspension was sonicated in an ice-cold sonication bath (4 °C) for 5 min in duplicates and was mixed with 1 ml 100% tricarboxylic acid (TCA) and 8 ml 100% ice-cold acetone. The mixture was vortexed for 10 min and centrifuged at 18,000 g for 15 min at 4 °C. The supernatant was discarded, resuspended the pellet in 1 ml ice-cold acetone, and washed it at 18,000g for 15 min at 4 °C. The above step was repeated to remove all TCA. All acetone was removed, and the sample was dried completely before dissolving in 50 mM ammonium bicarbonate with 1% SDS. Protein concentration was estimated by Bradford method (Bradford 1976), and 100 µg protein was used for trypsin digestion. The sample was treated with 10 mM DTT at 56 ^{o}C for 1h followed by 55 mM iodoacetamide (IDA) at room temperature in the dark for 45 min. The sample was then digested with Trypsin (1:100 enzyme/protein concentration) and incubated overnight at 37 ^{o}C. The resulting sample was vacuum dried and dissolved in 10 µl of 0.1% formic acid in water. After centrifugation at 10000 g, the supernatant was injected on C18 Nano-LC column (75µm x 150cm x 1.7µm BEHC18 column) for separation of peptides followed by analysis on the Waters Synapt G2 Q-TOF instrument (Applied Biosystems, Foster City, CA, USA).

Liquid chromatography-mass spectrometry analysis

The acquired raw data was processed by MassLynx 4.1 WATERS. The individual peptides MS/MS spectra were matched to the database sequence for protein identification on PLGS (Protein Lynx Global Server) software, WATERS, and MASCOT. The parameters chosen for identification against UNIPROT databases of *Triticum aestivum* included carbamidomethylation of cysteine as fixed modification and oxidation of methionine as variable modification, one missed cleavage, peptide mass tolerance set at 50 ppm (parts per million), fragment mass tolerance set at 0.8 Da, and peptide charges set at +2, +3, and +4. Mouse scoring algorithm was used to derive significance of the protein match with the ion score, which was calculated as $-10 \times LOG_{10}(P)$. The P in above formula represents an absolute probability of the observed match being a random event. It, thus, indicates that the match of a given protein and MS/MS spectra having relatively small P-value is not a random event. Expression of different proteins was compared between different treatments by calculating ratio of peptides in two samples based on ion-abundance data of peptides. Proteins/peptides having ratio of more than 1.5 was considered as up-regulated, whereas values below 0.75 were kept as the threshold for down-regulated proteins/peptides. Differentially expressed proteins were classified according to Gene Ontology (http://www.geneontology.org) for their molecular function, biological processes, and cellular processes involved in response to salt stress. According to the known or predicted cellular localization and molecular function of the proteins, as determined by Blast2Go (http://www.blast2go.com), specific groups of proteins were selected and analyzed on the basis of stimulus responses, chloroplasts proteins, and enzymes.

Hierarchical cluster analysis of differentially expressed protein

The expression profile of 79 differential expressed proteins (Enlisted in Table 5.1) common to three tested treatments was constructed through the two-way hierarchical clustering according to the Permut-Matrix software. Rows were mean centered, and euclidean distance as well as average linkage was used for data aggregation. The customized sets of parameters are employed for the analysis, i.e. low and high expression levels are shown with green and red colors respectively.

Result

Proteomic analysis

A total of 301 proteins were identified in the control plants treated without and with salt stress (200 mM NaCl) in treatment T-1. In the bacterial inoculated plants, treated with and without NaCl, a total of 286 (treatment T-3) and 307 (treatment T-2) proteins were identified respectively. To study the effects of bacterial inoculation and salinity effect, different levels of protein expression and differentially accumulated proteins were assigned to major functional groups, and it was further subcategorized for greater clarity (Fig. 5). All identified proteins were classified by gene ontology (GO) annotation software and then classified into three functional groups: molecular function, biological process, and cellular component. The results of the GO analyses for the various treatments are shown in Fig. 5. Most of the annotated molecular functions were found to relate to binding, transporter and receptor activity, while most of the annotated biological processes were found related to metabolic and signalling processes.

Among the differentially expressed proteins categorized under biological process, 25 proteins were classified as binding proteins for DNA, protein, or nucleotide, eighteen were involved in transporter activity, and 13 were classified as receptor proteins in T-1 experimental plants (Fig. 5.1 A). Proteins related to stress responses (12), regulation (8) and signal transduction (13) were also identified. For the T-2 treatment, 15 proteins were categorized for receptor activity, thirteen was involved in the transporter, and 21 were classified as binding proteins for DNA, protein, and nucleotide (Fig. 5.1 B). Bacterial inoculation slightly increases the regulatory (11) and signal transduction (15) proteins. For the T-3 treatment, 23 were recognized as binding proteins, 16 for receptor activity, and 12 for catalytic activity (Fig. 5.1 C)

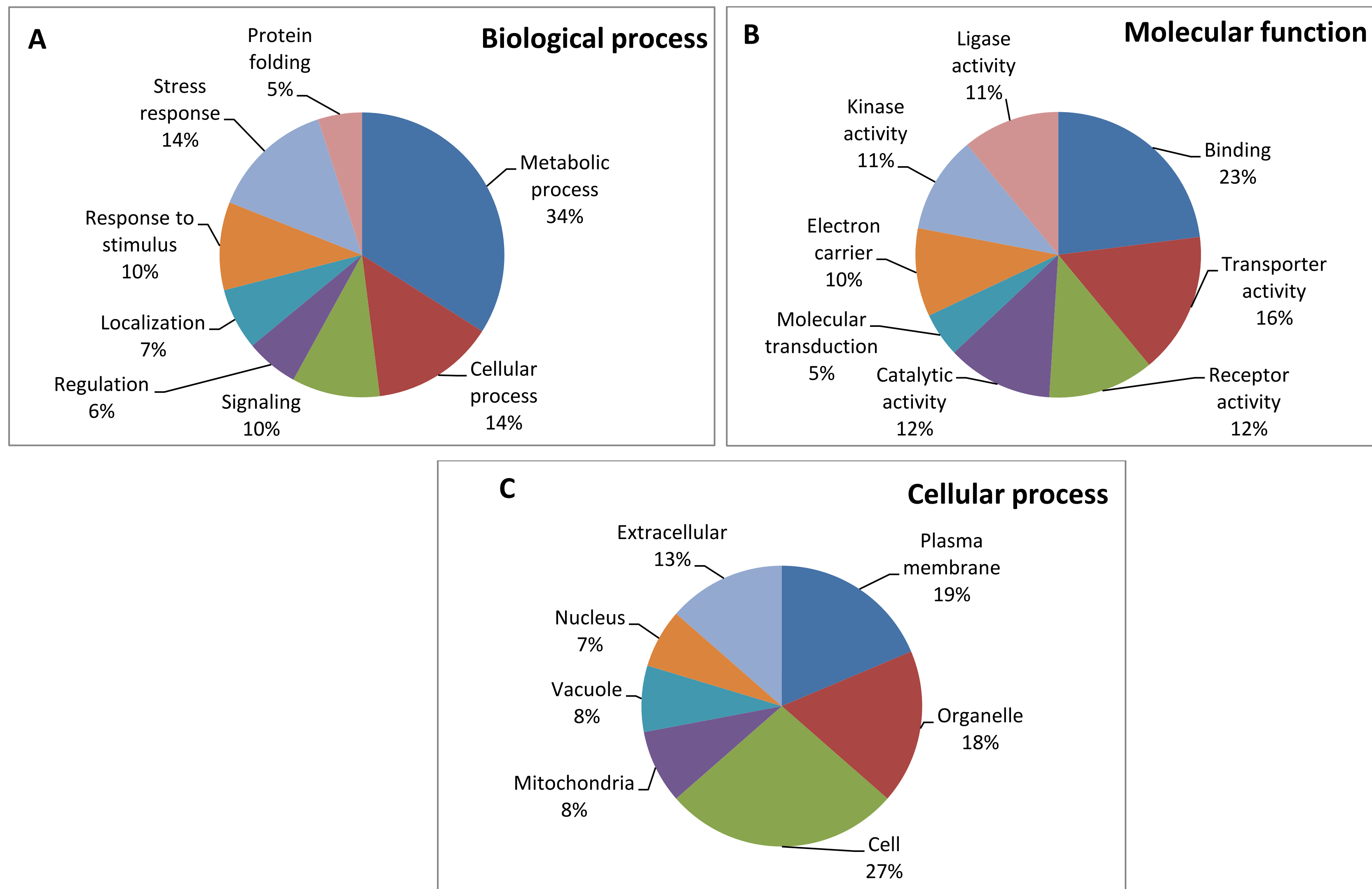


Fig. 5.1 A. Pie charts showing the distribution of differentially expressed proteins based on their predicted biological process (A), molecular functions (B), and cellular process (C) in Treatment T-1.

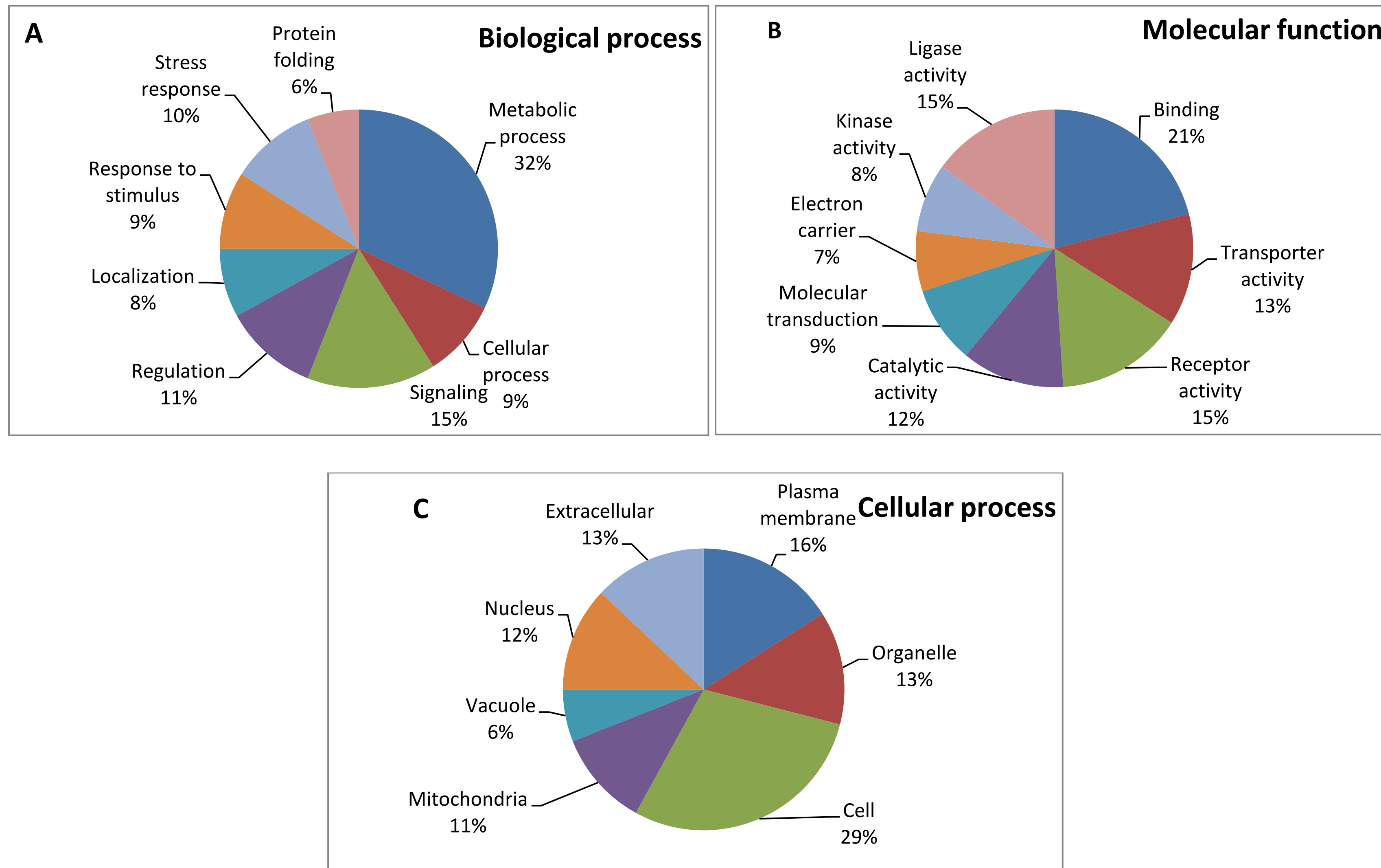


Fig. 5.1 B. Pie charts showing the distribution of differentially expressed proteins based on their predicted biological process (A), molecular functions (B), and cellular process (C) in Treatment T-2.

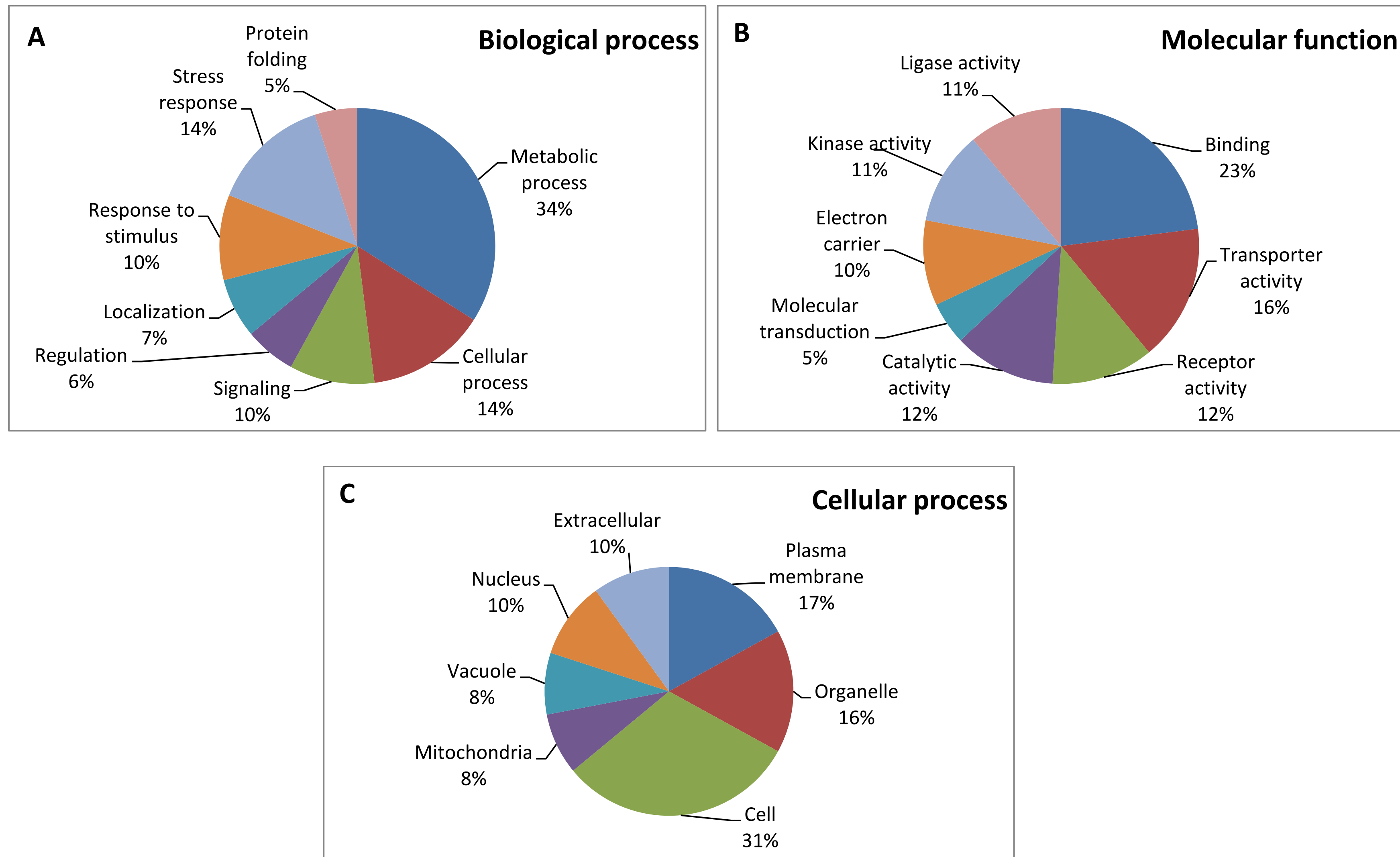


Fig. 5.1 C. Pie charts showing the distribution of differentially expressed proteins based on their predicted biological process (A), molecular functions (B), and cellular process (C) in Treatment T-3.

Functional annotation and classification of identified proteins

The fifteen functional groups included the proteins involved in cell division, chromatin-associated protein, defence and pathogenic protein, ion transport, lipid biosynthesis, metabolic pathway, photosynthesis, plant growth and development, protease inhibitor, protein synthesis, protein degradation, seed storage, stress-related, transcription control, and some others with known biological functions. The majority of the identified proteins were related to a metabolic pathway, photosynthesis, and stress mechanisms.

Following bacterial inoculation, the major changes occurred for the proteins involved in primary metabolism and stress mechanisms. Bacterial inoculation up-regulates the level of the proteins involved in defense, a protease inhibitor, and protein synthesis. In the presence of NaCl treatment, the level of proteins involved in cell division, defense, protein synthesis and stress-related were down-regulated. The highest protein up-regulation was observed for seed storage protein (581%), followed by the protein related to cell division (547%) in treatment T-1 (Fig. 5.2 A). It is evident from Fig. 5.2 A that salt stress down-regulated proteins related to plant growth and development, stress proteins, and transcriptional control by 62%, 57%, and 42% respectively.

In response to bacterial inoculation (T-2), the highest increase in up-regulation of protein was observed for ion transport (640%), transcriptional control (379%), photosynthesis (356%), and chromatin-associated protein (319%) (Fig. 5.2 B). Besides, the down-regulated protein belonged to lipid biosynthesis (60%), plant growth and development (73%), protein degradation (61%) and stress-related protein (51%) (Fig. 5.2 B). In other treatment (T-3), it was observed that proteins related to defense response were up-regulated by (2065%), photosynthesis (792%), and ion transport (765%). The down-regulated proteins were chromatin-associated protein (53%), lipid biosynthesis (47%), protein degradation (58%), and transcriptional control (51%) (Fig. 5.2 C)

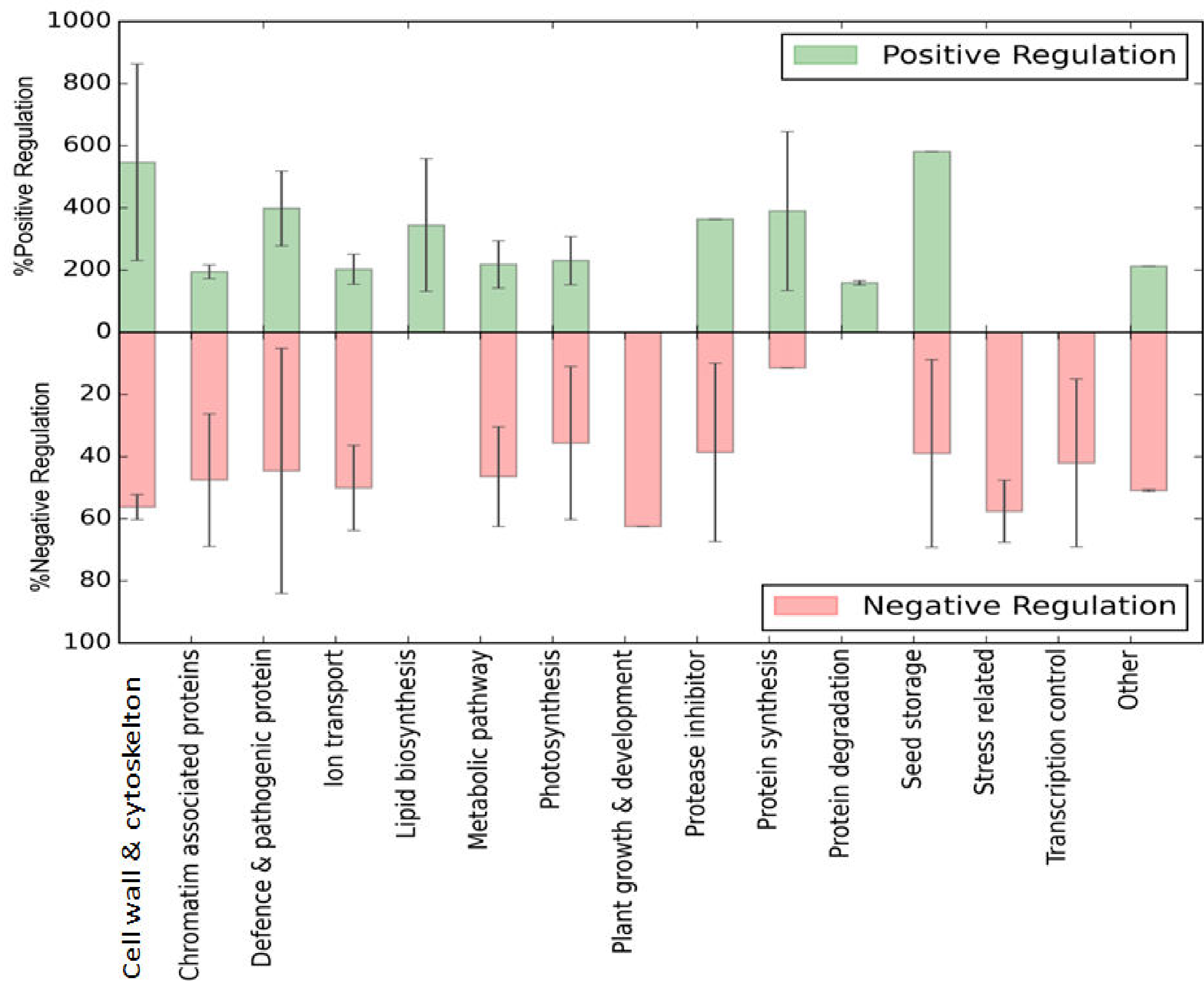


Fig. 5.2 A. The major functional groups of proteins identified in treatment T-1 (control vs uninoculated plants with salt stress). Standard deviation in each functional category was calculated by measuring the expression level of the entire proteins in given category.

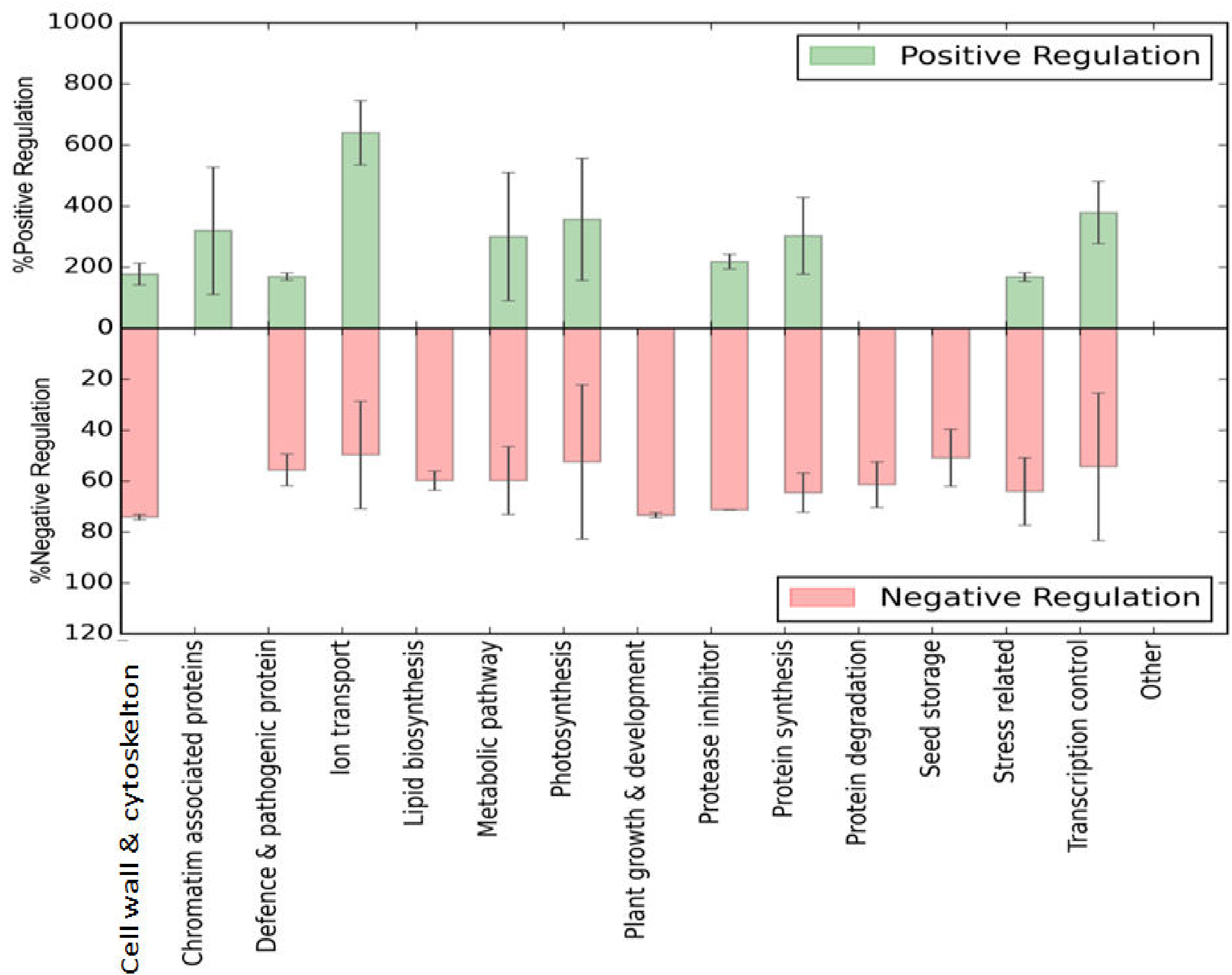


Fig. 5.2 B. The major functional groups of proteins identified in treatment T-2 (control vs inoculated plants). Standard deviation in each functional category was calculated by measuring the expression level of the entire proteins in given category.

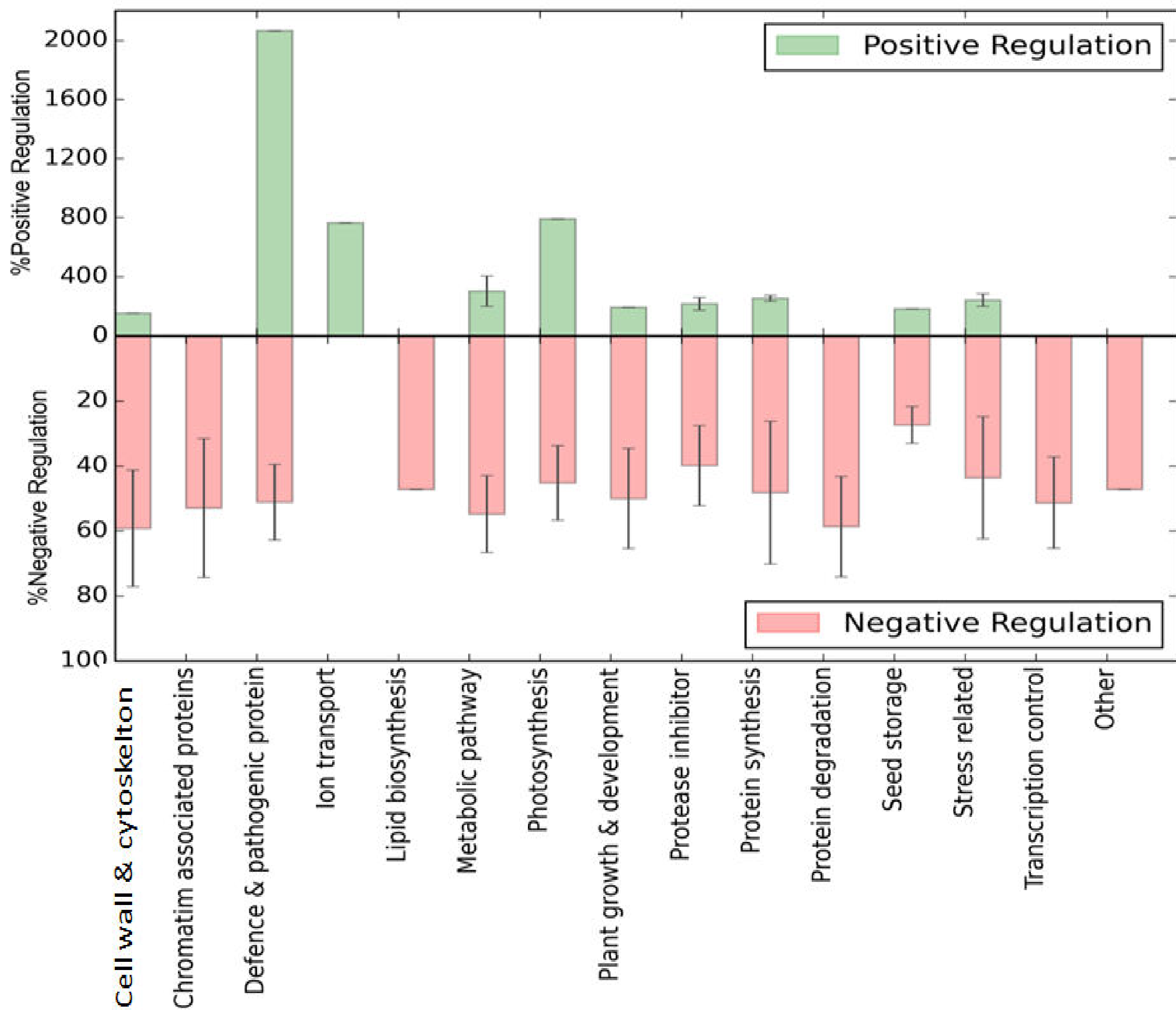


Fig. 5.2 C. The major functional groups of proteins identified in treatment T-3 (bacteria-inoculated vs salt stress). Standard deviation in each functional category was calculated by measuring the expression level of the entire proteins in given category.

Following expression analysis, based on significant changes of ≥ 1.5-fold or ≤ 0.75, proteins belonging to different category were differentiated. Among 96 proteins that showed predominant changes in treatment T-1, 38 were up-regulated, and 58 were down-regulated (Fig. 5.3 A). The higher increase in protein number was observed for protein synthesis (7), followed by photosynthesis (6) and metabolism (6). In response to bacterial inoculation (T-2), the expression level of 110 proteins showed significant changes, of which 43 were up-regulated, and 67 were down-regulated (Fig. 5.3 B). The metabolic proteins (13) were observed in higher number as compared to others. The expression level of 111 proteins showed significant changes in treatment T-3, of which 21 were up-regulated, and 90 were down-regulated (Fig. 5.3 C). The proteins related to metabolic and protein synthesis (5) was predominantly present as compared to others.

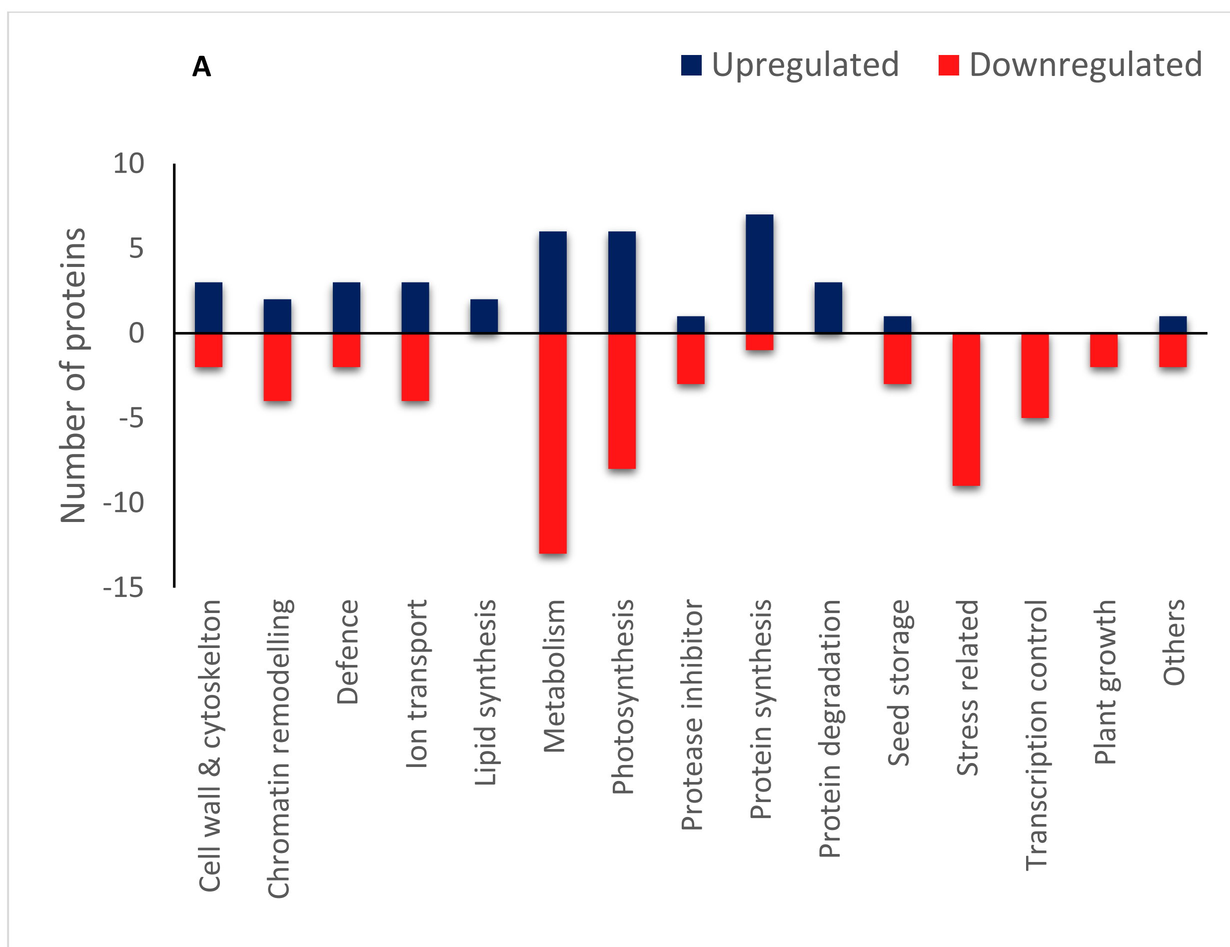


Fig. 5.3 A. Number of proteins up-regulated/down-regulated in each functional category in treatment T-1.

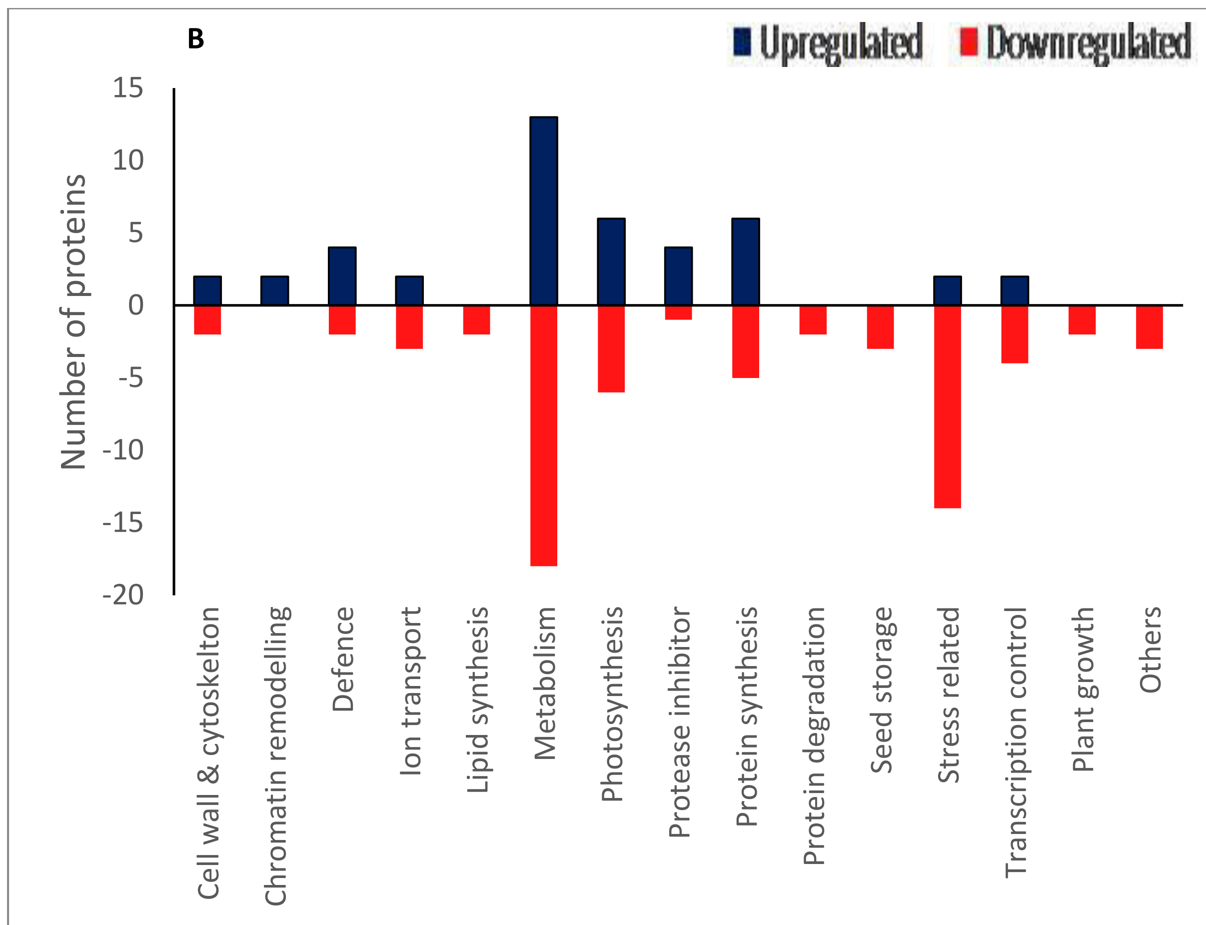


Fig. 5.3 B. Number of proteins up-regulated/down-regulated in each functional category in treatment T-2.

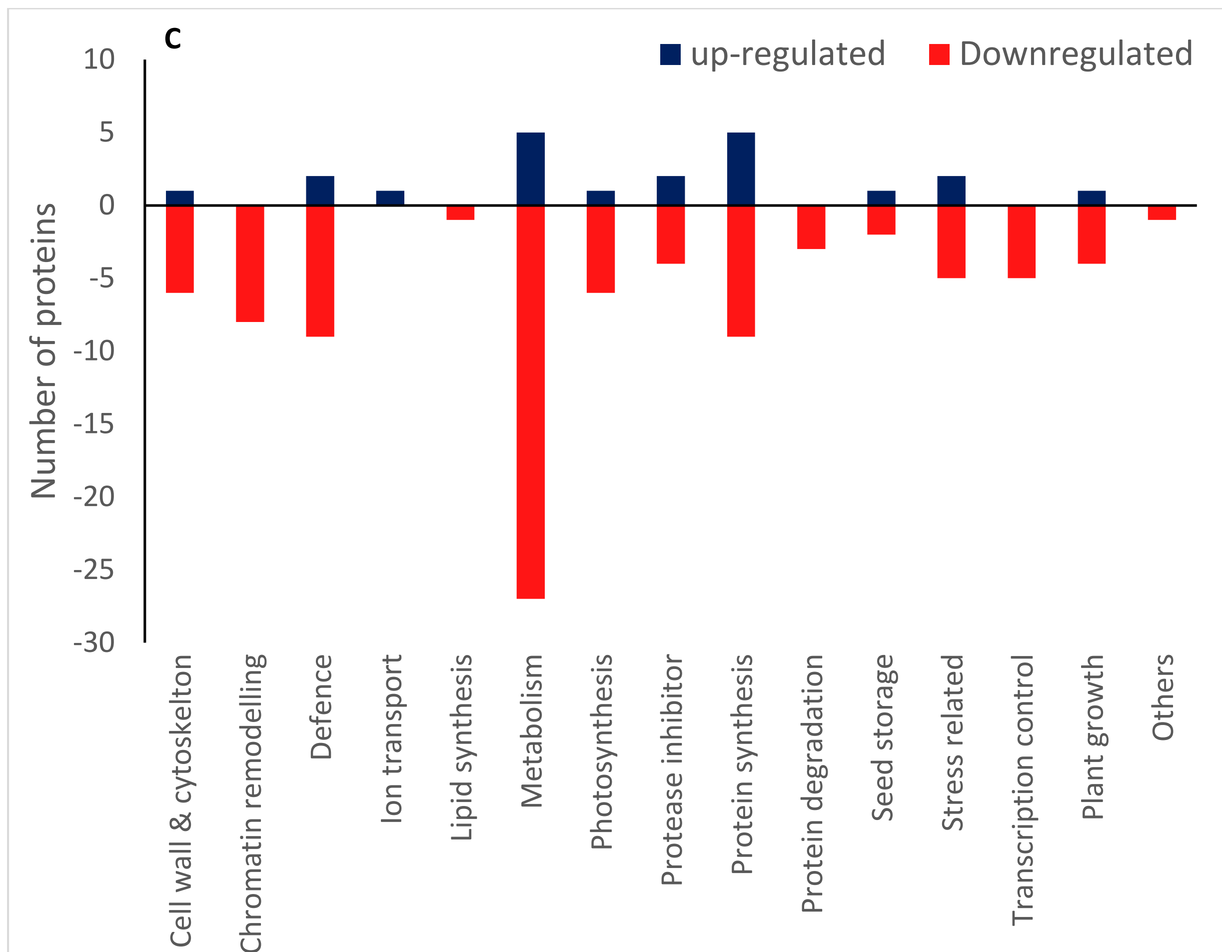


Fig. 5.3 C. Number of proteins up-regulated/down-regulated in each functional category in treatment T-3.

Identification of differentially expressed proteins

To depict the common and unique proteins identified in each treatment, we constructed the Venn diagram, where the numbers of proteins in response to bacterial inoculation and NaCl stress are reported. A total of 301 proteins observed in treatment T-1 (control vs. control with salt stress), 307 in treatment T-2 (control vs bacterial inoculated), and 286 in treatment T-3 (bacterial inoculated vs. bacterial inoculated with salt stress). After comparison, 278 proteins were common in treatment T-1 & T-2, whereas 256 and 266 proteins were common to treatment T-1 & T-3, and between T-2 & T-3 respectively (Fig. 5.4). Among the identified proteins, 243 proteins were identified under all treatments.

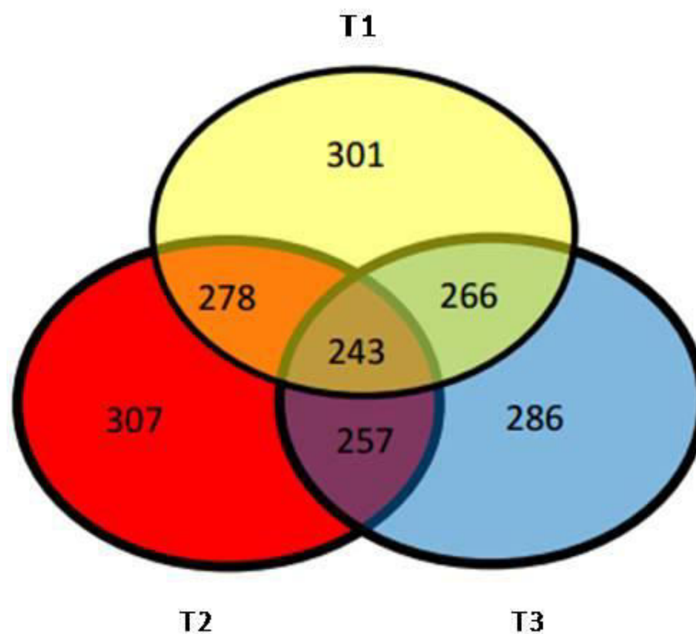


Fig. 5.4. Venn diagram representing the presence and common proteins in each treatments (T-1, T-2, T-3).

Cluster analysis of differentially expressed proteins

A total of 79 differentially expressed proteins common to all treatments (T-1, T-2, T-3) were used for hierarchical cluster analysis under different treatments (Table 5.1) protein with UNIPROT-ID). These 79 proteins were chose based on their higher abundance. In the T-1 treatment, the cluster contained the 19 up-regulated and 38 down-regulated proteins. Examples of proteins that were majorly decreased under salt stress are Photosystem I chlorophyll a (spot 43), Trypsin alpha-amylase inhibitor (spot 62), Glucose-1-phosphate adenyltransferase (spot 19) and Histone (spot 44). The other down-regulated protein were belonging to Peroxiredoxin protein (spot 65), Ribulose bisphosphate carboxylase (spot 28), and ATP synthase protein (spot 45). Bacterial inoculation enhanced the expression of the proteins belonging to ATP synthase (spot 18, 45), Ribulose bisphosphate carboxylase (spot 28), Translation initiation factor (spot 8), Glucose 1 phosphate adenyltransferase (spot 19), and Histone H3 (spot 44) under non-saline stress. Under the salt stress of 200 mM, bacterial inoculation enhanced the expression of Phosphoglycerate kinase (spot 21), Photosystem I (spot 43), Glucose-1- phosphate adenyltransferase (spot 20) and Cold shock protein (spot 40). The down-regulated proteins were belonging to Chloroplast envelope membrane protein (spot 46), NADP-dependent glyceraldehyde 3 phosphate dehydrogenase (spot 68), Thaumatin-like protein (spot 29), and Ribosomal protein (spot 64).

Table 5.1 The identified proteins with their UNIPROT-ID used for cluster analysis

UNIQID	Protein Name	Sample Name	T1	T2	T3
A9UL13	Retinoblastoma related protein 1	S1	3.490	0.733	0
B0LXM0	S adenosylmethionine synthase	S2	3.387	0	0.175
B1B5D4	Ninja family protein 2	S3	0.663	0.343	0
B1B5D5	Ninja family protein 3	S4	0.543	0.683	0
B8YG97	Avenin like b11	S5	0.105	0.778	0
B6DZC8	Fructan 1-exohydrolase	S6	0	0.748	0.6831
O04705	Gibberellin 20 oxidase 1 D	S7	0	0.740	0.346
O24473	Eukaryotic translation initiation factor 2 subunit beta	S8	1.616	8.414	0
O64392	Wheatwin 1	S9	0	1.822	0.511
P00413	Cytochrome c oxidase subunit 2	S10	0.360	0.307	0
P05312	NAD P H quinone oxidoreductase subunit I chloroplastic	S11	1.733	6.889	0.637
P05151	Cytochrome f	S12	1.803	6.685	0
P08488	Glutenin high molecular weight subunit 12	S13	0.733	0.410	0
P09195	Fructose 1 6 bisphosphatase chloroplastic	S14	0	0.657	0.527
P10387	Glutenin high molecular weight subunit DY10	S15	0.329	0	1.822

P11515	Serine carboxypeptidase 3	S16	0.463	0	0.511
P11534	50S ribosomal protein L2 chloroplastic	S17	0.582	0	0.559
P12112	ATP synthase subunit alpha chloroplastic	S18	1.521	2.181	0.683
P12298	Glucose 1 phosphate adenyltransferase large subunit Fragment	S19	0.543	7.924	0.423
P12300	Glucose 1 phosphate adenyltransferase large subunit chloroplastic amyloplastic Fragment	S20	1.858	1.537	3.669
P12782	Phosphoglycerate kinase chloroplastic	S21	0	0.726	4.566
P12783	Phosphoglycerate kinase cytosolic	S22	0	0.582	0.690
P16347	Endogenous alpha amylase subtilisin inhibitor	S23	0	0.711	0.272
P22701	Em protein CS41	S24	0.511	0.755	0.339
P23923	Transcription factor HBP 1b c38	S25	0.145	0.748	0
P25032	DNA binding protein EMBP 1	S26	0.486	0.670	0.070
P26304	NAD P H quinone oxidoreductase subunit K chloroplastic	S27	0.343	0.631	1.896
P26667	Ribulose bisphosphate carboxylase small chain PW9 chloroplastic	S28	0.427	2.013	0.740
P27357	Thaumatin-like protein PWIR2	S29	4.349	9.487	0
P27572	NADH ubiquinone oxidoreductase chain 4	S30	0.449	0.733	0
P27736	Granule bound starch synthase 1 chloroplastic	S31	0	0.477	0.307
P27806	Histone H1	S32	1.786	1.716	0.477
P27807	Histone H2B 1	S33	0.631	0	0.718
P30523	Glucose 1 phosphate adenyltransferase small subunit chloroplastic	S34	0.477	0.650	0
P31251	Ubiquitin activating enzyme E1 2	S35	1.552	0.683	0
P31252	Ubiquitin activating enzyme E1 3	S36	1.665	0	0.4538
P33432	Puroindoline A	S37	4.953	0	0.003
P38076	Cysteine synthase	S38	1.716	3.596	0
P46525	Cold shock protein CS120	S39	0	1.803	0.755
P46526	Cold shock protein CS66	S40	0	0.588	2.718
P52589	Protein disulfide isomerase	S41	0.657	0.286	0
P55313	Catalase	S42	0	0.755	0.554
P58311	Photosystem I P700 chlorophyll a apoprotein A1	S43	0	1.698	7.924
P68428	Histone H3 2	S44	0	7.315	0.501
P68538	ATP synthase protein MI25	S45	0.307	1.716	0.463
P69373	Chloroplast envelope membrane protein	S46	84.774	4.789	0.081
P80602	2 Cys-peroxiredoxin BAS1 chloroplastic	S47	0.516	0.600	0.506
P93692	Serpin Z2B	S48	0.165	0	0.711
Q01148	NADH ubiquinone oxidoreductase chain 1	S49	0.323	0.548	0.418
Q02066	Abscisic acid inducible protein kinase	S50	3.781	0.657	0.657
Q02879	TATA box binding protein 2	S51	0.326	4.481	0
Q03033	Elongation factor 1 alpha	S52	0	0.637	1.973
Q03968	Late embryogenesis abundant protein group 3	S53	0	0.644	0.677
Q1W374	Phosphomannomutase	S54	0.018	0.697	0.261
Q1XIR9	4 hydroxy 7 methoxy 3 oxo 3 4 dihydro 2H 1 4 benzoxazin 2 yl glucoside beta D glucosidase 1a chloro	S55	0.726	0	0.644

Q2QKB3	Splicing factor U2af large subunit A	S56	0.690	0	0.307
Q2UXF7	Fructan 6 exohydrolase	S57	0	0.367	0.748
Q41593	Serpin Z1A	S58	0	1.993	1.822
Q43206	Catalase 1	S59	0.677	1.568	0
Q43215	Histone H2B 4	S60	0.179	0	0.733
Q43217	Histone H2B 3	S61	0.453	0	0.733
Q43691	Trypsin alpha amylase inhibitor CMX2	S62	0.246	1.733	1.803
Q41558	Transcription factor HBP 1b c1 Fragment	S63	2.095	0	0.600
Q5I7K9	60S ribosomal protein L30	S64	17.993	0	0.027
Q6W8Q2	1 Cys-peroxiredoxin PER1	S65	0.650	0	0.733
Q84N28	Flavone O-methyltransferase 1	S66	0.436	0	0.650
Q84N29	Probable non specific lipid transfer protein 3	S67	0.511	0.625	0.472
Q8LK61	NADP dependent glyceraldehyde 3 phosphate dehydrogenase	S68	0	1.552	0.058
Q95H42	NADPH quinone oxidoreductase subunit H chloroplastic	S69	0	0.496	0.554
Q95H43	NADPH quinone oxidoreductase subunit 1 chloroplastic	S70	7.845	0.763	0
Q95H53	30S ribosomal protein S11 chloroplastic	S71	3.819	0.477	0
Q9S7U0	Inositol 3 phosphate synthase	S72	0.543	0.548	0
Q9XPS6	Photosystem II reaction center protein M	S73	0.117	0	0.440
Q9XPS9	DNA directed RNA polymerase subunit beta	S74	0.726	0.718	0
Q9ZRB1	Tubulin beta 2 chain	S75	0	0.748	0.733
Q9ZRB7	Tubulin alpha chain	S76	0.594	0	1.537
Q9XPS8	Photosystem II CP43 reaction center protein	S77	0	0.670	0.600
Q9SWW5	Glutathione gamma glutamyl cysteinyltransferase 1	S78	0	0.755	0.254
Q9ST58	Serpin Z1C	S79	0	2.075	0.588

Proteins having ratio >1.5 was considered as up-regulated, whereas values <0.75 were kept as down-regulated. Letter S denotes the identified proteins common to all treatments based on UNIPROT-ID.

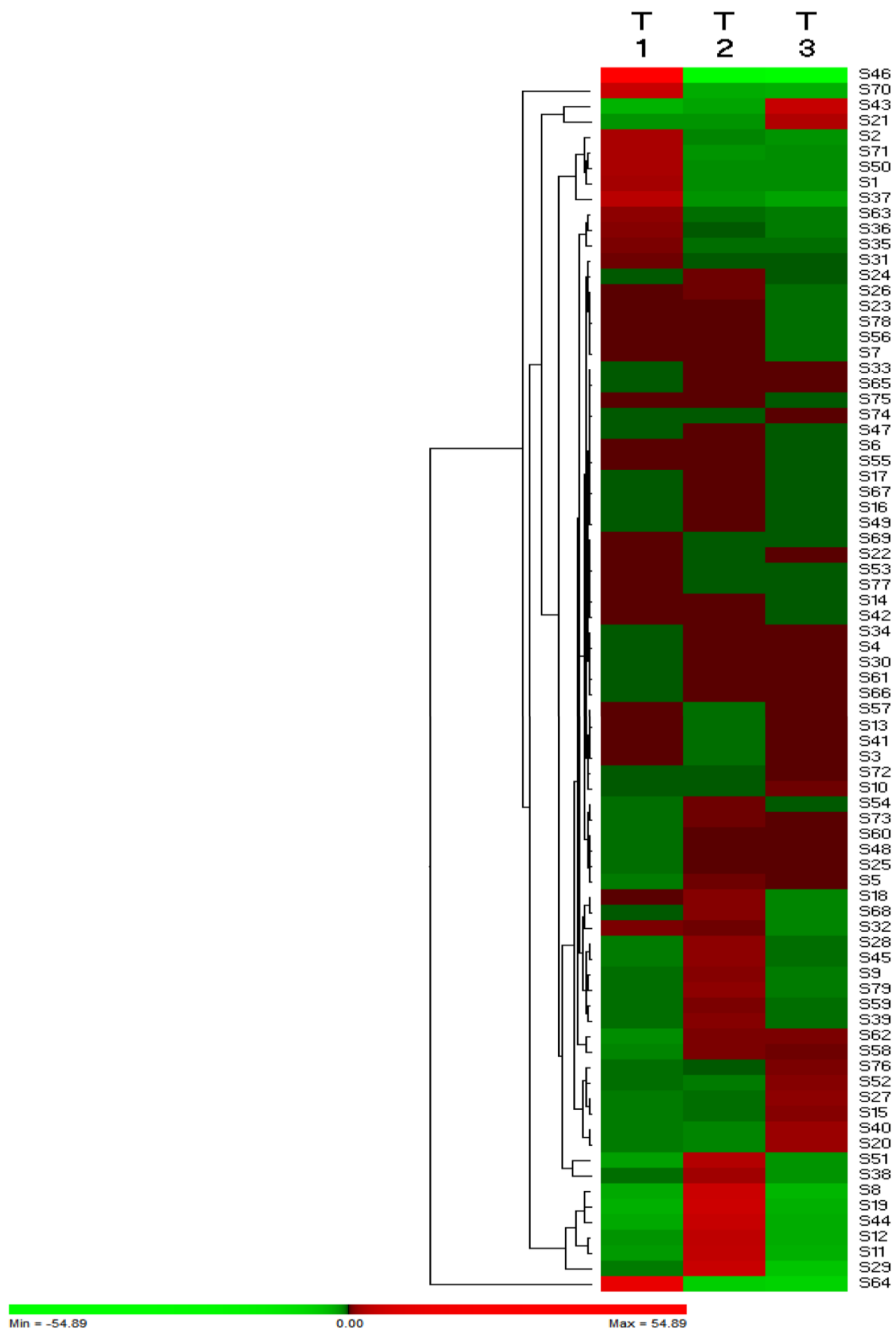


Fig. 5.5. Hierarchical cluster analysis of 79 differentially expressed proteins common to the three experimental treatments (T-1-T-3). T-1 treatment represents the untreated control plant versus salt stress (200 mM NaCl). T-2 treatment represents the control plant against the bacteria inoculated plant. T-3 is the comparison against bacterial inoculated plant against salt stress in the presence of bacterial inoculum. Up-regulation or down-regulation is indicated by the green and red color respectively. The intensity of the colors increases as the expression differences increase, as shown in the bar at the bottom.

Discussion

Previous studies have demonstrated that inoculation of PGPR can up-regulate or down-regulate expression of the proteins in response to salinity and other stresses (Gagné-Bourque et al., 2016; Subramanian et al., 2016). In this study, the variation in expression of the proteome of wheat plants due to NaCl treatment was analyzed in plants inoculated with PGPR *Klebsiella* sp. SBP-8. It is well documented that on exposure to different stresses such as temperature, drought, and salinity plants develop one or several other mechanisms which are regarded as adaptive mechanisms to sustain in given stress conditions. These adaptive features (modifications) can be exhibited at genetic, molecular, membranous and cellular levels. These modifications can occur either at one or many levels as mentioned above. The present study demonstrated that plants possess several different adaptive mechanisms in response to NaCl stress to cope with imposed stress by regulating the genes for proteins involved in stress, photosynthesis, transcriptional control, and protein synthesis (Subramanian et al., 2016). This altered gene expression in response to NaCl stress results in up-regulation/down-regulation of various stress-related proteins and protects the plant from stress. Thus an understanding of these differential expressions could provide an insight into plant's response to NaCl stress. In the present study, we used the gel-free proteomics protocol for the identification of proteins, as it allows major changes and even deeper analysis of the proteome. The functional groups related to different categories are discussed in detail in following sections.

Cell structure maintenance, cell division, and chromatin-associated protein

Maintenance of cellular integrity is of paramount importance for the organisms thriving in high osmotic stress conditions. In the present study, this was supported from the up-regulation of few cytoskeletal proteins such as 'Tubulin' which is required for the maintenance of cell integrity (Shoji et al., 2006). Protection of cell integrity under salt stress was also guaranteed by the increase in the levels of 'Profilin' that binds to actin and affects the structure of the cytoskeleton. Another up-regulated gene was one encoding 'Retinoblastoma' which is involved in cell-cycle progression, endoreplication, transcriptional regulation, chromatin remodeling, and cell growth were observed in the bacterial inoculated plants.

Bacterial inoculation enhanced the expression of 'Casparian strip membrane protein'(CASP) and 'Xyloglucan endotransglycosylase (XET)' as compared to uninoculated plants treated with salt stress. CASP regulates membrane-cell wall junctions and prevents

lateral diffusion of molecules by recruiting the lignin polymerization machinery in the endodermis (Roppolo et al., 2014) whereas XET relegates xyloglucan polymers, an essential constituent of the primary cell wall, and thereby participates in cell wall construction of growing tissues. In spite of the critical role of the endodermis development, very little is known about the biosynthetic mechanism of Casparian strip formation. XET integrates the newly secreted xyloglucan chains into an existing wall-bound xyloglucan restructuring existing cell wall material by catalyzing transglycosylation between previously wall bound xyloglucan molecules (Munoz-Bertomeu et al., 2013). Sometimes XET act as hydrolase (XEH), hydrolyzing one xyloglucan molecule, depending on the nature of the xyloglucan donor and acceptor substrates (Fry et al., 1992; Rose et al., 2002). Furthermore, XET may be important for regulating the polymer length and insertion of xyloglucans into the cell wall, which could alter the extensibility of the cell wall (Fry et al., 1992). A decrease in XET activity was reported in primary roots of maize with low water potential, which was correlated with a decrease in cell wall extensibility and cell elongation in that region (Pritchard et al., 1993). In contrast to this, Wu et al. (1994, 1996) found enhanced XET activity under abiotic stress like drought and heat in durum wheat that was correlated with an increase in cell wall extensibility.

Ion-transporters and lipid biosynthesis

In response to bacterial inoculation, the differential level of expression of ion transporter proteins was observed under salinity stress. In the ion-transporter category, four proteins were down-regulated under 200 mM NaCl stress with respect to the control and three proteins were up-regulated. As compared to control, bacterial inoculation enhanced the expression of ‘Malate transporter’ and ‘Two pore calcium channel protein’, whereas down-regulates the expression of ‘Mitochondrial outer membrane porin’. At high salinity stress of 200 mM NaCl, bacterial inoculation enhanced the ‘calcium channel protein’ that acts as the major ROS-responsive Ca^{2+} channel and mediates the salinity -induced Ca^{2+} influx in leaf cells. Ca^{2+} acts as the second messenger in response to environment stimuli under salt stress. A diversity of Ca^{2+} responsive proteins facilitates the regulation of their target proteins by coordinating the diverse signaling pathways (DeFalco et al., 2010). It has been demonstrated in an earlier study that the induced expression of calcium channel protein plays a pivotal role in regulating calcium homeostasis and protein folding in the endoplasmic reticulum in rice leaves under osmotic stress (Zang and Komatsu, 2007). Therefore, signaling pathway

mediated by calcium seems to be an important strategy of wheat seedlings in coping with salt stress.

The protein involved in the lipid biosynthesis provides the stability of cellular membranes and increases the ability to bind and/or carry hydrophobic molecules across the membrane (Brien et al., 2005). Various lipid biosynthesis proteins such as 'Obtusifoliol 14 alpha-demethylase' (CYP51) and 'Puroindoline' were up-regulated at 200 mM NaCl with respect to the bacterial inoculation. CYP51 are involved in the steroid biosynthesis pathway, whereas 'Puroindoline' forms monovalent cation-selective ion channels in membranes and also act as membrane toxin to protect the plants against predators. CYP51 is the most widely distributed cytochrome P450 gene family being found in all biological kingdoms. It catalyzes the first step following cyclization in sterol biosynthesis, leading to the formation of precursors of steroid hormones, including brassinosteroids, in plants (Brien et al., 2005). The increase in the sterol/brassinosteroids is essential for plant growth and reproduction. In addition, these are capable of increasing plant tolerance to both biotic stresses like pathogen attack and abiotic stress like drought, salinity, heat etc. (Divi and Krishna, 2009).

Bacterial inoculation enhances the 'Non-specific lipid transfer protein' (nsLTPs) expression that transfers phospholipids as well as galactolipids across membranes and plays a role in wax or cutin deposition in the cell walls of expanding epidermal cells and certain secretory tissues. The nsLTPs are small, basic proteins present in abundance in higher plants. They are involved in key processes of plant cytology, such as the stabilization of membranes, cell wall organization, and signal transduction. These are also known to play important roles in resistance to biotic and abiotic stress and in plant growth and development, such as sexual reproduction, seed development and germination (Liu et al., 2015). Although nsLTPs have been extensively studied, their modes of action in intact cells have not yet been fully elucidated.

Defense/stress-related proteins (including pathogenesis protein also)

The data showed that few of the defense proteins such as Clp protease, Thioredoxin H, 2 Cysperoxiredoxin, Catalase and Ninja family protein were down-regulated at 200 mM NaCl as compared to control plants. However, in the presence of bacterial inoculation, few of the defense related proteins belonging to Ninja family were up-regulated under salt stress. The increase in accumulation of defense proteins could be due to increase in the expression of these proteins in bacteria inoculated plants. Clp protease shows the chymotrypsin-like activity

and plays a major role in the degradation of misfolded proteins, but a physiological role in plants has not been well established yet. Thioredoxin H probably behaves as an antioxidant enzyme particularly important in the developing shoot and photosynthesizing leaf under stress. 2 Cys-peroxiredoxin may be an antioxidant enzyme particularly important in the developing shoot and photosynthesizing leaf. Catalase is an antioxidant enzyme involved in different processes, such as H_2O_2 detoxification, stress response, and senescence (Almagro et al., 2009).

The role of Ninja family protein in the plant has not been well understood. However, it is postulated that it acts as a negative regulator of abscisic acid (ABA) response during germination through the ubiquitin-mediated proteolysis of ABI5/DPBF1. Bacterial inoculation up-regulates the expression of Transcription factor HBP 1a and Cold shock protein CS120 (belong to ninja family of proteins) as compared to uninoculated plants. HBP 1a is a putative transcription factor which regulates histone gene expression. Cold shock protein CS120 may reduce intracellular freezing damage during winter by hydrogen-bonding to the lattice of the nascent ice crystals, thus modifying the structure and/or propagation of ice crystals. In addition, inoculation of bacterium up-regulated the expression of Hsp70, Hsp90 organizing protein, and Cold shock protein CS66 at 200 mM NaCl stress, as compared to control plants with respective salt stress. Many of the other stress-related proteins were down-regulated, illustrating that in the presence of bacterial inoculums, plants did not face the stress conditions.

Besides these, other proteins related to pathogenesis such as Thaumatin-like protein (TLPs), Alpha amylase trypsin inhibitor, Purothionin, Puroindoline B, Wheatwin, and Serpin Z1A were found to be up-regulated in the presence of bacterial inoculation under salt stress (T-3). The expression of these proteins provides insight into the understanding of cross-tolerance mechanism in wheat plants in response to biotic and abiotic stress. The pathogenesis-related protein plays a crucial role in response to pathogens. However, their involvement in salinity stress has been demonstrated in several crops (Guo et al., 2012; Wang et al., 2011). TLPs are reported to be widely distributed PR proteins across kingdoms including gymnosperm, angiosperm, and have been isolated and characterized from different plants and tissues. These are involved in the formation of disulfide linkages, which impart stability to the protein under varied thermal and pH conditions and are shown to be involved effectively in alleviating both biotic and abiotic stress tolerance (Das et al., 2011, Goel et al., 2010). Alpha amylase trypsin inhibitor could be involved in insect defence mechanisms.

Purothionin in conjunction with thioredoxin, affects proper protein folding, cytotoxic, presumably by forming pores in the cytoplasmic membrane. Their precise function is not known. Puroindoline B has antimicrobial activity against several bacterial and fungal pathogens. Wheatwins are pathogenesis-related proteins of the PR-4 family and shows antifungal activity towards the wheat-specific pathogenic fungi *Fusarium culmorum* and *F. graminearum.* In addition, wheatwin has been demonstrated to possess an RNAse activity that may be part of a mechanism for inhibiting invading pathogens (Caporale et al., 2004). The expression of wheatwin genes has been investigated in a number of tissues, particularly in response to pathogen challenge (Bertini et al., 2003). However, the potential role of wheatwin in response to abiotic stress has not been investigated. The wheat serpins are suicide substrate inhibitors of chymotrypsin and cathepsin A that may serve to inactivate serine proteases of grain-boring insects (Østergaard et al., 2000). They have not yet been assigned to specific genetic loci on the wheat chromosomes. Additionally, in presence of bacterial inocula decrease in the expression of Glutathione –S-transferase (GST) was noted.

Protein synthesis/degradation

Increase in NaCl stress causes a significant suppression of protein synthesis and its intermediate pathways (Jiang and Deyholos, 2006). We found that levels of Elongation factor 1 and Protein disulfide isomerase were decreased at NaCl, whereas activation of Ubiquitin-activating enzyme E1 1, E1 2, E1 3, Eukaryotic translation initiation factor 2, Translation initiation factor IF 1 and Ribosomal protein were increased. Activation of ubiquitin enzymes illustrates the proteolytic activity in response to NaCl stress. Many of the enzymes responsible for protein degradation and proteolytic complexes involved in recognizing and removing abnormal proteins were found to be up-regulated at NaCl stress. Ubiquitin enzymes play a central role in metabolism under abiotic stress as they are involved in protein inactivation, degradation of damaged proteins, and release of amino acids for metabolism (Capriotti et al., 2013). The levels of various ribosomal subunits were differentially decreased with respect to salt stress. Nevertheless, eukaryotic translation initiation factor 4B1, 4E 1 and 2 were significantly increased in response to bacterial inoculation. Therefore, it can be assumed that regulation of the translational machinery is an important component of stress response in plants (Ndimba et al., 2005).

Photosynthesis

Photosynthesis is one of the physiological processes that are very sensitive to salt stress. The primary effect of salt stress is the reduction of stomatal aperture in leaves, which leads to the reduction of CO_2 availability and thus minimizes the energy for plant growth. We found a significant increase of the level of Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO) large subunit at 200 mM NaCl. The increase in the levels of RuBisCO subunits seems to partially offset the energy reduction that naturally occurs under salinity stress (Capriotti et al., 2013). RuBisCO is the key enzyme for CO_2 assimilation and catalyzes the carboxylation of D-ribulose 1,5-bisphosphate, the primary event in CO_2 fixation in the Calvin cycle. It is stroma-localized protein and constitutes up to 50% of all chloroplast proteins. Salt stress increases oxidation and decreases carboxylation activities of RuBisCO, and causes to decrease in severity of CO_2 fixation (Sivakumar et al., 1998).

The other enzymes that increased included Cytochrome f, Photosystem II protein D1, Cytochrome b, Photosystem II CP47 reaction center protein, and Chloroplast envelope membrane protein. Cytochrome f mediates electron transfer between Photosystem II (PSII) and Photosystem I (PSI) as well as cyclic electron flow around photosystem I (PSI) (Willey et al., 1984). Photosystem II protein D1 forms the reaction core of PSII as a hetero-dimer with the D2 protein and withdraws electrons from water, leading to the splitting of water and the formation of molecular oxygen. Cytochrome b is the component of the ubiquinol-cytochrome c reductase complex (complex III or cytochrome b-c1 complex), which is a respiratory chain that generates an electrochemical potential coupled to ATP synthesis (Saalaoui et al., 1990). Photosystem II CP47 reaction centre proteins are the intrinsic transmembrane antenna proteins CP43 (PsbC) and CP47 (PsbB) found in the reaction centre of PSII, to cope with light limitations and stress conditions. The molecular function of Chloroplast envelope membrane protein (CemA) is unknown, however partially, involved in light-induced Na^+-dependent proton extrusion and has been implicated in CO_2 transport (Greiner et al., 2008). We found various differentially expressed proteins related to energy and metabolism after NaCl stress in the presence of bacterial inoculation. The down-regulated proteins belonging to photosystem category were of Chlorophyll a, b binding protein, Cytochrome b6, Photosystem II reaction centre protein M, Ribulose bisphosphate carboxylase small chain PW9, Cytochrome b6 f complex iron-sulfur subunit, Photosystem I P700, Photosystem II, and 50S ribosomal protein.

Proteins of metabolism

Several differentially expressed proteins related to metabolism were observed. Proteins those were up-regulated were as follows: Imidazole glycerol phosphate dehydratase, Fructan-1-exohydrolase, Fructan-6-exohydrolase, Fructan-1-exohydrolase, NADPH quinoneoxido-reductase, adenosyl homocysteinase, and Glucose-1-phosphate adenylyltransferase, and Phosphoglycerate kinase. The up-regulation of gene for the synthesis of Fructan-1-exohydrolase seems obvious, as apart from encoding storage compounds, fructans might have an important role in protection against stress (Hincha et al., 2002). These accumulated low molecular weight water-soluble compounds are known as “compatible solutes” or “osmolytes” is the common strategy adopted by several organisms to combat the environmental stresses. Osmotic adjustments at physiological levels are key to survive under salinity stress. The osmoregulators protect plant cells during extreme stress conditions of salt. Furthermore, genes related to osmoprotectant biosynthesis are up-regulated under salinity stress (Rontein et al., 2002). Also, their accumulation is preferentially favoured under salt stress because they provide tolerance against stress (Chen et al., 2002). The level of accumulation of osmo-protectants in different species provides different levels of protection during abiotic stresses. Genes responsible for the synthesis of different types of compatible solutes have been isolated from various organisms. Genetic engineering attempts are being made with these endogenous or ectopic genes to successfully use to synthesize compatible solutes in target organisms to improve stress tolerance (Umezawa et al., 2006; Chen et al., 2008).

The present study identified the adaptive mechanisms conferred by plant growth promoting rhizobacteria *Klebsiella* sp. SBP-8 on the wheat plant to counteract the salinity stress by proteomic profiling. The tested bacterium possesses the ACC deamianse activity that has certain advantages on plant growth responses under stress conditions. In the presence of bacterial inoculation, the identified over-expressed proteins have been shown to provide plant resistance to salinity stress. In addition, the expressed proteins following rhizobacterial inoculation govern the osmotic homeostasis, defense activation, cell wall strengthening, ion transportation, and photosynthesis functioning to confer stress resistance. The higher accumulation of metabolic pathway proteins illustrates that different metabolic pathways could be involved in the protection of wheat plant to salt stress. Therefore, by using the proteomic approaches the observed evidence supports that application of a beneficial PGPR to wheat seedlings could be used as an effective tool to overcome the salinity stress. The

identified proteins can be useful for genetic transformation to improve the salt tolerance mechanism in wheat-like cereal crops. The future study should be directed to quantitatively analyze the differentially expressed proteins under different salinity treatments to elucidate the proper salt tolerance mechanism.

CHAPTER-VI

Genome analysis of plant growth promoting bacterium *Enterobacter cloacae* SBP-8

6.1 Introduction

The generation of annotated genomic information/data from the prokaryotic organism in recent years has helped to facilitate the identification and characterization of useful genes in the microbes (Amineni et al., 2010; Dutta et al., 2006). Subtractive genomics together with bioinformatics analysis can find out previously unknown cellular functions/features in microbes based on the understanding of similar biological processes in other microbes (Vetrivel et al., 2011; Koteswara et al., 2010). The appearance of large data sets coming out from the whole genome sequencing projects leads to the identification of new antibacterial targets in bacteria pathogenic to plants that still an untouched area of in-silico research. However, whole genome analysis has identified a number of drug targets for phytopathogenic as well as human pathogenic bacteria (Barh et al., 2011). Moreover, systematic analysis of the genomic data has elucidated the understanding of the molecular genetics of many bacterial strains (MacLean et al., 2009).

Based on the plant growth promoting potential, strain SBP-8, emerged as the most promising isolate as described in previous chapters. Based on partial 16S rRNA sequence, the strain was identified as *Klebsiella* sp. SBP-8. However, based on whole genomic analysis and full length 16S rRNA sequence, this was identified as *Enterobacter cloacae* SBP-8. The plant rhizosphere harbours several beneficial bacteria having potential to stimulate the plant growth, even under stress conditions (Kloepper et al., 1978). *Enterobacter cloacae* SBP-8 is a gram-negative, facultative anaerobic rod-shaped Proteobacteria, a genus of *Enterobacteriaceae* widely found in all natural environments. Strain SBP-8 promoted plant growth under salt and temperature stresses through its multifarious activities. Thorough understanding of different properties this isolate will be useful for its extensive exploitation for enhancing agricultural productivity. Genome sequencing of this bacterial genome will allow the investigation of its entire genomic content with respect to plant growth promotion and its molecular interactions with the host plant. Complete genome annotation and interpretation of a few *Enterobacter cloacae* is available. However, most of the analyses were focused on their endophytic as well as pathogenic behaviour (Liu et al., 2012; Ren et al., 2010; Taghavi et al., 2010). Recently, the draft genome sequence of a chromium-resistant bacterium, *Enterobacter cloacae* B2-DHA was analyzed to search the genes potentially involved in bioremediation of chromium and other toxic metals in polluted environments (Rahman et al., 2016). The genes that code for putative proteins involved in root adhesion, colonization, and protection of the plant against fungal and bacterial infections have been

characterized in a few strains and shown to be of agricultural importance, as *Enterobacter cloacae* has been studied to increased crop yields (Taghavi et al., 2010).

The plant pathogenic effects of *Enterobacter cloacae* strains have been reported (Nishijima et al., 2004; 2007). A few *Enterobacter* species are human opportunistic pathogens responsible for nosocomial infections such as osteomyelitis, cholecystitis, and neonatal meningitis (Ren et al., 2010). However, information about plant associative *Enterobacter* sp. effectively behaving as PGPR (plant growth promoting rhizobacteria) is scarce. The plant growth promoting rhizobacteria use direct or indirect mechanisms to stimulate the plant growth, stress tolerance, and protection from phytopathogens (Glick 2012; Lugtenberg and Kamilova 2009). The strain SBP-8 can be considered as safer and more practical that exhibit direct antagonistic activities against phytopathogens. Moreover, it has the advantage that it is salt tolerant and significantly promotes plant growth in saline soil. Therefore, sequencing and genome information of PGPR *Enterobacter cloacae* SBP-8 would provide the valuable information related to the mechanism of plant growth promotion, antagonistic activity, and its pathogenecity. Major objective of the genome sequence is to analyze overall genomic analysis of PGPR *Enterobacter cloacae* SBP-8, detail description of plant growth promoting properties, and colonizing potential of the test strain.

6.2 Methodology

6.2.1 DNA extraction, sequencing, and genome assembly

Genomic DNA of isolate *Enterobacter cloacae* SBP-8 was extracted by standard method using bacterial DNA extraction kit (Qiagen, USA). De-novo whole genome sequencing was done using Illumina Paired-end sequencing platform technology. The Illumina paired-end raw reads were quality checked using FastQC. Illumina raw reads were processed by in-house pearl script for adapters and low-quality bases trimming towards 3'-end. De-novo assembly of Illumina PE data was performed using SPAdes assembler. SPAdes assembler is intended for *de novo* assembly after error-correction of sequenced reads. Assembled contigs were further scaffolded using SSPACE program. Genes from assembled scaffolds were annotated using NCBI genomic tools and pathways were determined using online available program RAST server.

6.2.2 Annotation of genome

The *Enterobacter cloacae* SBP-8 genome sequence was first annotated using web-based automated pipelines including Bacterial Annotation System (BASys) v1.0 (Van Domselaar et al., 2005). Glimmer 3.0 (www.cbcb.umd.edu/software/glimmer) software was used to predict the number of genes and tRNA scan - SE v1.3.1 software to forecast the rRNA and tRNA of the genome. Putative CDS were identified by Glimmer v3.02 and Prokaryotic Dynamic Programming Gene finding Algorithm (Prodigal) v2.5 (Delcher et al., 2007). The functional annotation of genome sequence was performed against the databases of KEGG (http://www.genome.jp/kegg/), COG (http://www.ncbi.nlm.nih.gov/COG/) and GO (http://www.geneontology.org/). The draft genome was provided to BASys (Bacterial Annotation System), a web-based annotating pipeline to predict functional elements in the genome and attach biological information to them (Van Domselaar et al., 2005). The final annotated chromosome and plasmid were plotted using CIRCOS to show gene locations, GC-skew, and GC content (Krzywinsk et al., 2009). Genomic islands (GIs) were detected using Island Viewer (Langille and Brinkman, 2009).

6.2.3 Genome comparison

The genome sequence of *Enterobacter cloacae* SBP-8 was compared to other assembled *Enterobacter* genomes using progressive Mauve aligner using default settings (Darling et al., 2010). The published genomes used in the alignment were obtained from PATRIC database (http:// patricbrc.vbi.vt.edu) (Snyder et al., 2007). The sequence alignment file generated by the aligner was parsed to calculate pairwise similarity. Briefly, we first extracted the conserved blocks from the alignment file and then regions with, 50 continuous gaps were considered for computing similarity score based on a pairwise sequence similarity percentage and coverage score which represents the percentage of the genome that could be aligned pairwise.

6.2.4 Phylogeny of *AcdS* gene sequence and protein modeling

To obtain the *AcdS* sequence of closely related strains, BLAST analysis was performed in the NCBI databases using the *AcdS* sequence of *Enterobacter cloacae* SBP-8 as the queries. Similarly, NCBI genomic BLAST search (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) was also performed to retrieve the *AcdS* encoded protein completely sequenced organisms Default BLAST parameters were used when obtaining the sequences. Sequence identities and

similarities were analyzed using SIAS (http://imed.med.ucm.es/Tools/sias.html) with default parameters. Similarly, the protein sequences were aligned with MAFFT tool and the alignments were further refined by using Gblocks. Phylogenetic trees were built with PhyML 3.0 under the best model predicted by ProtTest3 with bootstrap values for branch support resulting from 1000 bootstrap replicates

The upstream regulatory elements were analyzed by the SCOPE (Suite for computational identification of promoter elements, genei.dartmouth.edu/scope) (version 2.1.0) tool using default parameters. The sequenced *AcdS* gene (1 kb) of *Enterobacter cloacae* SBP-8 was converted to the corresponding protein sequences by Expasy translate tool (web.expasy.org/translate) using default parameters for all the 6 reading frames. Then one protein sequence from one reading frame was selected by comparing it with other homologous protein sequences. This selected protein sequence was then BLASTed against PDB database. The top hit (lowest E-value, highest score) (PDB ID: 4D96, D-Cysteine desulfhydrase from *Salmonella typhimurium* complexed with 1-amino-1-carboxycyclopropane (ACC)) was used as a template to model our protein. It was modelled by using Modeler v9.15 taking 4D96 as a template. Out of 100 model generated the best 3D model was selected based on the lowest Normalized Dope Score (-1.35270).

Estimates of evolutionary divergence between *AcdS* sequences and 16S rRNA in groups of bacterial strains were computed using MEGA software 6.06. The number of base substitutions per site from between sequences was calculated, and analyses were conducted using the NJ-model with 1000 bootstrap replications. All positions containing gaps and missing data were removed. In another study, the evolutionary divergence between *AcdS* sequences and other house-keeping (*rpoB, rpoD*) was also established.

6.2.5 Sequence retrieval of 16S rRNA and other house-keeping genes

The 16S rRNA gene sequences of *Enterobacter cloacae* SBP-8 and other closely related organisms were obtained from the NCBI database (www.ncbi.nlm.nih.gov/) and aligned by the multiple sequences alignment program. A neighbour-joining tree was build using the MEGA 6.0 software using the 1000 bootstrap pseudo-replications (Tamura et al., 2013). All positions containing gaps and missing data were removed. Similarly, the other house-keeping (*rpoB, rpoD*) gene sequences of test strain and other related strains were retrieved from NCBI database and the phylogenetic relationship was established to evaluate the evolutionary relationship.

6.3 Results

6.3.1 Genome characteristics

The draft genome of *Enterobacter cloacae* SBP-8 contains a circular chromosome (4,854,065 bp) and a plasmid (85,398 bp). The draft genome sequence of *Enterobacter cloacae* SBP-8 was deposited in the Genbank with the accession number CP016906 (chromosome) and CP017413 (plasmid). Altogether 939 scaffolds were analyzed based on which 4192 genes were predicted which encodes 4052 proteins. The genome consists of 73 genes for tRNA and 14 for rRNAs (Table 6.1).The genomic analysis identified the presence of pseudogenes (44) and non-coding rRNA (9). The average contig length of the WGS library turned out to be 6241 bp with median contig length of 782 bp. The average G+C content was found to be 55%. The protein-coding genes have an average length of 915 bp and account for 87.5% of the chromosome. The chromosome of *E. cloacae* SBP-8 displays two clear GC skew transitions, which corresponds with its oriC and terminus (Fig. 6.1). Similar to the chromosome, plasmid map was constructed (Fig. 6.2). Twenty-three putative GIs were identified by Island-Viewer, which integrates two prediction methods Island Path (DNA composition comparison) and SIGI-HMM (codon usage) (Fig. 6.3). The size of putative islands ranged from 4176 bp to 54,345 bp.

Table 6.1 General properties of genome of PGPR *Enterobacter cloacae* SBP-8

Genome Summary	***Enterobacter cloacae* SBP-8**
Genome Size	5.8Mb (both genome + plasmid)
Total number of Scaffolds	939
Total number of genes predicted	4192
Total number of protein encoding genes	4052
Total number of tRNA genes	73
Total number of rRNA genes	14
Pseudogenes	44
Non-coding rRNA	9
G-C content	55%

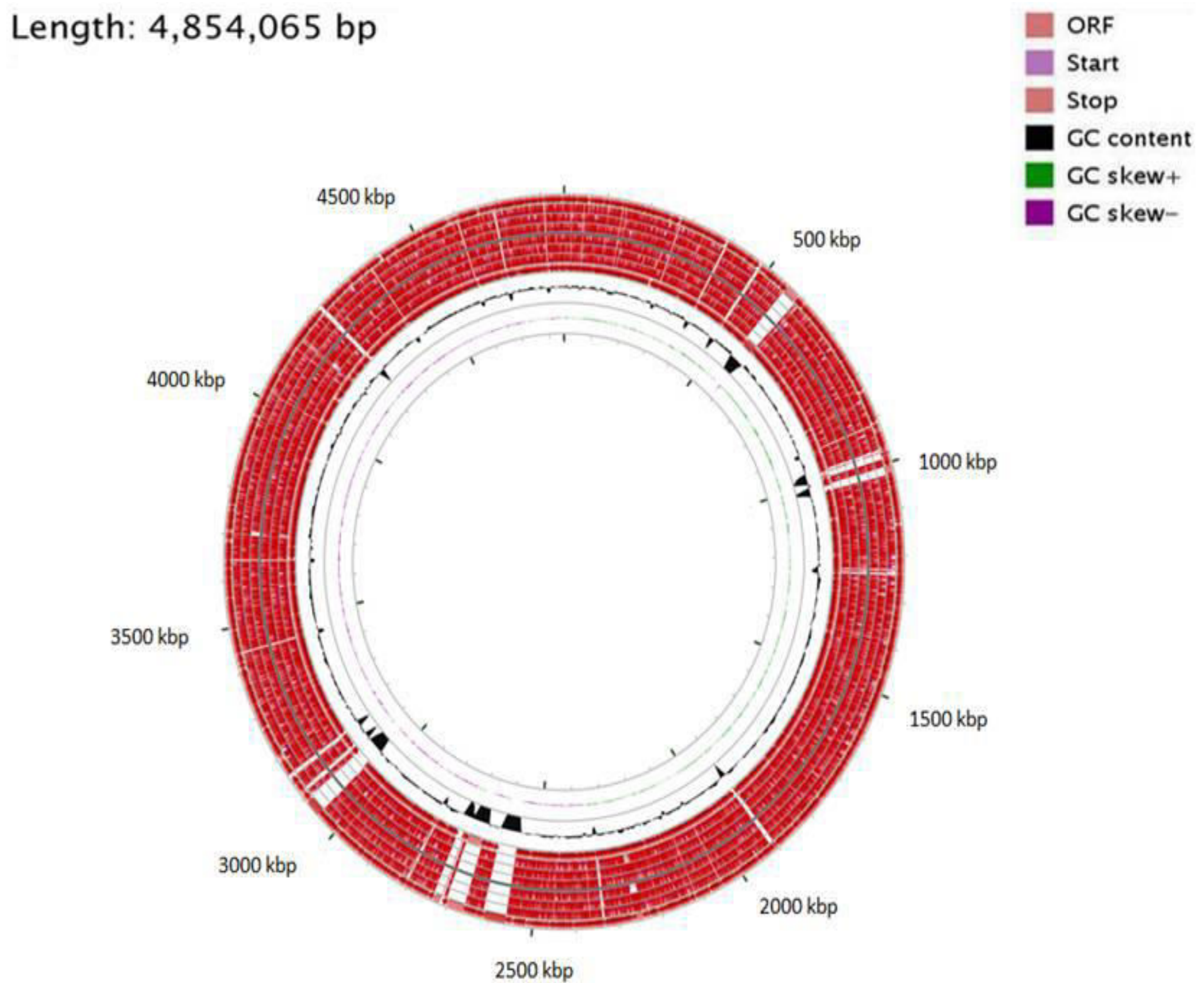


Fig. 6.1. Cicular map representation of *Enterobacter cloacae* SBP-8 complete genome. Rings show from outside (1) Nucleotide sequences (2) GC content, and (3) GC skew. GC content and GC skew were calculated using a 10-kb window in steps of 200 bp. Genomic map was constructed using the BLAST Ring Image Generator (BRIG, version 0.95).

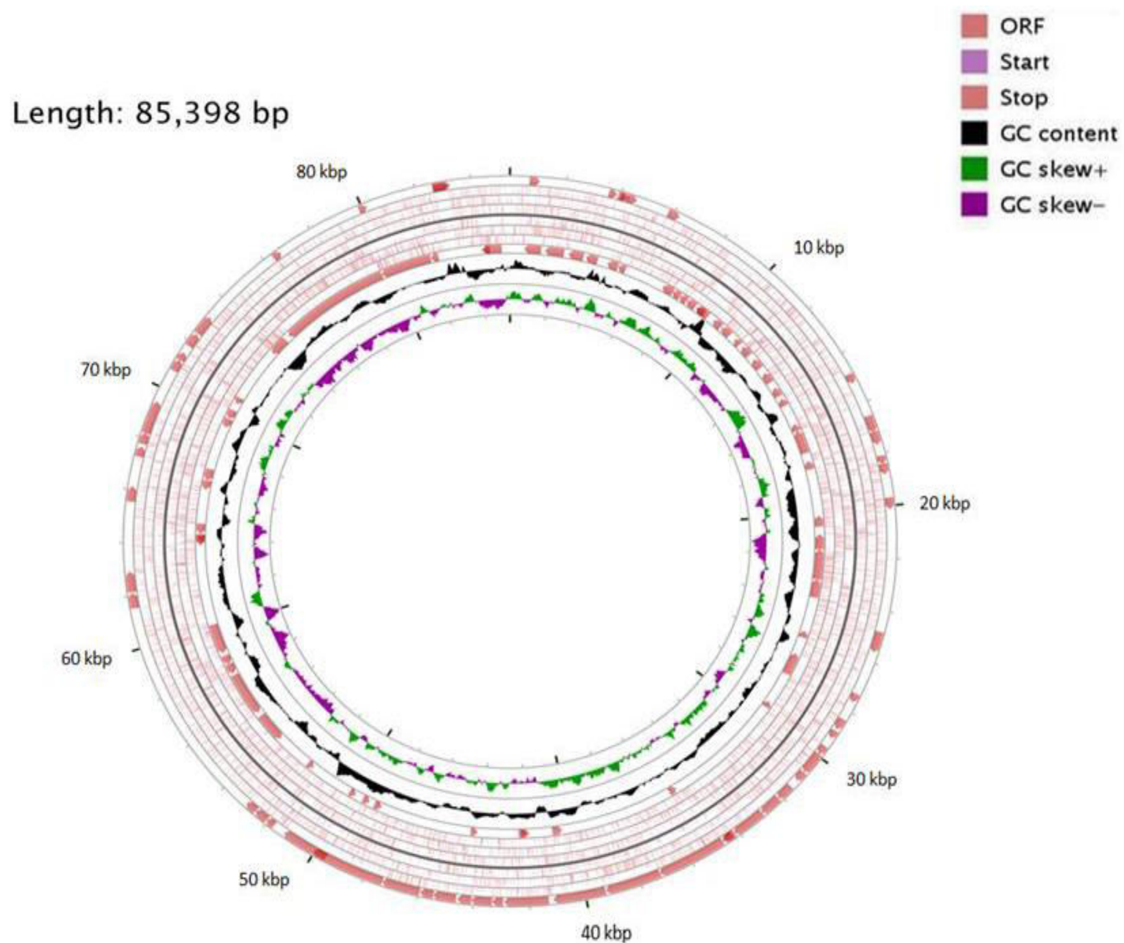


Fig. 6.2. Plasmid map representation of *Enterobacter cloacae* SBP-8 complete genome. Rings show from outside (1) Nucleotide sequences (2) GC content, and (3) GC skew. GC content and GC skew were calculated using a 10-kb window in steps of 200 bp. The genomic map was constructed using the BLAST Ring Image Generator (BRIG, version 0.95).

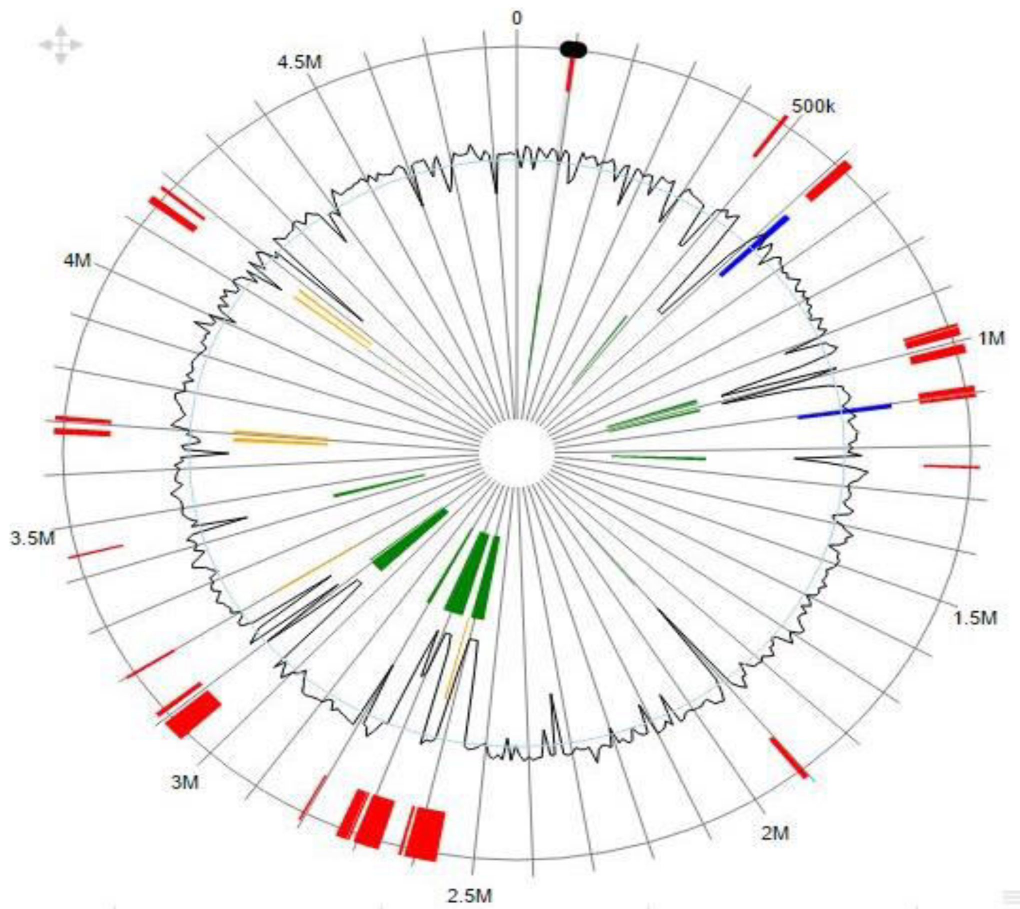


Fig. 6.3. Genomic islands of *Enterobacter cloacae* SBP-8 predicted by Island Viewer. The outer black circle shows the scale line in Mbps. Predicted genomic islands are colored based on the following methods: SIGI-HMM, orange; IslandPath-DIMOB, blue; Integrated detection, red. Black plot represents the GC content.

6.3.2 Gene distribution

RAST server based annotation of the whole genome describes the subsystem distribution of *Enterobacter cloacae* SBP-8 (Fig. 6.4). Genes responsible for carbohydrate metabolism (735 ORFs), amino acids and derivatives (528 ORFs), cofactors and vitamin synthesis (276) and cell wall and capsule formation (232 ORFs) were abundant among the subsystem categories. It also possessed several genes for heavy metals (Zn, As, and Cu) resistance (187), protein metabolism (327), membrane transport (224) and RNA metabolism (248). The presence of higher number of motility and chemotaxis genes (100) in the test isolate might be involved in the mechanisms of colonization. The test isolate showed the salinity and alkali tolerant behaviour and it contains a large number of stress-responsive genes (187).

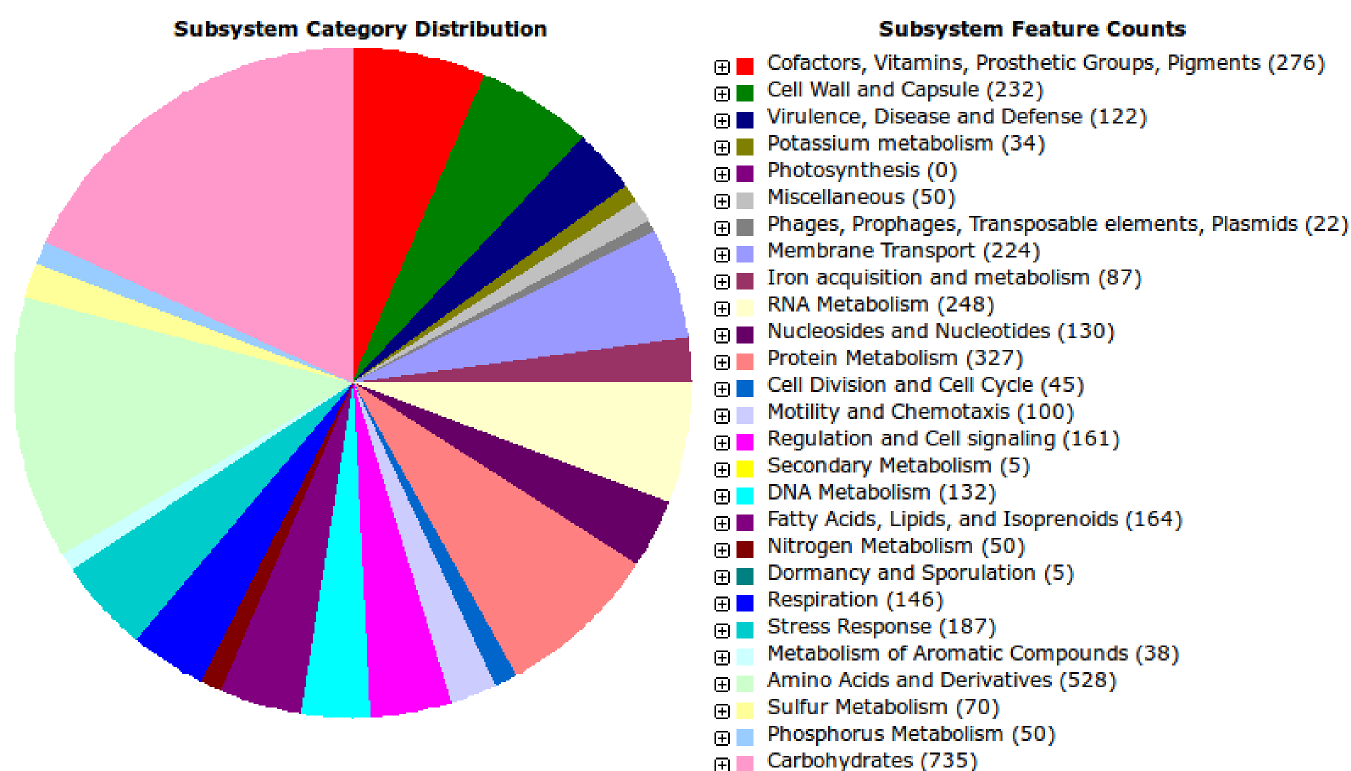


Fig. 6.4. Subsystems distribution statistic of *Enterobacter cloacae* SBP-8 based on genome annotations performed according to RAST server. The pie chart presents the abundance of each subsystem category and the count of each subsystem feature is listed in parentheses at the chart legend.

6.3.3 Motility, chemotaxis, adhesion, and root colonization

Genomic sequencing revealed the genes required for chemotaxis, motility, and adhesion that favours a rhizobacterium for its strong competitive colonization ability of plant roots (Kamilova et al., 2005). For motility, the genes involved in the flagellar biosynthesis protein (*FliL, FliP, FliR, FliC, FlhB, FliZ, FliS, FlgN, FlhA, FliQ, FliT*), and for flagellar motor switch protein (*FliN, FliG, FliM*) were found (Table 6.2). For chemotaxis, chemotaxis protein (*CheV*), and other genes related to regulation and signal transduction (*CheW, CheB, CheR, CheZ, CheA, CheY*) are present.

Table 6.2 Gene distribution for motility, chemotaxis, adhesion and root colonization in genome of PGPR *Enterobacter cloacae* SBP-8

Genes	Proteins
Motility and Chemotaxis	
FliL, FliP, FliR, FliC, FlhB, FliZ, FliS, FlgN, FlhA, FliQ, FliT	Flagellar biosynthesis protein
FliN	Flagellar motor switch protein
FliG, FliM	Flagellar motor switch protein
CheV	Chemotaxis protein
CheW	Positive regulator of CheA protein activity
CheB	Chemotaxis response regulator protein-glutamate methylesterase
CheR	Chemotaxis protein methyltransferase
CheZ	Chemotaxis response - phosphatase
CheA	Signal transduction histidine kinase
CheY	Chemotaxis regulator - transmits chemoreceptor signals to flagellar motor components
Adhesion & Colonization	
YidE	Mediator of hyper adherence
YjbEFGH	Exopolysaccharide Production
Genes for Secretions systems I, II, V and VI	Attachment and infection to host
Establishment in host organism	

lsr *ACDBFGE* operon	Autoinducer 2 (AI-2) transport and processing
Genes for Biofilm Adhesin Biosynthesis	Biofilm PGA synthesis auxiliary protein PgaD, Biofilm PGA outer membrane secretin PgaA, Biofilm PGA synthesis deacetylase PgaB, Biofilm PGA synthesis N-glycosyltransferase PgaC

6.3.4 Plant growth promotion and protection

Among the plant growth promoting genes, *AcdS* (1-aminocyclopropane-1-carboxylate (ACC) deaminase gene was found, that lowers the level of the plant stress ethylene. Full-length sequence of *AcdS* (987 bp) and its 1000 bp upstream sequence were used for detailed analysis of the gene and its regulatory components. It is evident from Fig. 4 that regulatory elements consist of two rpoD elements (position 1409, 1438), two argR, argR1, argR2 (1413, 1414), ihf (1415), P2 (1416), P1 (1436), and CRP (1453) (Fig. 6.5). Detail about the *AcdS* gene/protein phylogeny and protein modeling has been described in proceeding section 6.3.8.

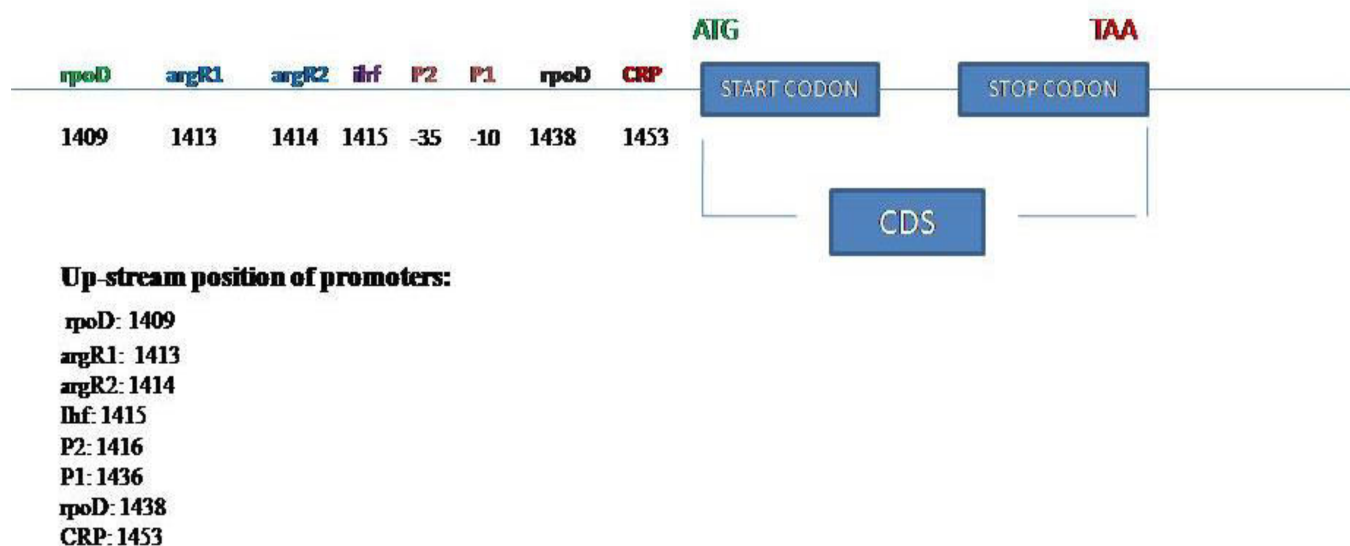


Fig. 6.5. Upstream regulatory elements of *AcdS* structural gene of *Enterobacter cloacae* SBP-8. The regulatory elements were analyzed by the SCOPE (Suite for computational identification of promoter elements, genei.dartmouth.edu/scope) (version 2.1.0) tool using default parameters.

Siderophore production is a typical characteristic possessed by many of the rhizobacteria. *Enterobacter cloacae* SBP-8 was found to produce the siderophore. Consistent with this observation, several siderophore biosynthesis pathways and transport were found, such as those for siderophore aerobactin (*FhuA, FhuB, IucB, IucD, IucA, FhuC, FhuD*), siderophore enterobactin (*FepE, FepG, FepD, FepC, FepB, EntS, EntB*), iron transporter (*EfeB, EfeU*), and iron transport regulator protein (*FuR, PiuB, FeoC*). For phytohormone IAA production, a gene related to Tryptophan synthase (alpha and beta chain) catalyzes the last step in the biosynthesis of tryptophan was found. The gene related to biosynthesis of tryptophan (phosphoribosyl anthranilate isomerase) was found as a single copy. Similarly, anthranilate phosphoribosyltransferase, that catalyzes the most fundamental biochemical reactions in the aromatic amino acid biosynthesis pathway specifically the tryptophan synthesis were present.

The strain SBP-8 is salt (NaCl) tolerant and have the ability to promote the plant growth in saline soil. Following this observation, a large number of genes related to osmoprotectant production were identified by genome sequencing. The genes involved in the biosynthesis and accumulation of osmoprotectants were found, such as those for carnitine (*OpuAB, opuCB*), choline (*ChoV, choW),* glycine betaine (*GbsA*), and proline (*ProP, ProV, ProW, ProX, ProY*). The strain was found to be effective for protection against bacterial and fungal pathogens and also the production of chitinase, cellulase was observed in *Enterobacter cloacae* SBP-8. It illustrates that mechanism for fungal disease suppression by SBP-8 would rely on these pathways found in other closely related *Enterobacter* genome.

6.3.5 Secretion system and biofilm formation

Genomic analysis indicated the presence of secretion systems, I, II, IV and VI which are known to play an important role in infection process. Type I secretion system comprised of an outer membrane protein, and membrane secretory protein. Genes involved in the type II secretion system (GspG, GspH, GspI, and GspJ) encodes proteins for promoting the assembly of pilus subunits. In type IV secretion system, genes encoding for pilus biogenesis protein (PilC, PilM, PilN, PilP PilQ), twitching motility protein (PilT), Type IV pilin assembly protein (PilA) and Type IV fimbrial assembly ATPase (PilB) was identified. Type VI secretion system of *Enterobacter cloacae* SBP-8 is composed of five hypothetical protein, lysozyme protein, ImpG protein involved in plant root infection and few others with unknown function (Table 6.3).

The presence of curli and other genes required for biofilm formation suggest that the bacterial isolate SBP-8 can form a biofilm on or in plants for its maintenance. The biofilm formation can be mediated by quorum sensing mechanism which is supported by the presence of genes encoding AI-2 mediated quorum-sensing in the genome of SBP-8. The linear homopolymer poly-β-1,6-*N*-acetyl-d-glucosamine (β-1,6-GlcNAc; PGA) serves as an adhesin for the maintenance of biofilm structural stability in diverse eubacteria. Its function in *Enterobacter cloacae* SBP-8 illustrates the presence of *pgaABCD* operon, all of which are necessary for biofilm formation. *PgaA* mediates the protein-protein interactions, implying that it forms the outer membrane secretin for PGA synthesis. *PgaB* contains carbohydrate binding and polysaccharide *N*-deacetylase domains. The over-expression of pgaB increased the primary amine content (glucosamine) of PGA. *PgaC* is an apparent glycosyltransferase that is required for PGA synthesis.

Table 6.3 Presence of secretion system and their gene/protein distribution

Secretion system type	Gene/Protein
Type I	Outer membrane protein (omp)
Type I	Target domain containing protein
Type I	TolC/ Type I secretion outer membrane protein
Type II	GspE/ Hexameric protein having ATPase activity
Type II	GspC/ IM platform, secretin interaction
Type II	GspD/ OM secretin
Type II	GspF/ IM platform
Type II	GspG/ Major pseudopilin
Type II	GspH/ Minor pseudopilin
Type II	GspI/ Minor pseudopilin
Type II	GspJ/ Minor pseudopilin
Type II	GspL/ IM platform
Type IV	PilC, PilM, PilN, PilP PilQ, PilT
Type VI	Rhs protein
Type VI	Hcp1/ Formed inner lining of needle
Type VI	T6SS Lysozyme like protein/ Uncharacterized protein similar to VCA0109
Type VI	T6SS associated lipoprotein
Type VI	T6SS associated protein/ VasD (unknown function)
Type VI	ImpG/ involve in Plant root infection
Type VI	EvpB family type VI secretionprotein
Type VI	type VI secretion system-associated protein/ Hypothetical Protein
Type VI	type VI secretion-associated protein/ Hypothetical Protein
Type VI	type VI secretion system protein ImpL/ Hypothetical Protein
Type VI	type VI secretion system-associated protein/ Hypothetical Protein
Type VI	T6SS associated lipoprotein/ Unknown function

6.3.6 Metal and other stress resistance

The draft genome sequence of *Enterobacter cloacae* SBP-8 identified the various heavy metal resistance genes. Five genes that might be involved in the copper resistance of SBP-8 were identified. Copper translocating P-type ATPase (CopA) is involved in the copper resistance of bacteria. However, its presence in *Enterobacter cloacae* is still unknown. The gene encoding CopC is a periplasmic protein involved in copper resistance. Copper resistance protein D (CopD) is the trans-membrane protein found in many Proteobacteria, however, its exact function in unknown. Metal binding copper oxidizing (CueO) probably involved in periplasmic detoxification of copper by oxidizing Cu^{+} to Cu^{2+} and thus preventing its uptake into the cytoplasm. Similarly, Copper-sensing two-component system response regulator (CusR) is the plasmid-borne system that is involved in metal-responsive gene regulation. Besides copper, SBP-8 also possesses resistance to other heavy metals, such as zinc and cobalt. Among the zinc resistance, the gene for zinc efflux and zinc transporter (ZitB) was noted. Similarly, multiple metal (cobalt/zinc/cadmium) resistance transporter proteins belonging to CzcB family was also identified in the genome of SBP-8. Arsenic resistance determinants were found in the genome of *Enterobacter cloacae* SBP-8, including operon *ArsRBCH* and *ArsB*, *ArsC* genes. Multiple antibiotic resistance genes (*MarA, MarB, MarC*, *MarR*) were identified in the genome of *Enteroabcter cloacae* SBP-8. Besides, multidrug transporters are a large and diverse group of proteins capable of protecting cells against a wide variety of environmental toxins by active extrusion of noxious compounds. In the genome of SBP-8, six copy multidrug transporters (*Mdt ABCD*) were identified. In addition to this, multiple copies of multidrug-resistance efflux pumps including *AcrAB* operon were observed in the genome of SBP-8.

6.3.7 Genome comparisons and phylogeny

A phylogenetic tree was constructed using comparisons of genome sequences of *Enterobacter cloacae* SBP-8 to other completely sequenced *Enterobacter* sp. The resulting phylogenetic tree clearly illustrates its similarity to other strains of *Enterobacter cloacae* available in genome database (Fig. 6.6). The analysis revealed that SBP-8 showed the maximum identity to *Enterobacter cloacae* 13047 and *Enterobacter cloacae* GGT036.

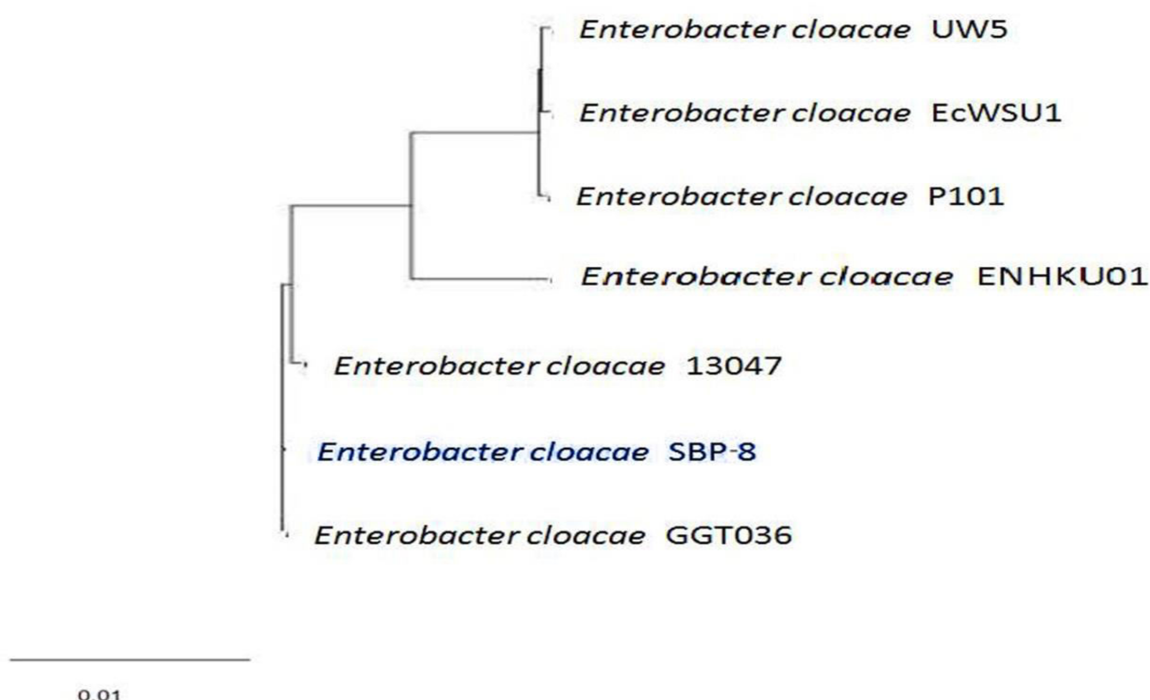


Fig.6.6. Phylogenetic analysis of *Enterobacter cloacae* SBP-8 based on whole genome sequences, with the available genome sequence of *Enterobacter cloacae*. The evolutionary history was inferred by the Neighbour-joining method with bootstrap value 1000.

6.3.8 Phylogeny of *AcdS* gene and protein modeling

After searching the NCBI database using *Enterobacter cloacae* SBP-8 as the query sequence (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi), it was observed that the gene is not commonly seen in most sequenced organisms. A phylogenetic tree was constructed using comparisons of *AcdS* gene in other sequenced organisms and revealed that it was found to be grouped with sequences of *Enterobacter sacchari* SP-1 and *shigella flexneri* 2a (Fig. 6.7). The nucleic acid sequence was converted to protein sequence for bioinformatics analysis of

ACC deaminase. The generated *AcdS* protein sequence of isolate SBP-8 showed 87.2% identity with the template (*Salmonella typhimurium;* PDB ID: 4D96) sequence. This identity shows a high level of conservation of protein sequences (or amino acid) among the two protein sequences. As the target protein (our protein) was modeled, to look into the accuracy of the modeled protein the two proteins (our modeled protein and 4D96) were superimposed on each other using Chimera software (Fig. 6.8). After superimposition, the root mean square deviation (RMSD) value was 0.241 which showed that the two structures are quite close to each other and have less number of variations. Similar to nucleotide sequence, a phylogenetic tree was constructed for the protein sequences as well (Fig. 6.9). While evaluating the evolutionary distance between the 16S rRNA and *AcdS* gene in specific bacterial species groups, it was found that the ratio between 16S rRNA and *AcdS* sequence divergence is not always identical between strains and groups (Fig. 6.10, 6.11).

Fig. 6.7. A phylogenetic tree of ACC deaminase (*AcdS*) gene of *Enterobacter cloacae* SBP-8 and other closely related bacterial genera. The evolutionary history was inferred by the Neighbour-joining method with bootstrap value 1000.

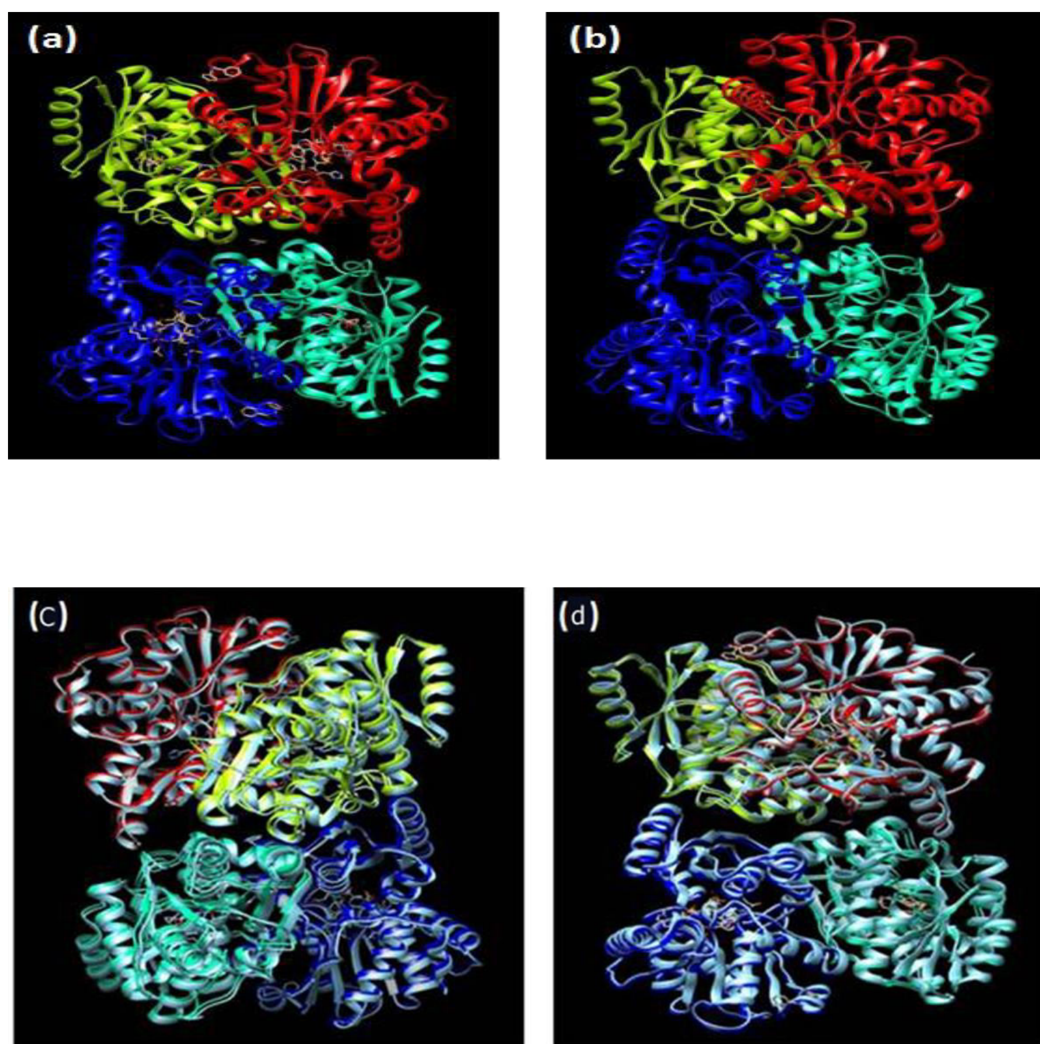


Fig. 6.8. Modeling of *AcdS* protein. (a) Template-4D96 (experimental, from *Salmonella*), (b) Model generated for our protein from *Enterobacter cloacae* SBP-8, (c) Superimposed proteins (our protein and 4D96 from Angel 1), (d) Superimposed proteins (our protein and 4D96 from Angel 2).

Fig. 6.9. A phylogenetic tree of *AcdS* protein sequence of *Enterobacter cloacae* SBP-8 and other closely related bacterial genera. The evolutionary history was inferred by the Neighbour-joining method with bootstrap value 1000.

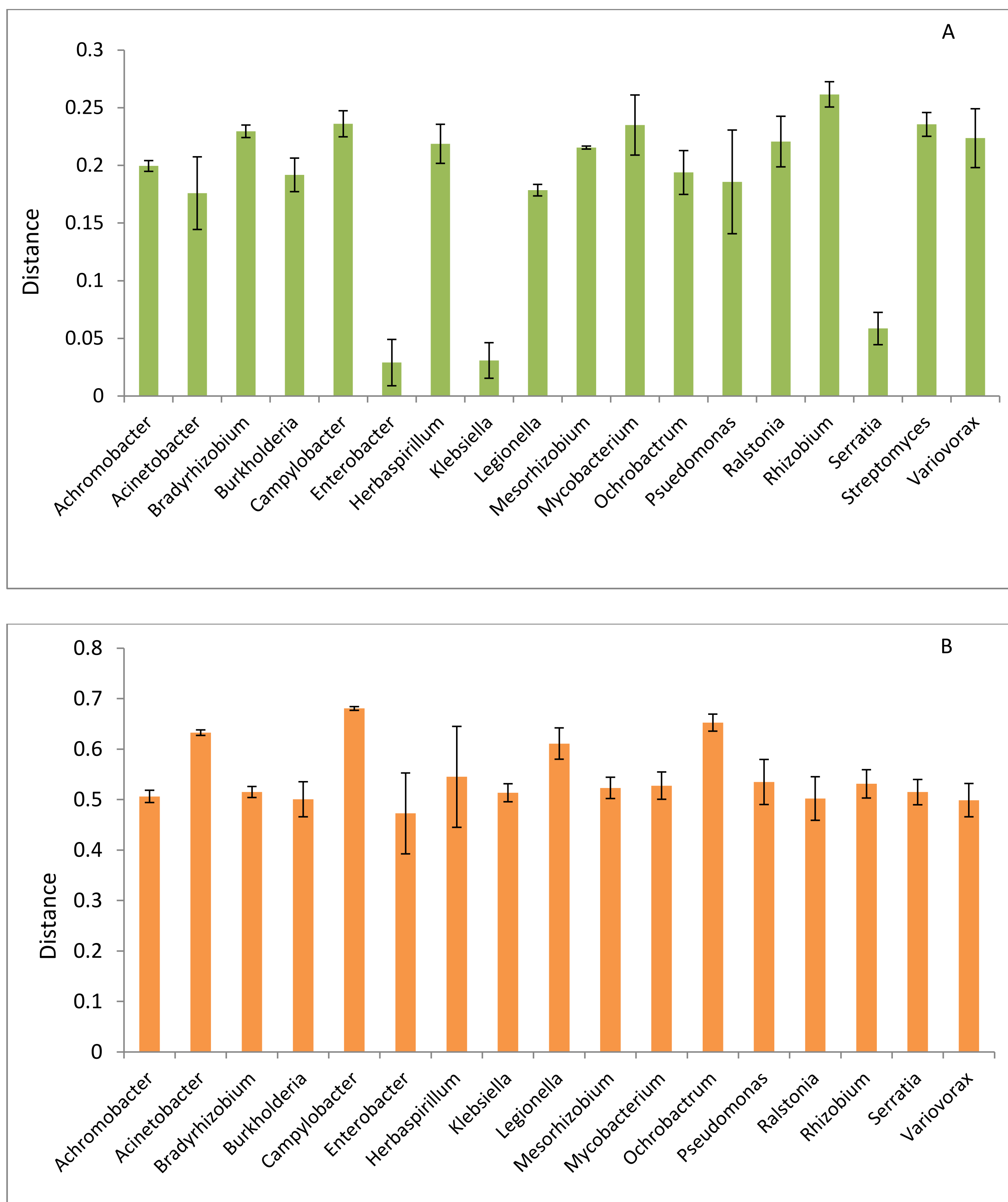


Fig. 6.10. Evaluation of evolutionary distance between the 16S rRNA (A) and *AcdS* gene (B) in the selected bacterial genera. The nucleotide sequence of selected bacteria was aligned in ClustalW, and the distance was calculated in mega 6.0. Error bar represents the standard error of means of triplicates.

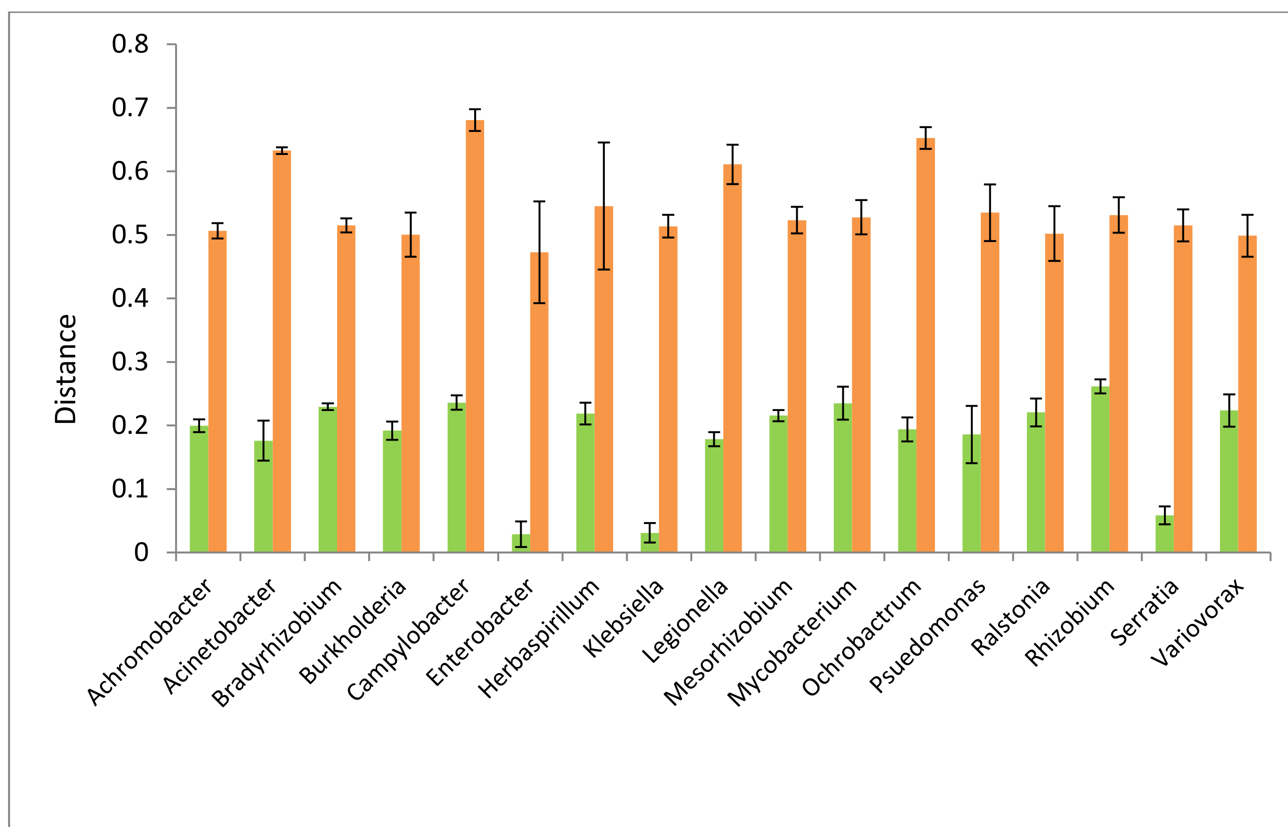


Fig. 6.11. Comparison of evolutionary distance between the 16S rRNA and *AcdS* gene in the selected bacterial genera. The values were relative to *Enterobacter cloacae* SBP8.

6.3.9 Taxonomical relationship

The 16S rRNA gene sequences is frequently used to identify and classify microorganisms and found to be highly conserved. However, the copy number of rRNA genes in prokaryotic genomes are highly variable. A Neighbour-joining (NJ) phylogenetic tree was constructed for the 16S rRNA genes of *Enterobacter* genomes with the test isolates and SBP-8 was found to group with those of other *Enterobacter* sp. (Fig. 6.12). Additional analysis was conducted for other housekeeping genes (*rpoD,* and *rpoB*) of 140 *Enterobacter* strains (Fig. 6.13, 6.14). The phylogenetic tree revealed that SBP-8 fell into *Enterobacter* group.

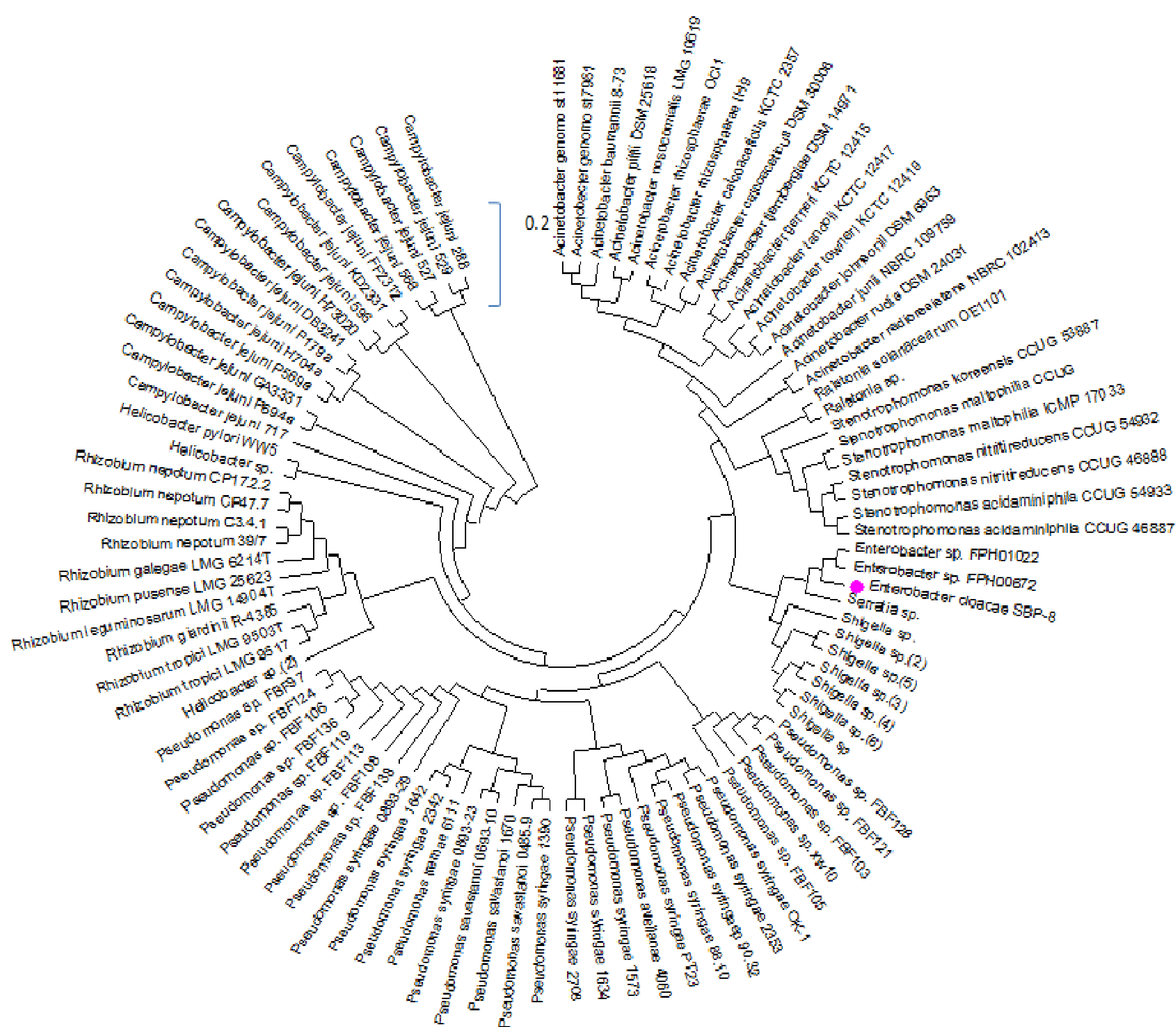


Fig. 6.12. Phylogenetic tree showing the relationship of *Enterobacter cloacae* SBP-8 to closely related bacteria. The 16S rRNA gene sequence of closely related species was obtained from NCBI GenBank database. The tree was obtained using neighbor joining method of software package Mega version 6.0 at the bootstrap value of (n= 1000).

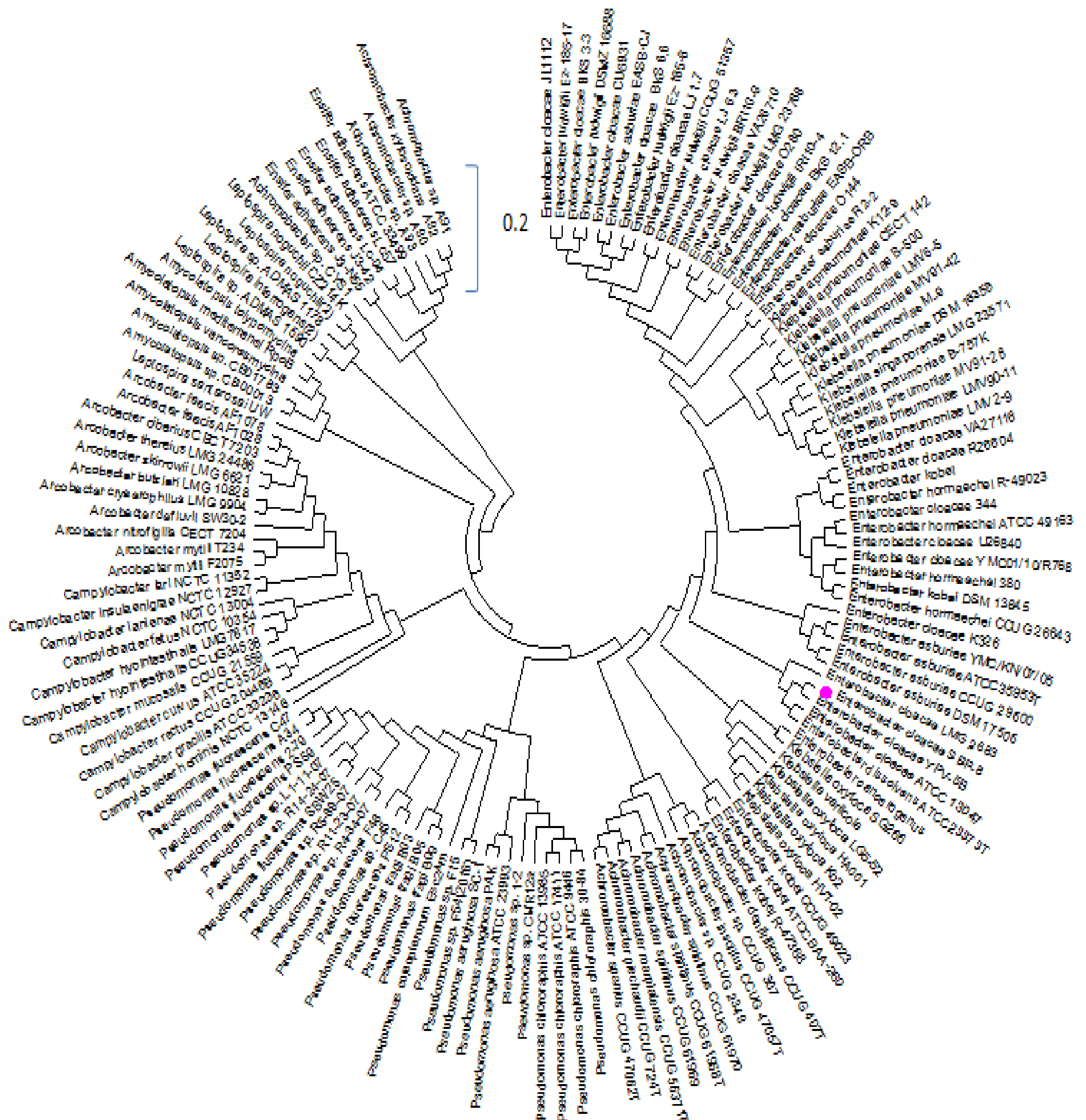


Fig. 6.13. Phylogenetic tree showing the relationship of *Enterobacter cloacae* SBP-8 to closely related bacteria. The house-keeping *rpoB* gene sequence of closely related species was obtained from NCBI GenBank database. The tree was obtained using neighbor joining method of software package Mega version 6.0 at the bootstrap value of (n= 1000).

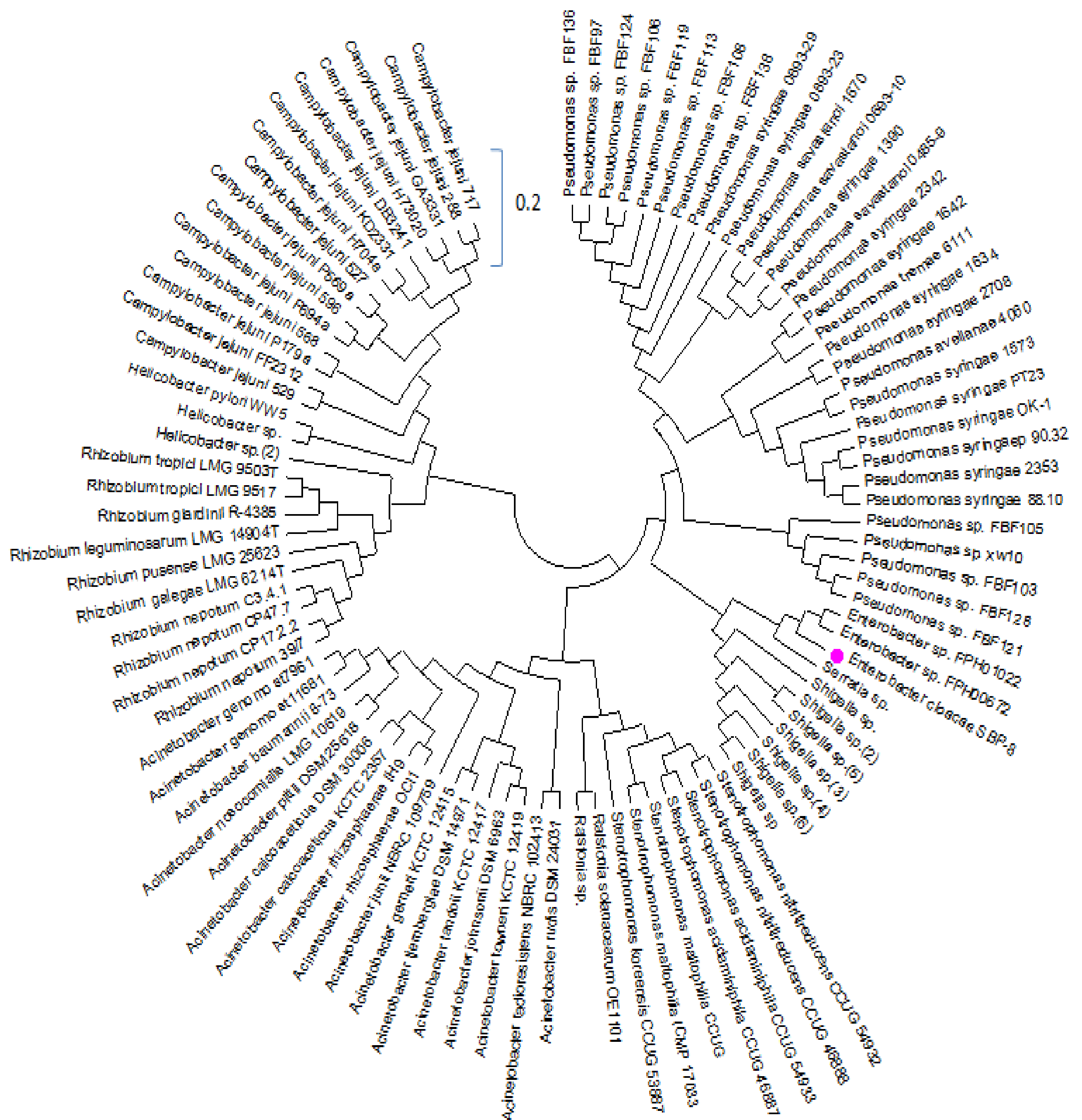


Fig. 6.14. Phylogenetic tree showing the relationship of *Enterobacter cloacae* SBP-8 to closely related bacteria. The house-keeping *rpoD* gene sequence of closely related species was obtained from NCBI GenBank database. The tree was obtained using neighbor joining method of software package Mega version 6.0 at the bootstrap value of (n= 1000).

6.4 Discussion

The analysis of the draft genome sequence of *Enterobacter cloacae* SBP-8 provides the information about a set of genes related to plant growth promotion, biocontrol, motility, biofilm formation, chemotaxis, and secretion system. The genome sequence of SBP-8 contains several large and small coverage missing regions (gaps) which need to be analyzed to ascertain the presence or absence of certain genes. Therefore, a combination of mapping and *de novo* assembly of the whole genome paired-end reads will be a good approach for finding missing or novel genes, resolving complex repetitive regions, and filling the gaps. Previous studies have shown the importance of these genes for a rhizobacterium that exhibits colonization ability of plant roots (Lugtenberg et al., 2001; De Weert et al., 2002). Annotation of the genome sequence revealed that SBP-8 also contain gene clusters responsible for the production of vitamin B complex. Furthermore, gene clusters involved in the production of antimicrobial compounds and stress resistance are also present in the genome. However, their conservation in SBP-8 and other *Enterobacter* genomes assumed to demand additional analysis.

The genome sequence of *Enterobacter cloacae* SBP-8 reveals its potential for production of various plant growth promoting traits along with ACC deaminase. ACC deaminase was initially identified in the yeast *Hansenula saturnus* and the bacterium *Pseudomonas* sp. ACP (Honma and Shimomura, 1978). After that, many groups have reported the presence of *AcdS* gene in a wide range of different organisms, mostly bacteria and fungi (Glick et al., 1998). Environmental variations lead to different gene mutation, loss, acquisition, and genome rearrangements that might play a crucial role in bacterial adaptation and survival (Boussau et al., 2004; Dobrindt et al., 2010). The bacteria adapted to different environmental conditions may have different *AcdS* divergence rates. Therefore there is the possibility of some of the variance in *AcdS* genes in bacteria from the same species. Phylogenetic analysis of *AcdS* gene and proteins showed its closest homology to the *Enterobacter* groups. Studies regarding the mechanisms regulating the *AcdS* gene expression have been reported for some Proteobacteria. Binding sites for CRP (cAMP receptor protein), FNR (fumarate-nitrate reduction regulatory protein) and LRP (leucine-responsive regulatory protein) were present in the promoter region of the *Pseudomonas* sp. UW4 *AcdS* gene and were shown to function in regulating the *AcdS* expression (Grichko and Glick, 2000; Cheng et al., 2008). Beside of *AcdS* gene, the strain was found to possess the gene for siderophore biosynthesis. The ability of bacteria to produce siderophores surely benefits these organisms,

as they may function in different environments, making them more competitive against other organisms in the same niche. Similarly many bacteria are able to synthesize the phytohormone IAA. However the amounts produced vary significantly between different bacterial strains. Depending on the concentration, bacterially produced IAA can either promote or inhibit plant growth. In the genome of *Enterobacter cloacae* SBP-8, two potential IAA biosynthesis pathways, the indole-3-acetamide (IAM) and indole-3-acetonitrile (IAN) pathways, were identified. In the IAM pathway, tryptophan is converted to IAM by tryptophan 2-monooxygenase and then to IAA by amidase. In the IAN pathway, tryptophan is converted to indole-3-acetaldoxime and then to IAN by indole acetaldoxime dehydratase. Next, IAA can be produced directly through IAN by nitrilase. In an alternate way, IAN can be first converted to IAM by nitrile hydratase and then IAM is converted to IAA by amidase (Duan et al., 2013). However, future work will need an experimental confirmation of the putative functions of the above-mentioned genes in IAA biosynthesis. Many studies have shown that numerous bacterial strains possess multiple IAA synthesis pathways. Therefore, to study the role of each gene in IAA biosynthesis of a particular bacterium, it is necessary to construct a large number of mutants, single or multiple, and test the function of each one.

Bacterial biofilms are multicellular communities that are formed in many natural habitats and provide protection against hostile conditions, including immunological defenses (Costerton et al., 1995; Donlan and Costerton, 2002). A distinguishing feature of biofilms is its extracellular polymeric matrix, containing a variety of components that affect its architecture, ion selectivity, and other properties (Sutherland, 2001). The structural integrity of biofilm often depends upon polysaccharides like cellulose, or poly-β-1,6-*N*-acetyl-d-glucosamine (poly-β-1,6-GlcNAc; PGA). PGA subsequently depends on the *pgaABCD* operon for its production. This operon is present in diverse bacterial species and presence of a *pgaABCD*-homologous locus in the genome of gram-negative species has been reported (Itoh et al., 2005; Izano et al., 2007). The genome of *Enterobacter cloacae* SBP-8 has identified the operon and curli gene responsible for biofilm formation that may also help in the colonization process. In addition, the presence of the genes for the secretion system I, IV and VI assist in the adhesion and colonization processes. The presence of genes for osmo-protectant improves the bacteria strain for abiotic stress tolerance. In the genome of *Enterobacter cloacae* SBP-8 several genes for metal resistance and efflux transporter were identified that can extrude the heavy metal and prevent the bacterial cell from abiotic stresses.

When calculating the evolutionary distance between the 16S rRNA and *AcdS* gene in specific bacterial species groups, it was found that the ratio between 16S rRNA and *AcdS* sequence divergence is not always identical between strains. Sequence analysis of 16S rRNA gene for phylogenetic studies shows that SBP-8 is closely related to *Enterobacter* sp. Although 16S rRNA gene is used as a basic tool for the bacterial classification, however very closely related species of bacteria cannot be differentiated based on this gene (Dutta and Gachhui, 2007; Zurdo-Pineiro et al., 2007; Wink et al., 2003). Therefore, many studies have shown that other genes, such as ‘‘housekeeping’’genes *rpoB, rpoD* should be used to assist bacterial species classification. The analyzed evolutionary relationship of SBP-8 supports the hypothesis for the conservation of house-keeping genes and is likely to be the consequence of an ancestral gene transfer event. Phylogenetic analysis shows that the house-keeping genes may have been acquired by an ancestor close to *Enterobacter* sp. and subsequently lost in some lineages. Moreover, based on the whole genome phylogeny, among other sequenced *Enterobacter* genome shows that the strain SBP-8 closely belongs to the non-pathogenic *Enterobacter cloacae* GGT036 and human opportunistic pathogen *Enterobacter cloacae* ATCC 13047.

Thus the genome sequence of *E. cloacae* SBP-8 provides a future opportunity to gain insights into the differences between the diverse group of *E. cloacae* involving endophytic-plant and human pathogenic strains through comparative genome analysis. Moreover, the strain SBP-8 harbors several important genes whose functionality and pathogenic behaviour of strain has yet to be revealed. Further analysis is required to understand which genes made the strain SBP-8 capable enough to confer better plant protection without producing too many effective antifungal/antibacterial compounds. Completion of the SBP-8 genome will reveal its potential of producing secondary metabolites for developing agro-biotechnological agents. The comparative genomics analyses with other related strains would reveal the extensive genetic variations among the diverse plant-associated rhizobacteria with the potential for biotechnology applications. Finally, this study provides a foundation for the future genetic investigation of this bacterium and its molecular interactions with the plant hosts.

Summary & conclusion

Plant growth promoting rhizobacteria has achieved special attention from the agricultural point of view as these are actively involved in the various processes of plant growth and enhancement. These rhizobacteria reside in the rhizospheric region of plant roots and can benefit the host plant by providing nutrition, phytohormone production, biocontrol, and the induced systemic tolerance (IST). IST refers to the ability of plant-associated bacteria to protect from deleterious effects of different abiotic stressors. Among different mechanisms of IST, bacterial ACC deaminase produced by plant-associated bacteria ameliorates different stressors by reducing the level of ‘stress ethylene,' which is inhibitory to plant growth. A systematic approach is required for the screening of these potential bio-inoculants to harness beneficial plant-microbe association to utilize in stress agriculture. Under stress like salinity, these rhizobacteria modulate the ionic balance through differential expression of transporters favoring the level of K^+/Na^+, enhancing the antioxidant activity for scavenging the ROS molecules, elevated the level of various osmolytes and thus minimize the toxic ionic effect on plants. Understanding the mechanism of colonization behavior and analysis of various developmental processes could be utilized to exploring the development of better bio-fertilizer/biocontrol agents in sustainable agriculture. Therefore, the present study was aimed to characterize ACC deaminase producing bacteria at physiological and molecular level, and to study the rhizobacteria-mediated mechanism of stress protection to plants growing under salinity/temperature stress conditions through polyphasic approaches like a screening of colonization behavior, exploring the ability to augment induced systemic tolerance, etc. Throughout the study the major achievements are as follows:

1. A total of twenty-five (25) ACC deaminase producing rhizobacterial isolates were recovered from various plants growing in Thar Desert of Rajasthan. Most of the isolates were found to possess the plant growth promoting features like phosphate solubilization, phytohormone production, siderophore production, nitrogen fixation, and ammonia production. based on qualitative and quantitative screening. These isolates were identified as a member of different bacterial genera namely *Enterobacter*, *Klebsiella*, *Bacillus*, *Serratia, Stenotrophomonas, Mesorhizobium,* and *Pantoea* sp. etc. based on partial sequence analysis of 16S rRNA gene. The recovered isolates were screened for various biochemical tests, quantitative analysis of ACC deaminase, other plant growth promoting properties, and antagonistic behavior against

phytopathogens. The ACC deaminase activity varied in different isolates ranging from 115 to 390 nm α-ketobutyrate/mg protein/h. The highest ACC deaminase activity was observed in *Klebsiella* sp. SBP-8 with 396±21 nm α-ketobutyrate/mg protein/h. Based on the suitable plant growth promoting features, twelve isolates were selected for further analysis of ACC deaminase, detection of *AcdS* gene that encodes ACC deaminase, and effect of these bacteria on growth of the wheat plant.

2. The selected twelve isolates were tested for their ability to cross-infect the wheat plants. Before plant growth test, different bacterial isolates were tested for their tolerance to a different level of salt, and temperature stress and it was observed that most of the isolates were able to tolerate salt stress (2 to 8% NaCl) and temperature stress up to 40 °C of temperature. Results of ACC deaminase activity of various isolates under different physiological conditions indicated that optimum activity was observed at pH (8.0), temperature (30 °C), ACC concentration (3 mM), and in the presence of ammonium chloride used as a nitrogen source. The presence of ACC deaminase activity was further confirmed at the molecular level by the amplification, sequencing and phylogenetic analysis of *AcdS* gene in isolates *Enterobacter cloacae* ZNP-2, *Klebsiella* sp. SBP-8 and *Bacillus licheniformis* HSW-16. Plant growth promoting test was performed under salt and temperature stress conditions to study the protective nature of these rhizobacteria through evaluating the various physiological growth parameters, biomass, and photosynthetic pigments. Among the tested isolates, *Klebsiella* sp. SBP-8 was found to be most effective for increasing the growth parameters of the wheat plant as well as for protecting the wheat plants from salt and temperature stress.
3. Based on the experimental observations *Klebsiella* sp. SBP-8 (later described as *Enterobacter cloacae* based identification by full-length 16S rRNA and other housekeeping genes) was selected to study changes in its membrane lipid composition and to evaluate the mechanism of growth promotion through polyphasic approaches. Fatty acid analysis (FAME) of extracts of bacteria exposed to different concentration of NaCl (salt stress) was analyzed by GC-MS, which revealed the presence of higher amount of unsaturated fatty acids and fatty alcohol under salt stress which can increase membrane fluidity to modulate the distribution of transport to protect from osmotic and ionic stress. Further, isolate SBP-8 was inoculated to wheat seeds and grown under different concentration of NaCl. Ionic analysis of treated plants

demonstrated that inoculation with SBP-8 decreases the accumulation of Na^+ and increases the concentration of K^+ and Ca^{2+} to maintain favourable Na^+/K^+ ratio in wheat plants under salt stress. Bacteria-treated plant showed better accumulation of osmoprotectants (proline, total soluble sugar), and antioxidant activity (CAT, SOD, POX) to counteract the toxic ionic effect. Since, effective colonization is required for optimum mutual interaction; we tested colonization ability of the isolate. Results of fluorescence microscopy of roots of treated plants following acridine orange staining, CFU counting of bacteria recovered from treated plants, and identical ERIC-profile of isolated bacteria to that of a pure culture of SBP-8 confirmed colonization of the isolate in plant root. Moreover, to ensure the role of ACC deaminase in amelioration of salt stress, a null mutant of *AcdS* gene was generated by transposon mutagenesis in the test isolate and was used to inoculate wheat plant grown with 200 mM NaCl. Decrease in growth parameters and plant biomass in *AcdS*$^-$ inoculated plants suggested the involvement of ACC deaminase activity in plant protection from salt stress.

4. To understand the bacterial-mediated stress protection, differential expression of proteins in wheat plant following inoculation with *E. cloacae* SBP-8 was analyzed by Q-TOF analysis. Protein analysis showed the expression of 280 to 300 proteins in different treatments. The highest up-regulated proteins were belonging to the category of metabolic (13), followed by protein synthesis (5), and photosynthesis (4). Bacterial treatment resulted in the increased level of various proteins belonging to the category of stress tolerance, photosynthesis, defense mechanism, ion transportation, protein synthesis, metabolic pathways proteins, and chromosome remodeling proteins. The over-expressed proteins following rhizobacterial inoculation govern the osmotic homeostasis, activation of defense response, strengthening of cell-wall, transportation of ions, and photosynthesis functioning to confer stress resistance. The higher accumulation of metabolic pathway proteins illustrates that different metabolic pathways could be involved in the protection of wheat plant to salt stress. Therefore, by using the proteomic approaches the observed evidence supports that application of a beneficial PGPR to wheat seedlings could be used as an effective tool to overcome the salinity stress. This investigation confirmed that tested ACC deaminase-producing PGPR *E. cloacae* SBP-8 can be exploited as a promising biofertilizer with multifarious properties.
5. Genomic analysis of selected bacterium *Enterobacter cloacae* SBP-8 identified the genes responsible for plant growth promotion and protection from abiotic stress. The genome of

Enterobacter cloacae SBP-8 is 5.8 Mb (both genome and plasmid) with 4052 protein coding genes. Genes responsible for carbohydrate metabolism (735 ORFs), amino acids and derivatives (528 ORFs), cofactors and vitamin synthesis (276) and cell wall and capsule formation (232 ORFs) were abundant among the subsystem categories. The *Enterobacter cloacae* SBP-8 genome also contain genes contribute to salinity resistance including those for proline and glycine-betaine synthesis, trehalose synthesis, and a number of the osmoprotectants. Genome analysis showed the presence of genes potentially involved in plant growth promotion (ACC deaminase), phytohormone production (IAA), siderophore synthesis and various other useful genes. The analysis of upstream regulatory element of *AcdS* gene showed the presence of two rpoD, two argR, ihf, and CRP elements. However their role in *AcdS* gene regulation needs to be studied in future. The genome of SBP-8 contains the several genes for colonization and establishment in host plant which included genes encoding proteins for chemotaxis, adherence, biofilm formation, and invasion. It showed the presence of secretion systems (I, II, IV, VI) which can have an important role in the establishment of bacteria-plant interaction. Therefore, genomic analysis of test isolate *Enterobacter cloacae* SBP-8 unravel the various useful genes whose regulation and functionality can be exploited in future studies.

The result of the present study indicates that bacteria equipped with ACC deaminase activity and several other plant growth promoting traits was successful for protecting the wheat plant by several approaches under abiotic stress like salinity. The plant protecting effect of bacterial strain was characterized by a physiological, biochemical and molecular method of proteomic analysis. In addition, the genome analysis identified the presence of genes contributing directly or indirectly to enable PGPR effects on plants, salt tolerance, rhizocompetence, colonization and elucidation of metabolic pathways aiming. The sum of these results will be useful to better understand the mode of interaction of the bacterium and plants. Furthermore, it will be helpful for a comparative genome study with other PGPR strains sequenced genomes to analyze singularity and/or co-occurrence of genes involved in plant growth promotion.

Future scope of work

The use of plant growth promoting bacteria with ACC deaminase activity substantially decreases the elevated level of 'stress ethylene' following biotic and abiotic stress conditions, and therefore, serves as promising biofertilizers for stress agriculture. The ability of ACC deaminase bacteria in the present study has been tested under gradient salinity and temperature stress conditions on wheat plant and therefore few selected strains serves as future biofertilizers. The use of these bacterial bio-inoculants can be used as biotechnological alternative to chemical fertilizers and pesticides as agricultural supplements to mitigate the biotic and abiotic stress responses. The present work can be extended in future studies in following aspects:

(i) Since, *Enterobacter cloacae* is an opportunistic pathogen, the selected isolate SBP-8 must be checked for its virulence in a model organism before it can be used in field study.

(ii) Regulatory component of *AcdS* can be functionally characterized to understand regulation of ACC deaminase under various physiological conditions so that it can be manipulated for optimal exploitation.

(iii) Molecular mechanism of colonization can be studied by producing knock-outs of several important genes including that of secretion system VI which is least characterized in *E. cloacae.*

References

Abeles FB, Morgan PW, Saltveit ME (1992) Ethylene in plant biology. San Diego:Academic press. 41: 1710‑1716.

Aghaleh M, and Niknam V (2009) Effect of salinity on some physiological and biochemical parameters in explants of two cultivars of soybean (*Glyicine max* L.). Journal of Phytology 1: 86-94.

Ahmad S., Anwar M., UlJah H (1998) Wheat seed presoaking for improved germination. Journal of Agronomy and Crop Science 181: 125–127.

Ahmad M, Zahir ZA, Asghar HN, Ashgar M (2011) Inducing salt tolerance in mung bean through co-inoculation with rhizobia and plant growth-promoting rhizobacteria containing 1-aminocyclopropane- 1-carboxylate deaminase. Canadian Journal of Microbiology 57: 578-589.

Ait bakra E, Nowak J, Clement C (2006) Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth promoting rhizobacterium *Burkholderia phytofirmans* strain PsJN. Applied Environmental Microbiology 72: 7246–7252.

Al-Abdalall AHA (2010) Pathogenicity of fungi associated with leguminous seeds in the Eastern kingdom of Saudi Arabia. African Journal of Agricultural Research 5: 1117-1126.

Alizadeh O, Namazi L (2011) Effect of different irrigation levels on nutrient uptake by mycorrhizal and non-mycorrhizal corn plants as affected by different soil phosphorus content. Advances in Environmental Biology 5: 2317‑2321.

Ali S, Charles TC, Glick BR (2014) Amelioration of high salinity stress damage by plant growth-promoting bacterial endophytes that contain ACC deaminase. Plant Physiology and Biochemistry 80: 160-167.

Almagro L, Gómez Ros LV, Belchi-Navarro S, Bru R, Ros Barceló A, Pedreño MA (2009) *gacA* derivative CHA96 on their growth-promoting and disease-suppressive capacities. Canadian Journal of Microbiology 46: 898-907.

Ames BN (1964) Assay of inorganic phosphate, total phosphate and phosphatases. Methods in Enzymology 8: 115-118.

Amineni U, Pradhan D, Marisetty H (2010) In silico identification of common putative drug targets in *Leptospira interrogans*. Journal of Chemical Biology 3:165–173

Andreae WA, Ysselstein VMWH (1959) Studies on 3-Indoleacetic Acid Metabolism. V. Effect of calcium ions on 3-indoleacetic acid uptake and metabolism by Pea roots. Plant Physiology 35: 220-224.

Arshad M, Frankenberger WT (1990) Ethylene accumulation in soil in response to organic amendments. Soil Science Society of America Journal 54: 1026-1031.

Arshad M, Shaharoona B, Mahmood T (2008) Inoculation with *Pseudomonas* spp. containing ACC-deaminase partially eliminates the effects of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.). Pedosphere 18: 611–620.

Ashraf M, Hasnain S, Berge O, Mahmood T (2004) Inoculating wheat seedlings with exopolysaccharide-producing bacteria restricts sodium uptake and stimulates plant growth under salt stress. Biology and Fertility of Soil 40: 157‑162.

Babalola OO, Osir EO, Sanni AI, Odhiambo GD, Bulimo WD (2003) Amplification of 1-amino-cyclopropane-1-carboxylic (ACC) deaminase from plant growth promoting bacteria in Striga-infested soil. Africal Journal of Biotechnology 2: 157–160.

Barh D, Tiwari S, Jain N, Ali A, Santos AR, Misra AN, Azevedo V, Kumar A (2011) In silico subtractive genomics for target identification in human bacterial pathogens. Drug Development Research 72: 162–177.

Barlow KM, Christy BP, O'Leary GJ, Riffkin PA, Nuttall JG (2015) Simulating the impact of extreme heat and frost events on wheat crop production: are- view. Field Crops Research 171: 109–119.

Barnawal D, Bharti N, Maji D, Chanotiya CS, Kalra A (2014) ACC deaminase-containing *Arthrobacter protophormiae* induces NaCl stress tolerance through reduced ACC oxidase activity and ethylene production resulting in improved nodulation and mycorrhization in *Pisum sativum*. Journal of Plant Physiology 171: 884–894.

Baset Mia MA, Ashraf Shah R, Shamsuddin ZH (2010) Nitrogen fixation and transportation by rhizobacteria: A scenario of rice and banana. International Journal of Botany 6: 235 – 242.

Bates L, Waldren RP, Teare ID (1973) Rapid determination of free proline for water-stress studies. Plant & Soil 39: 205-207

Belimov AA, Safronova VI, Sergeyeva TA, Egorova TN, Matveyeva VA,Tsyganov VE, Borosov AY, Tikhonovich IA, Kluge C, Preisfeld A, Dietz KJ, Stepanok VV (2001) Characterization of plant growth promoting rhizobacteriaisolated from polluted soils and containing 1- aminocyclopropane-1-carboxylate deaminase. Canadian Journal of Microbiology 47: 242-252.

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S et al. (2005) Cadmium tolerant plant growth-promoting bacteria associated with roots of Indian mustard (*Brassica juncea* L Czern.). Soil Biology and Biochemistry 37: 241–250.

Belimov AA, Safronova VI, Mimura T (2002) Response of spring rape to inoculation with plant growth-promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase depends on nutrient status of the plant. Canadian Journal of Microbiology 48: 189–199.

Bertini L, Leonardi L, Caporale C et al. (2003) Pathogen-responsive wheat PR4 genes are induced by activators of systemic acquired resistance and wounding. Plant Science 164: 1067–1078.

Blaha D, Prigent-Combaret C, Mirza MS, Moenne-Loccoz Y (2006) Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene acdS in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. FEMS Microbiology & Ecology 56: 455–470.

Boller T (1991) Ethylene in pathogenesis and disease resistance. *In*AK Mattoo, JC Suttle, eds, The Plant Hormone Ethylene. CRCPress, Boca Raton, FL, pp 293-314.

Boussau B, Karlberg EO, Frank AC, Legault B-A, Andersson SGE (2004) Computational inference of scenarios for alpha-proteobacterial genome evolution. Proceeding of Natural Academy of Science USA 101: 9722–9727.

Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254.

Bray EA, Bailey-Serres J, Weretilnyk E. Responses to abiotic stresses (2000) In W Gruissem, B Buchannan, R Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 1158–249.

Bruce RJ, and West CA (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of castor bean. Plant Physiology 91: 889-897.

Bui EN (2013) Soil salinity: A neglected factor in plant ecology and biogeography. Journal of Arid Environment 92: 14-25.

Burd GI, Dixon DG, Glick BR (1998) A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. Applied and Environmental Microbiology 64: 3663–3668.

Burd GI, Dixon DG, Glick BR (2000) Plant growth promoting bacteria that decrease heavy metal toxicity in plants. Canadian Journal of Microbiology 46: 237-245.

Burrows LL (2012) *Pseudomonas aeruginosa* twitching motility: type IV pili in action. Annual Review of Microbiology 66: 493-520.

Campbell SA, and Close TJ (1997) Dehydrins: genes, proteins, and associations with phenotypic traits. New Phytologist 137: 61–74.

Caporale C, Di Berardino I, Leonardi L, Bertini L, Cascone A, Buonocore V, Caruso C (2004). Wheat pathogenesis-related proteins of class 4 have ribonuclease activity. FEBS Letters 575: 71-76.

Cappuccino JG, Sherman N (1992) Biochemical activities of microorganisms. In: Microbiology, A Laboratory Manual. The Benjamin / Cummings Publishing Co. California, USA. Chalutz, pp. 171-180.

Capriotti AL, Borrelli GM, Colapicchioni V, Roberto Papa R, Piovesana S, Samperi R, Stampachiacchiere S, Laganà A (2014) Proteomic study of a tolerant genotype of durum wheat under salt-stress conditions. Analytical and Bioanalytical Chemistry 406: 1423–1435.

Castro RO, Cornejo H.AC, Rodriguez LM, Bucio JL (2009) The role of microbial signals in plant growth and development. Plant Signalling Behaviour 4: 701-712.

Chen N, Goodwin PH, Hsiang T (2003) The role of ethylene during the infection of *Nicotiana tabacum* by *Colltotrichum destructivum.* Journal of Experimental Botany 54: 2449-2456.

Chen TH, Murata N (2008) Glycine betaine: an effective protectant against abiotic stress in plants. Trends in Plant Science 13: 499–505.

Cheng Z, Park E, Glick BR (2007) 1-Aminocyclopropane-1-carboxylate deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. Canadian Journal of Microbiology 53: 912-918.

Cheng Z, Duncker BP, Mc Conkey BJ, Glick BR (2008) Transcriptional regulation of ACC deaminase gene expression in *Pseudomonas putida* UW4. Canadian Journal of Microbiology 54: 128-136.

Christensen JH, Overney S, Rohde A, Diaz WA, Bauw G, Simon P, van Montagu M, Boerjan W (2001) The syringaldazine-oxidizing peroxidase PXP 3-4 from poplar xylem: cDNA isolation, characterization and expression. Plant Molecular Biology 47: 581-593.

Christie WW (2003) Lipid Analysis. 3rd edition The Oily Press, Bridgwater, UK.

Cleyet-Marcel JC, Larcher M, Bertrand H, Rapior S, Pinochet X (2001) Plant growth enhancement by rhizobacteria. In: Morot- Gaudry, J.-F. (Ed.), Nitrogen Assimilation by Plants:

Physiological, Biochemical, and Molecular Aspects. Science Publishers Inc., Plymouth, UK, pp. 185–197.

Colmer TD, Epstein E, Dvorak J (1995) Differential solute regulation in leaf blades of various ages in salt sensitive wheat and a salt-tolerant wheat × *Lophopyrum elongatum* (Host.) A. Love amphiploid. Plant Physiology 108**:** 1715‑1724.

Compant S, Duffy B, Nowak J, Clément C, Barka EA (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: Principles, mechanisms of action, and future prospects. Applied and Environmental Microbiology 71: 4951-4959.

Connelly MB, Young GM, Sloma A (2004) Extracellular proteolytic activity plays a central role in swarming motility in *Bacillus subtilis*. Journal of Bacteriology 186: 4159–4167.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995) Microbial biofilms. Annual Review of Microbiology 497: 11-745.

Darling AE, Mau B, Perna NT (2010) ProgressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5: e11147.

Das M, Chauhan, H, Chhibbar A, Haq QMR, Khurana P (2011) High-efficiency transformation and selective tolerance against biotic and abiotic stress in mulberry, *Morus indica* cv. K2, by constitutive and inducible expression of tobacco osmotin. Transgenic Research 20: 231–246.

DeFalco TA, Bender KW, Snedden WA (2010) Breaking the code: Ca^{2+} sensors in plant signalling. Biochemistry Journal 425: 27–40.

De Leij FAAM, Suttom EM, Whipps JM, Linch JM (1995) Impact of field release of genetically modified *Pseudomonas fluorescens* on indigenous microbial populations of wheat. Applied and Environment Microbiology 61: 3442-3453.

De Weert S, Vermeiren H, Mulders IHM, Kuiper I, Hendrickx N, Bloemberg GV et al. (2002) Flagella-driven chemotaxis towards exudate components is an important trait for tomato root colonization by *Pseudomonas fluorescens*. Molecular Plant Microbe Interaction 15: 1173–1180.

Delcher AL, Bratke KA, Powers EC, Salzberg SL (2007) Identifying bacterial genes and endosymbiont DNA with Glimmer. Bioinformatics 23: 673–679.

Dell'Amico E, Cavalca L, Andreoni V (2005) Analysis of rhizobacterial communities in perennial *Graminaceae* from polluted water meadow soil, and screening of metal-resistant, potentially plant growth-promoting bacteria. FEMS Microbiology and Ecology 52: 153–162.

Denich TJ, Beaudettle LA, Lee H, Trevors JT (2003) Effects of selected environmental and physico-chemical factors on bacterial cytoplasmic membranes. Journal of Microbiolgical Methodology 52: 149.

Di Gregorio S, Barbafieri M, Lampis S, Sanangelantoni AM, Tassi E, Vallini G (2006) Combined application of Triton X-100 and *Sinorhizobium* sp. Pb002 inoculum for the improvement of lead phytoextraction by *Brassica juncea* in EDTA amended soil. Chemosphere 63: 293–299.

Dimkpa C, Weinan T, Asch F (2009) Plant‐rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell and Environment 32: 1682‐1694.

Divi UK, Krishna P (2009) Brassinosteroid: A biotechnological target for enhancing crop yield and stress tolerance. New Biotechnology 26: 131–136.

Dobbelaere S, Croonenborghs A, Thys A, Ptacek D, Vanderleyden J, Dutto P et al. (2001) Responses of agrinomically important crops to inoculation with *Azospirillum.* Australian Journal of Plant Physiology 28: 871–879.

Dobrindt U, Chowdary MG, Krumbholz G, Hacker J (2010) Genome dynamics and its impact on evolution of Escherichia coli. Medical Microbiology and Immunology 199: 145–154.

Dodd IC, Zinovkina NY, Safronova VI, Belimov AA (2010) Rhizobacterial mediation of plant hormone status. Annals of Applied Biology 157: 361–379

Donlan RM, and Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Review 15: 167-193.

Duan J, Muller KM, Charles TC, Vesely S, Glick BR (2009) 1-Aminocyclopropane-1-carboxylate (ACC) deaminase genes in rhizobia from Southern Saskatchewan. Microbial Ecology 57: 423-436.

Dunbar J, Barns SM, Ticknor LO, Kuske CR (2002) Empirical and theoretical bacterial diversity in four Arizona soils. Applied Environment and Microbiology 68: 3035–3045.

Dutta A, Singh SK, Ghosh P, Mukherjee R, Mitter S, Bandyopadhyay D (2006) In silico identification of potential therapeutic targets in the human pathogen *Helicobacter pylori.* In Silico Biology 6:43–47.

Dutta D and Gachhui R (2007) Nitrogen-fixing and cellulose-producing *Gluconacetobacter kombuchae* sp. nov., isolated from *Kombucha* tea. International Journal of Systematic and Evolutionary Microbiology 57: 353–357.

Dworkin M, and Foster J (1958) Experiments with some microorganisms which utilize ethane and hydrogen. Journal of Bacteriology 75: 592–603.

eGara L, de Pinto MC, Arrigoni O (1997) Ascorbate synthesis and ascorbate peroxidase activity during the early stage of wheat germination. Physiologia Plantarum 100: 894–900.

Ehrlich HL (1990) Geo-microbiology, 2nd edn. Dekker, New York, p 646.

Falkenmark M (2013) Growing water scarcity in agriculture: future challenge to global water security. Philosophical Transactions of the Royal Society 371: 20120410.

FAO (2008) Land and plant nutrition management service. www.fao.org/ag/agl/agll/spush.

FAO (2012) Food and Agricultural Organization of the United Nation. FAO statistical database. Availableonlineat:http://faostat.fao.org/faostat/collections?Subset=agriculture.

Farajzadeh D, Aliasgharzad N, Sokhandan Bashir N, Yakhchali B (2010) Cloning and characterization of a plasmid encoded ACC deaminase from an indigenous *Pseudomonas fluorescens* FY32. Current Microbiology 61: 37–43.

Farwell AJ, Vesely S, Nero V, Rodriguez H, McCormack K, Shah S et al. (2006) The use of transgenic canola (*Brassica napus*) and plant growth-promoting bacteria to enhance plant biomass at a nickel- contaminated field site. Plant and Soil 288: 309–318.

Farwell AJ, Vesely S, Nero V, Rodriguez H, McCormack K, Shah S, Dixon DG, Glick BR (2007) Tolerance of transgenic canola plants (*Brassica napus*) amended with plant growth-promoting bacteria to flooding stress at a metal contaminated field site. Environmental Pollution 147: 540–545.

Feng Y, Shen D, Song W (2004) Rice endophyte *Pantoea agglomerans* YS19 promotes host plant growth and affects allocations of host photosynthates. Journal of Applied Microbiology 100: 938-945.

Fridlender M, Inbar J, Chet I (1993) Biological control of soilborne plant pathogens by a ß-1, 3 glucanase-producing *Pseudomonas cepacia.* Soil Biology and Biochemistry 25: 1211-1221.

Fry SC, Smith RC, Renwick KF, Martin DJ, Hodge SK, Matthews KJ (1992) Xyloglucan endotransglycosylase, a new wall-loosening enzyme activity from plants. Biochemistry Journal 282: 821–8.

Fujino A, Ose T, Yao M, Tokiwano T, Honma M, Watanabe N, Tanaka I (2004). Structural and enzymatic properties of 1-aminocyclopropane-1-carboxylate deaminase homologue from *Pyrococcus horikoshii*. Journal of Molecular Biology 341: 999–1013.

Gagné-Bourque F, Bertrand A, Claessens A, Aliferis KA, Suha Jabaji S (2016) Alleviation of drought stress and metabolic changes in Timothy (*Phleum pratense* L.) colonized with *Bacillus subtilis* B26. Frontier in Plant Science http://dx.doi.org/10.3389/fpls.2016.00584.

Geddie JL, Sutherland IW (1993) Uptake of metals by bacterial polysaccharides. Journal of Applied Bacteriology 74: 467–472.

Gill SS, and Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry 48: 909–930.

Glick BR, Karaturovíc D, Newell P (1995) A novel procedure for rapid isolation of plant growth-promoting rhizobacteria. Canadian Journal of Microbiology 41: 533–536.

Glick BR, Patten CL, Holguin G, Penrose GM (1999) Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London.

Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiology Letters 251: 1–7.

Glick BR (2007) Promotion of plant growth by bacterial ACC deaminase. Critical Review in Plant Science 26: 227–42.

Glick BR (2012) Plant growth-promoting bacteria: Mechanisms and Applications. Hindawi Publishing Corporation, Scientifica.

Glick BR, and Bashan Y (1997) Genetic manipulation of plant growth promoting bacteria to enhance biocontrol of phytopathogens. Biotechnology Advances 15: 353-378.

Glick BR, Penrose D, Mand Li J (1998) A model for lowering plant ethylene concentrations by plant growth promoting rhizobacteria. Journal of Theoretical Biology190: 63-68.

Glick BR, Li J, Ovakim DH, Charles TC (2000) An ACC deaminase minus mutant of *Enterobacter cloacae* UW4 no longer promotes root elongation. Current Microbiology 4: 101–105.

Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Scientifica 1–15.

Glick BR, Nascimento FX, Vicente CSL, Barbosa P, Espada M et al. (2013) Evidence for the involvement of ACC deaminase from *Pseudomonas putida* UW4 in the biocontrol of pine wilt disease caused by *Bursaphelenchusx ylophilus*. Biocontrol 58: 427–433.

Goel D, Singh AK, Yadav V, Babbar SB, Bansal KC (2010) Overexpression of osmotin gene confers tolerance to salt and drought stresses in transgenic tomato (*Solanum lycopersicum* L.). Protoplasma 245: 133–141.

Gordon SA, Weber RP (1951) Colorimetric estimation of indole acetic acid. Plant Physiology 26: 192–195.

Goryshin IY, Jendrisak, J, Hoffman, LM, Meis R, and Reznikoff, WS (2000) Insertional transposon mutagenesis by electroporation of released Tn5 transposition complexes. Nature Biotechnology 18: 97–100.

GrichkoVP, and Glick BR (2001) Amelioration of flooding stress by ACC deaminase containing plant growth-promoting bacteria. Plant Physiology and Biochemistry 39: 11–17.

Griffiths BS, Ritz K, Ebblewhite N, Dobson G (1999) Soil microbial community structure: effects of substrate loading rates. Soil Biology and Biochemistry 31: 145–153.

Grogan DW, and Cronan JE (1997) Cyclopropane ring formation in membrane lipids of bacteria. Microbiology and Molecular Biology Reviews 61: 429–441.

Greenway H, and Munns R (1980) Mechanisms of salt tolerance in non halophytes. Annual Reviewof Plant Physiology 31: 149-190.

Greiner S, Wang X, Rauwolf U, Silber MV, Mayer K, Meurer J, Haberer G, Herrmann RG (2008) The complete nucleotide sequences of the five genetically distinct plastid genomes of *Oenothera*, subsection *Oenothera*: I. Sequence evaluation and plastome evolution." Nucleic Acids Research 36: 2366-2378.

Grichko VP, and Glick BR (2001) Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. Plant Physiology and Biochemistry 39: 11–17.

Grobbelaar N, Clarke B, Hough MC (1971) The nodulation and nitrogen fixation of isolated roots of *Phaseolus vulgaris* L. Plant and Soil 215–223.

Guinel FC, and Geil RD (2002) A model for the development of the rhizobial and arbuscular mycorrhizal symbioses in legumes and its use to understand the roles of ethylene in the establishment of these two symbioses. Canadian Journal of Botany 80: 695–720.

Guo G, Ge P, Ma C, Li X, Lv D, Wang S et al. (2012a) Comparative proteomic analysis of salt response proteins in seedling roots of two wheat varieties. Journal of Proteomics 75: 1867–1885.

Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Canadian Journal of Microbiology 43: 895-914.

Hammerschmidt R, Nuckles EM, Kuć J (1982) Association of enhanced peroxidase activity with induced systemic resistance of cucumber to *Colletotrichum lagenarium*. Physiological Plant Pathology 20: 73-82.

Hampson CR, and Simpson GM (1990) Effect of temperature, salt and osmotic potential on early growth of wheat (*Trticum aestivum* L.). I. Germination. Canadian Journal of Botany 68: 524-528.

Han HS, and Lee KD (2005) Plant growth promoting rhizobacteria effect on antioxidant status, photosynthesis, mineral uptake and growth of lettuce under soil salinity. Research Journal of Agricultural and Biological Science 1: 210-215.

Hanson AD, and Nelson CE (1978) Betaine accumulation and (^{14}C) formate metabolism in water stressed barley leaves. Plant Physiology 62: 305–312.

Hao Y, Charles TC, Glick BR (2007) ACC deaminase from plant growth promoting bacteria affects crown gall development. Canadian Journal of Microbiology 53: 1291–1299.

Hardoim PR, Van Overbeek LS, Elsas JD (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends in Microbiology 16: 463-471.

Harley JP, and Prescott LM (2002) Laboratory Exercises in Microbiology, 5th ed. McGraw-Hill Publishers, USA.

Hase S, Van Pelt JA, Van Loon LC, Pieterse CMJ (2003) Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection. Physiological and Molecular Plant Pathology 62: 219–226.

Hasnain S, and Sabri AN (1996) Growth stimulation of *Triticum aestivum* seedlings under Cr-stresses by non rhizospheric *Pseudomonad* strains. Abstracts of the 7th International Symposium on Biological Nitrogen Fixation with Non-Legumes. Kluwer Academic Publishers, the Netherlands, p.36.

Hincha DK, Zuther E, Hellwege EM, Heyer AG (2002) Specific effects of fructo-and gluco-oligosaccharides in the preservation of liposomes during drying. Glycobiology 12: 103–110.

Hiraga S, Sasaki K, Ito H, Ohashi Y, Matsui H (2001) A large family of class III plant peroxidases. Plant Cell Physiology 42: 462-468.

Hodges DM, Delong JM, Forney CF, Prange RK (1999) Improving the thiobarbituric acid reactive substances assay for estimating lipid peroxidation in plant tissue containing anthocyanin and other interfering compounds. Planta 207: 604-611.

Holbrook A, Edge W, Bailey F (1961) Spectrophotometric method for determination of gibberellic acid. Advance Chemical Science 28: 159–167.

Honma M, and Shimomura T (1978) Metabolism of 1-aminocyclopropane-1-carboxylic acid. Agricultural Biology and Chemistry 42: 1825–1831.

Honma M (1985) Chemically reactive sulfhydryl groups of 1-aminocyclopropane-1-carboxylate deaminase. Agricultural and Biological Chemistry 49: 567-571.

Honma M, Kawai J, Yamada M (1993) Identification of the sulfhydryl group of 1-aminocyclopropane-1-carboxylate deaminase. Bioscience Biotechnology and Biochemistry 57: 2090-3000.

Hontzeas N, Zoidakis J, Glick BR, Abu-Omar MM (2004) Expression and characterization of the 1-aminocyclopropane-1-carboxylic acid deaminase from the rhizobacterium *P.putida* UW4: a key enzyme in bacterial plant growth promotion. Biochimica et Biophysica Acta 1703: 11-19.

Hontzeas N, Richardson AO, Belimov AA, Safranova VI, Abu-Omar MM, Glick BR (2005) Evidence for horizontal gene transfer (HGT) of ACC deaminase genes. Applied and Environmental Microbiology 71: 7556–7568.

Horie T et al. (2008) Functions of HKT transporters in sodium transport in roots and in protecting leaves from salinity stress. Plant Biotechnology 25: 233‑239.

Huang X-D, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM (2004) Multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. Environment Pollution 130: 465–476.

Irigoyen JJ, Einerich DW, Sanchez-Diaz M (1992) Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia Plantarum 84: 55-60.

Itoh T, Aiba H, Baba T, Hayashi K, Inada T, Isono K, Kasai H, Kimura S, Kitakawa M, Kitagawa M, Kitagawa M, Makino K, Miki T, Mizobuchi K, Mori H, Mori T, Motomura K, Nakade S, Nakamura Y, Nashimoto H, Nishio Y, Oshima T, Saito N, Sampei G, Seki Y, Horiuchi T et al. (1996) A 460-kb DNA sequence of the *Escherichia coli* K-12 genome corresponding to the 40.1-50.0 min region on the linkage map (Supplement). DNA Research 3: 441-445.

Itoh Y, Wang X, Hinnebusch BJ, Preston JF, Romeo T (2005) Depolymerization of β-1,6-*N*-acetyl-d-glucosamine disrupts the integrity of diverse bacterial biofilms. Journal of Bacteriology 187: 382-387.

Izano EA, Sadovskaya I, Vinogradov E, Mulks MH, Velliyagounder K, Ragunath C, Kher WB, Ramasubbu N, Jabbouri S, Perry MB,. Kaplan JB (2007) Poly-*N*-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*. Microbial Pathology 431-439.

Jackson ML (1967) Soil chemical analysis. Prentice hall of Indian private limited, New Delhi.

Jackson D (1991) In situ hybridization in plants. In: Bowles DJ, Gurr SJ, McPherson M (eds) Molecular plant pathology: a practical approach, vol 1. IRC Press, Oxford, pp 163–174.

Jackman SC, Lee H, Trevors JT (1992) Survival, detection and containmant of bacteria. Microbe Releases 1: 125-154.

Jacobson CB, Pasternak JJ, Glick BR (1994) Partial purification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. Canadian Journal of Microbiology 40: 1019-1025.

Jha Y, Subramanian RB, Patel S (2011) Combination of endophytic and rhizospheric plant growth promoting rhizobacteria in *Oryza sativa* shows higher accumulation of osmoprotectant against saline stress. Acta Physiologia Plantarum 33: 797–802.

Jha CK, Annapurna K, Saraf M (2012) Isolation of Rhizobacteria from *Jatropha curcas* and characterization of produced ACC deaminase. Journal of Basic Microbiology 52: 285-295.

Jia YJ, Kakuta Y, Sugawara M, Igarashi T, Oki N, Kisaki M et al. (1999) Synthesis and degradation of 1-aminocyclopropane-1-carboxylic acid by *Penicillium citrinum*. Bioscience Biotechnology and Biochemistry 63: 542-549.

Jiang Y, Yang B, Harris NS, Deyholos MK (2006) Comparative proteomic analysis of NaCl stress-responsive proteins in *Arabidopsis* roots. Journal of Experimental Botany 58: 3591–3607.

Jiang CY, Sheng XF, Qian M, Wang QY (2008) Isolation and characterization of a heavy metal resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal polluted soil. Chemosphere 72: 157–164.

Jones JDG, and Dangl JL (2006) The plant immune system. Nature 444: 323-329.

Jorrin-Novo JV, Maldonado AM, Echavarría-Zomeno S, Valledor L, Castillejo MA, Curto M et al. (2009) Plant proteomics update (2007– 2008): second-generation proteomic techniques, an

appropriate experimental design, and data analysis to fulfil MIAPE standards, increase plant proteome coverage and expand biological knowledge. Journal of Proteomics 72: 285–314.

Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kiyokawa C, Kohara M, Matsumoto M, Matsuno A, Mochizuki Y, Nakayama S, Nakazaki N, Shimpo S, Sugimoto M, Takeuchi C, Yamada M, Tabata S (2000a) Complete genome structure of the nitrogen-fixing bacterium *Mesorhizobium loti*. DNA Research 7: 331-338.

Karthikeyan B, Joe MM, Islam MR, Sa T (2012) ACC deaminase containing diazotrophic endophytic bacteria ameliorate salt stress in *Catharanthus roseus* through reduced ethylene levels and induction of antioxidative defense systems. Symbiosis 56: 77–86.

Kasana R, Salwan R, Dhar H, Dutt S, Gulati A (2008) A rapid and easy method for the detection of microbial cellulases on agar plates using Gram's iodine. Current Microbiology 57: 503-507.

Khan M, Zaidi A, Wani P (2007) Role of phosphate solubilising microorganisms in sustainable agriculture. A. review. Agronomy and Sustainable Development 27**:** 29-43.

Kim JJ, Park CS, Park SH (2010) Genome sequence of the polymyxin-producing plant probiotic rhizobacterium *Paenibacillus polymyxa* E681. Journal of Bacteriology 192: 6103–6104.

Kirchhof G, Eckert B, Stoffels M, Baldani JI, Reis VM, Hartmann A (2001) *Herbaspirillum frisingense* sp. nov., a new nitrogen-fixing bacterial species that occurs in C4-fibre plants. International Journal of Systematics and Evolutionary Microbiology 51: 157-168.

Klee HJ, Hayford MB, Kretzmer KA, Barry GF, Kishore GM (1991) Control of ethylene synthesis by expression of bacterial enzyme in transgenic tomato plants. Plant Cell 3: 1187-1193.

Klee HJ, and Kishore GM (1992) Control of fruit ripening and senescence in plants. United States Patent Number 5: 702-933.

Kloepper JW, Lifshitz R, Zablotowicz RM (1989) Free-living bacterial inocula for enhancing crop productity. Trends in Biotechnology 7: 39–43.

Kloepper JW, Leong J, Teintze M, Schroth MN (1980) Enhanced plant growth by siderophores produced by plant growth-promoting rhizobacteria. Nature 286: 885–886.

Kohler J, Caravaca F, Alguacil MM, Roldán A (2009) Elevated CO_2 increases the effect of an arbuscular mycorrhizal fungus and a plant growth-promoting rhizobacterium on structural stability of a semiarid agricultural soil under drought conditions. Soil Biology and Biochemistry 41: 1710-1716.

Koike M, Okamoto T, Tsuda S, Imai R (2002) A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation. Biochemical Biophysical Research Communation 298: 46-53.

Koryo HW (2006) Effect of salinity on growth, photosynthesis and solute composition of the potential cash crop halophyte plantago. Environmental and Experimental Botany 56: 136-146.

Koteswara RG, Nagamalleswara RK, Phani RK, Aravind S (2010) In Silico identification of potential therapeutic targets in *Clostridium botulinum* by the approach subtractive genomics. Indian Journal of Bioinformatics Research 2: 12–16

Kpomblekou K, and Tabatabai MA (1994) Effect of organic acids on release of phosphorus from phosphate rocks. Soil Science 158: 442–453.

Krzywinski M, Schein J, Birol İ, Connors J, Gascoyne R, Horsman D, et al. (2009) Circos: an information aesthetic for comparative genomics. Genome Research 19: 1639–1645.

Kuan KB, Othman R, Abdul Rahim K, Shamsuddin ZH (2016) Plant growth-promoting rhizobacteria inoculation to enhance vegetative growth, nitrogen fixation and nitrogen remobilisation of maize under greenhouse conditions. PLoS One 24; e0152478.

Kumar KV, Singh N, Behl HM, Srivastava S (2008) Influence of plant growth promoting bacteria and its mutant on heavy metal toxicity in *Brassica juncea* grown in fly ash amended soil. Chemosphere 72: 678–683.

Kurt J (1991) Association of ß-1,3-glucanase activity and isoform pattern with systemic resistance to blue mould in tobacco induced by stem injection with *Peronospora tabacina* or leaf inoculation with tobacco mosaic virus. Physiology and Molecular Plant Pathology 39: 25-39.

Lalande R, Bissonnette N, Coutlee D and Antoun H (1989) Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. Plant and Soil 115: 7-11.

Lamb C, and Dixon RA (1997) The oxidative burst in plant disease resistance. Annual Review of Plant Biology 48: 251-275.

Langille MG, and Brinkman FS (2009) Island-Viewer: an integrated interface for computational identification and visualization of genomic islands. Bioinformatics 25: 664–665.

Larkindale J, Mishkind M, and Vierling E (2005) Plant responses to high temperature. pp. 100–144. In: Plant Abiotic Stress. Blackwell, Oxford, UK.

Lee KH, and La Rue TA (1992) Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv Sparkle. Plant Physiology 100: 1334–1338.

Lei CH, Lindstrom JT, Woodson WR (1996) Reduction of 1-aminocyclopropane-1-carboxylic acid (ACC) in pollen by expression of ACC deaminase in transgenic *Petunias*. Plant Physiology 111: 149.

Li J, and Glick BR (2001) Transcriptional regulation of the *Enterobactercloaceae* UW4 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene (*AcdS*). Canadian Journal of Microbiology 47: 259-267.

Li C-H, Zhao M-W, Tang C-M, Li S-P (2010) Population dynamics and identification of endophytic bacteria antagonistic toward plant-pathogenic fungi in cotton root. Microbial Ecology 59: 344-356.

Liu F, Zhang X, Lu C, Zeng X, Li Y, Fu D, Wu G (2015) Non-specific lipid transfer proteins in plants: presenting new advances and an integrated functional analysis. Journal of Experimental Botany doi: 10.1093/jxb/erv313.

Li W, Raoult D, Fournier P (2009) Bacterial strain typing in the genomic era. FEMS Microbiological Review 33: 892‑916.

Liu Y, Su C, Zhang H, Li X, Pei J (2014) Interaction of soil heavy metal pollution with industrialisation and the landscape pattern in Taiyuan city, China. PLoS One 9: e105798.

Liu F, Zhang X, Lu C, Zeng X, Li Y, Fu D et al. (2015) Non-specific lipid transfer proteins in plants: presenting new advances and an integrated functional analysis. Journal of Experimental Botany 66: 5663–5681.

Liu Y-H, Huang C-J, Chen C-Y (2008) Evidence of induced systemic resistance against *Botrytis elliptica* in lily. Phytopathology 98: 830–836

Liu WY, Chung KMK, Wong CF, Jiang JW, Hui RKH, Leung FCC (2012) Complete genome sequence of the endophytic *Enterobacter cloacae* subsp. *cloacae* strain ENHKU01. Journal of Bacteriology 194: 21.

Lugtenberg B, and Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annual Review of Microbiology 63: 541-556.

Lugtenberg BJJ, Dekkers L, Bloemberg GV (2001) Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annual Review of Phytopathology 39: 461–490.

Maathuis FJM (2014) Sodiumin plants: perception, signalling, and regulation of sodium fluxes. Journal of Experimental Botany 65: 849–858.

MacLean D, Jones JD, Studholme DJ (2009) Application of 'next-generation' sequencing technologies to microbial genetics. Nature Review in Microbiology 7: 287–296.

Madhaiyan M, Poonguzhali S, Ryu J, Sa T (2006) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carboxylate deaminase-containing *Methylobacterium fujisawaense*. Planta 224: 268-278.

Ma W, Guinel FC, Glick BR (2003a) *Rhizobium leguminosarum biovarviciae* 1-aminocyclopropane-1-carboxylate deaminase promotes nodulation of pea plants. Applied and Environmental Microbiology 69: 4396-4402.

Ma W, Sebestianova SB, Sebestian J, Burd GI, Guinel FC, Glick BR (2003b) Prevalence of 1-aminocyclopropane-1-carboxylate deaminase in *Rhizobium* spp. Antonie Van Leeuwenhoek 83: 285-291.

Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK (2002) A putative lipid transfer protein involved in systemic resistance signaling in *Arabidopsis*. Nature 419: 399-403.

Marasas WFO, Nelson PE, Toussoun TA (1984) Toxigenic *Fusarium* species. Identity and mycotoxicology. Pennsylvania State University, Park, pp 246.

Marinetti GV (1962) Chromatographic separation: identification and analysis of phospholipides. Journal of Lipid Research 3: 1–20.

Martin F, Winspear MJ, MacFarlane JD, Oaks A (1983) Effect of methionine sulfoximine on the accumulation of ammonia in C3 and C4 leaves: the relationship between NH_3 accumulation and photorespiratory activity. Plant Physiology 71: 177–181.

Martin MN, Saftner RA (1995) Purification and characterization of 1-aminocyclopropane-I-carboxylic acid N-malonyltransferase from tomato fruit. Plant Physiology 108: 1241-1249.

Mattoo AK, and Suttle JC (1991) The Plant Hormone Ethylene. CRC Press, Boca Raton, FL.

Mayak S, Tirosh T, Glick BR (1999) Effect of wild-type and mutant plant growth-promoting rhizobacteria on the rooting of mung bean cuttings. Journal of Plant Growth Regulation 18: 49–53.

Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiology and Biochemistry 42: 565–572.

Mayer AM, Harel E, Ben-Shaul R (1966) Assay of catechol oxidase—a critical comparison of methods. Phytochemistry 5: 783-789.

McDonnell L, Plett JM, Andersson-Gunnera˚s S, Kozela C, Dugardeyn J, Van Der SD, Glick BR, Sundberg B, Regan S (2009) Ethylene levels are regulated by a plant encoded 1-aminocyclopropane-1-carboxylic acid deaminase. Physiologia Plantarum 136: 94–109.

Mehta S, and Nautiyal CS (2001) An efficient method for qualitative screening of phosphate solubilizing bacteria. Current Microbiology 43: 51– 56.

Miller SH, Browne P, Prigent-Combaret C, Combes-Meynet E, Morrissey JP, O'Gara F (2010) Biochemical and genomic comparison of inorganic phosphate solubilization in *Pseudomonas* species. Environmental Microbiology Reports 2: 403–411.

Minami R, Uchiyama K, Murakami T, Kawai J, Mikami K, Yamada T, Yokoi D, Ito H., Matsui, H, Honma M (1998) Properties, sequence, and synthesis in *Escherichia coli* of 1-aminocyclopropane-1-carboxylate deaminase from *Hansenula saturnus*. Journal of Biochemistry 123: 1112–1118.

Mishra S, Singh RP, Raghuvanshi S, Gupta S (2015) Deducing the bio-perspective capabilities of Fe (II) oxidizing bacterium isolated from extreme environment. Biochemistry and Analytical Biochemistry 4: 166.

Mizutani F, Rabbany ABMG, Akiyoshi H (1998) Inhibition of ethylene production and 1-aminocyclo-propane-1-carboxylate oxidase activity by tropolo-nes. Phytochemistry 48: 31-34.

Mohite B (2013) Isolation and characterization of Indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. Journal of Soil Science & Plant Nutrition 13: 638-649.

Morgan PW, and Drew MC (1997) Ethylene and plant responses to stress. Physiologia Plantarum 100: 620-630.

Morris CE, Monier J, Jacques M (1997) Methods for observing microbial biofilms directly on leaf surfaces and recovering them for isolation of culturable microorganisms. Applied Environmental Microbiology 63: 1570‑1576.

Munns R, and Tester M (2008) Mechanisms of salinity tolerance. Annual Review of Plant Biology 59: 651–681.

Munoz-Bertomeu J, Miedes E, Lorences EP (2013) Expression of xyloglucan endotransglucosylase/hydrolase (XTH) genes and XET activity in ethylene treated apple and tomato fruits. Journal of Plant Physiology 170: 1194– 1201.

Munoz-Bertomeu J, Miedes E, Lorences EP (2013) Expression of xyloglucan endotransglucosylase/hydrolase (XTH) genes and XET activity in ethylene treated apple and tomato fruits. Journal of Plant Physiology 170: 1194.

Nadeem SM, Zahir ZA, Naveed M, Arshad M, Shahzad SM (2006) Variation in growth and ion uptake of maize due to inoculation with plant growth promoting rhizobacteria under salt stress. Soil and Environment 25: 78-84.

Nasri N, Saïdi I, Kaddour R, Lachaâl M (2015) Effect of salinity on germination, seedling growth and acid phosphatase activity in Lettuce. American Journal of Plant Sciences 6: 57-63.

Nascimento FX, Vicente CSL, Barbosa P, Espada M, Glick BR, Oliveira S, Mota M (2013) The use of the ACC deaminase producing bacterium *Pseudomonas putida* UW4 as a biocontrol agent for pine wilt disease. Biocontrol 58: 427–433.

Nascimento FX, Rossi MJ, Soares CRFS, McConkey BJ, Glick BR (2014) New insights into 1-aminocyclopropane-1-carboxylate (ACC) deaminase phylogeny, evolution and ecological significance. PLoS One 9: e99168.

Nayani S, Mayak S, Glick BR (1998) Effect of plant growth-promoting rhizobacteria on senescence of flower petals. Indian Journal of Experimental Biology 36: 836–839.

Ndimba BK, Chivasa S, Simon WJ, Slabas AR (2005) Identification of *Arabidopsis* salt and osmotic stress responsive proteins using two-dimensional difference gel electrophoresis and mass spectrometry. Proteomics 5: 4185–4196.

Nelson LM (2004) Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. 2004 Plant Management Network, online doi:10.1094/CM-2004-0301-05-RV.

Nie L, Shah S, Rashid A, Burd GI, Dixon DG, Glick BR (2002) Phytoremediation of arsenate contaminated soil by transgenic canola and the plant growth promoting bacterium *Enterobacter cloaceae* CAL2. Plant Physiology and Biochemistry 40: 355–361.

Nishijima KA, Alvarez AM, Hepperly PR, Shinta-ku MH, Keith LM, Sato DM, Bushe BC, Armstrong JW, Zee FT (2004) Association of *Enterobacter cloacae* with rhizome rot of edible ginger in Hawaii. Plant Disease 88: 1318-1327.

Nishijima KA, Wall MM, Siderhurst MS (2007) Demonstrating pathogenicity of *Enterobacter cloacae* on macadamia and identifying associated volatiles of gray kernel of macadamia in Hawaii. Plant Disease 91: 1221-1228.

Nukui N, Ezura H, Yuhashi K-I, Yasuta T and Minamisawa K (2000) Effects of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in *Lotus japonicus* and *Macroptilium atropurpureum.* Plant Cell and Physiology 41: 893–897.

Olsen SR, Cole CV, Watanabe FS, Dean LA (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. Circular 939 US Department of Agriculture, Washington DC, USA.

Onofre-Lemus J, Herna´ndez-Lucas I, Girard L, Caballero-Mellado J (2009) ACC (1-aminocyclopropane-1-carboxylate) deaminase activity, a widespread trait in *Burkholderia* species, and its growth-promoting effect on tomato plants. Applied Environment and Microbiology 75: 6581–6590.

Ordentlich A, Elad Y, Chet I (1988) The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. Phytopathology 78: 84-88.

O'Toole GA, and Kolter R (1998) Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis. Molecular Microbiology 28: 449-461.

Pan YB, Grisham MP, Burner DM (1997) A polymerase chain reaction protocol for the detection of *Xanthomonas albilineans*, the causal agent of sugarcane leaf scald disease. Plant Disease 81: 189-194.

Parida SK, and Das AB (2005) Salt tolerance and salinity effects on plants. Ecotoxicology and Environmental Safety 60: 324–349.

Parker R, Flowers TJ, Moore AL, Harpham NV (2006) An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. Journal of Experimental Botany 57: 1109–1118.

Patrignani F, Iucci L, Belletti N, Gardini F, Guerzoni ME, Lanciotti R (2008) Effects of sub-lethal concentrations of hexanal and 2-(E)-hexenal on membrane fatty acid composition and volatile compounds of *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella enteritidis* and *Escherichia coli*. International Journal of Food Microbioogy 123: 1–8.

Penrose DM, and Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiologia Plantarum 118: 10-15.

Peterson TA, Reinsel MD, Krizek DT (1991) Tomato (*Lycopersicon esculentum* Mill., cv. Better Bush) plant response to root restriction. II. Root respiration and ethylene generation. Journal of Experimental Botany 42: 1241–1249.

Peters NK, and Crist-Estes DK (1989) Nodule formation is stimulated by the ethylene inhibitor aminoethoxyvinylglycine. Plant Physiology 91: 690–693.

Pitman MG, and Läuchli A (2002) Global impact of salinity and agricultural ecosystems. In: Salinity: Environment–Plants–Molecules, A. Läuchli and U. Lüttge (Eds.). Kluwer Academic Publishers, Dordrecht, pp. 3–20.

Plett JM, McDonnell L, Regan S (2009) Plant encoded 1-aminocyclopropane-1-carboxylic acid deaminase activity implicated in different aspects of plant development. Plant Signalling Behaviour 4: 1186-1189.

Podile AR, and Laxmi VDV (1998) Seed bacterization with *Bacillus subtilis* AF 1 increases phenilalanine ammonia-lyase and reduces the incidence of *fusarial* wilt in *pigeon pea*. Journal of Phytopathology 146: 255- 259.

Porter JR, and Gawith M (1999) Temperature and growth and development of wheat: A review. Europian Journal of Agronomy 10: 23-36.

Pritchard J, Hetherington PR, Fry SC, Tomos A (1993) Xyloglucan endotransglycosylase activity, microfibril orientation and the profiles of cell wall properties along growing regions of maize roots. Journal of Experimental Botany 44: 1281–1289.

Quiroga M, Guerrero C, Botella MA, Barceló A, Amaya I, Medina MI, Alonso FJ, de Forchetti SM, Tigier H, Valpuesta V (2000) A tomato peroxidase involved in the synthesis of lignin and suberin. Plant Physiology 122: 1119-1127.

Rahman A, Nahar N, Olsson B, Mandal A (2016) Complete genome sequence of *Enterobacter cloacae* B2-DHA, a chromium-resistant bacterium. Genome Announcement 4(3): e00483-16. doi:10.1128/genomeA.00483-16.

Rajaei SV, Niknam S, Seyedi H, Razavi K (2009) Contractile roots are the most sensitive organ in *Crocus sativus* to salt stress. Biologia Plantarum 53: 523-529.

Rajkumar M, Ae N, Prasad MNV, Freitas H (2010) Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends in Biotechnology 28: 142–149

Ramadoss D, Lakkineni VK, Bose P, Ali, S Annapurna K (2013) Mitigation of salt stress in wheat seedlings by halotolerant bacteria isolated from saline habitats. Springer plus 2: 6.

Ramamoorthy V, Raguchander T, Samiyappan R (2002) Induction of defense-related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. *lycopersici*, 55-68.

Ramamoorthy V, Raguchander T, Samiyappan R (2002) Induction of defense-related proteins in tomato roots treated with *Pseudomonas fluorescens* Pf1 and *Fusarium oxysporum* f. sp. *lycopersici*. Plant and Soil 239: 55-68.

Rashid S, Charles TC, Glick BR (2012) Isolation and characterization of new plant growth-promoting bacterial endophytes. Agriculture Ecosystem and Environment 61: 217–224.

Raza W, Yang X, Wu H, Wang Y, Xu Y, Shen Q (2009) Isolation and characterisation of fusaricidin-type compound- producing strain of *Paenibacillus polymyxa* SQR-21 active against *Fusarium oxysporum* f. sp. *nevium.* Europian Journal of Plant Pathology 125: 471–483.

Reed AJ, Magin KM, Anderson JS, Austin GD, Rangwala T, Linde DC et al. (1995) Delayed ripening in tomato plants expressing the enzyme 1- aminocyclopropane-1-carboxylic acid deaminase: molecular characterization, enzyme expression, and fruit ripening traits. Journal of Agricultural Food Chemistry 43: 1954–1962.

Reed MLE, and Glick BR (2005) Growth of canola (*Brassica napus*) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. Canadian Journal of Microbiology 251: 1061–1069.

Reetha S, Bhuvaneswari G, Thamizhiniyan P, Mycin TR (2014) Isolation of indole acetic acid (IAA) producing rhizobacteria of *Pseudomonas fluorescens* and *Bacillus subtilis* and enhance growth of onion *(Allim cepa.*L). International Journal of Current Microbiology and Applied Sciences 3: 568-574

Reid MS, and Wu MJ (1992) Ethylene and flower senescence. Plant Growth Regulation 11: 37–43.

Riemenschneider A, Wegele R, Schmidt A, Papenbrock J (2005) Isolation and characterization of a D-cysteine desulfhydrase protein from *Arabidopsis thaliana*. FEBS Journal 272: 1291–1304.

Reinhold-Hurek B, Hurek T, Gillis M, Hoste B, Vancanneyt M, Kersters K, De Ley J (1993) *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. International Journal of Systematic Bacteriology 43: 574-584.

Ren Y, Ren Y, Zhou Z, Guo X, Li Y, Feng L, Wang L (2010) Complete genome sequence of *Enterobacter cloacae* subsp. *cloacae* Type strain ATCC 13047. Journal of Bacteriology 192: 2463–2464.

Robison MM, Shah S, Tamot B, Pauls KP, Moffatt BA, Glick BR (2001) Reduced symptoms of *Verticillium* wilt in transgenic tomato expressing a bacterial ACC deaminase. Molecular Plant Pathology 2: 135–145.

Rodrigues EP, Rodrigues LS, de Oliveira ALM, Baldani VLD, Teixeira KRS, Urquiaga S, Reis VM (2008) *Azospirillum amazonense* inoculation: effects on growth, yield and N_2 fixation of rice (*Oryzasativa* L.). Plant and Soil 302: 249–261.

Rontein D, Basset G, Hanson AD (2002) Metabolic engineering of osmoprotectant accumulation in plants. Metabolic engineering 4: 49-56.

Roppolo D, Boeckmann B, Pfister A, Boutet E, Rubio MC, Dénervaud-Tendon V, Vermeer JE, Gheyselinck J, Xenarios I, Geldner N (2014) Functional and evolutionary analysis of the casparian strip membrane domain protein family. Plant Physiology 11: 1709-1722.

Rose JKC, Braam J, Fry SC, Nishitani K (2002) The XTH family of enzymes involved in xyloglucanendotransglucosylation and endohydrolysis: current perspectives and a new unifying nomenclature. Plant Cell Physiology 43: 1421–35.

Saalaoui E, Litvak S, Araya A (1990) The apocytochrome b from an alloplasmic line of Wheat (*T. aestivum*, cytoplasm-T. timopheevi) exists in two differently expressed forms." Plant Science 66: 237-246.

Safronova VI, Stepanok VV, Engqvist GL, Alekseyev YV, Belimov AA (2006) Root-associated bacteria containing 1-aminocyclopropane-1-carboxylate deaminase improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. Biology and Fertility of Soils 42: 267–272.

Saha R, Saha N, Donofrio RS, Bestervelt LL (2012) Microbial siderophores: a mini review. Journal of Basic Microbiology 53: 303-317.

Sahay H, Mahfooz S, Singh AK, Singh S, Kaushik R, Saxena AK, Arora DK (2012) Exploration and characterization of agriculturally and industrially important halo-alkaliphilic bacteria from environmental samples of hypersaline Sambhar lake, India. World Journal of Microbiology and Biotechnology 28: 3207–3217.

Saleem, M, Arshad M, Hussain S, Bhatti AS (2007) Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. Journal of Indian Microbiology and Biotechnology 34: 635–648.

Saravanakumar D, and Samiyappan R (2007) ACC deaminase from *Pseudomonas Xuorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. Journal of Applied Microbiology 102: 1283–1292.

Scandalios JG, Guan L, Polidoros AN (1997) Catalases in plants: gene structure, properties, regulation and expression. In: Scandalios JG, ed. Oxidative stress and the molecular biology of antioxidants defenses. New York: Cold Spring Harbor Laboratory Press, 343–406

Schwyn B, and Neilands JB (1987) Universal chemical assay for the detection and determination of siderophores. Analytical Biochemistry 160: 47-56.

Sergeeva E, Shah S, and Glick BR (2006) Growth of transgenic canola (*Brassica napus* cv. *Westar*) expressing a bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene on high concentrations of salt. World Journal of Microbiology and Biotechnology 22: 277–282.

Shabala S, and Shabala L (2011) Ion transport and osmotic adjustment in plants and bacteria. Biomolecular Concepts 2: 407-419.

Shabala S, and Cuin TA (2008) Potassium transport and plant salt tolerance. Physiologia Plantarum 133: 651-669.

Shaharoona B, Arshad M, Khalid A (2007) Differential response of etiolated pea seedling to 1-aminocyclopropane-1-carboxylate and/or L-methionine utilizing rhizobacteria. Microbiology 45: 15-20.

Shahzad SM, Arif MS, Riaz M, Iqbal Z, Ashraf M (2013) PGPR with varied ACC-deaminase activity induced different growth and yield response in maize (*Zea mays* L.) under fertilized conditions. European Journal of Soil Biology 57: 27-34.

Sharma S, Kulkarni J, Jha B (2016) Halotolerant rhizobacteria promote growth and enhance salinity tolerance in peanut. Frontier in Microbiology doi: 10.3389/fmicb.2016.01600.

Sharma R, Manda R, Gupta S, Kumar S, Kumar V (2013) Isolation and characterization of osmotolerant bacteria from thar desert of western Rajasthan (India). Revista de Biología Tropical. 61: 1551-1562.

Shoji T, Suzuki K, Abe T, Kaneko Y, Shi H, Zhu JK, Rus A, Hasegawa PM, Hashimoto T (2006) Salt stress affects cortical microtubule organization and helical growth in *Arabidopsis*. Plant Cell Physiology 47: 1158–1168.

Siddikee MA, Tipayno SC, Kim K, Chung JB, Sa T (2011) Influence of varying degree of salinity-sodicity stress on enzyme activities and bacterial populations of coastal soils of Yellow Sea, South Korea. Journal of Microbiology and Biotechnology 21: 341–346.

Silva HSA, Romeiro R, Macagnan D, Halfeld-Viera B, Pereira MCB, Mounteer A (2004) Rhizobacterial induction of systemic resistance in tomato plants:non-specific protection and increase in enzyme activities. Biological Control 29: 288-295.

Singh VS, Pandey DN, Gupta AK, Ravindranath NH (2010) Climate Change Impacts, Mitigation and Adaptation: Science for Generating Policy Options in Rajasthan, India. http://hdl.handle.net/10535/5860.

Singh N, and Kashyap S (2012) In-silico identification and characterization of 1-aminocyclopropane-1-carboxylate deaminase from *Phytophthora sojae*. Journal of Molecular Modelling 18: 4101–4111.

Singh RP, Jha P, Jha PN (2015) The plant growth promoting bacterium *Klebsiella* sp. SBP-8 confers induced systemic tolerance in wheat (*Triticum aestivum*) under salt stress. Journal of Plant Physiology 184: 57‑67.

Sivakumar P, Sharmila P, Saradhi PP (1998) Proline suppresses Rubisco activity in higher plants. Biochemical Biophysical Research Communication 252: 428–432.

Snyder EE, Kampanya N, Lu J, Nordberg EK, Karur HR, et al. (2007) PATRIC: the VBI PathoSystems Resource Integration Center. Nucleic Acids Research 35: D401–406.

Šramková Z, Gregová E, Šturdík E (2009) Genetic improvement of wheat - a review, Nova Biotechnologica 9: 27-51.

Stearns J, and Glick BR (2003) Transgenic plants with altered ethylene biosynthesis or perception. Biotechnology Advances 21: 193–210.

Strobel GA, Kluck K, Hess WM, Sears J, Ezra D, Vargas PN (2007) *Muscodor albus* E-6, an endophyte of *Guazuma ulmifolia*, making volatile antibiotics: isolation, characterization and experimental establishment in the host plant. Microbiology 153: 2613–2620.

Subramanian S, Souleimanov A, and Smith DL (2016) Proteomic studies on the effects of lipo-chitooligosaccharide and thuricin 17 under unstressed and salt stressed conditions in *Arabidopsis thaliana*. Frontier in Plant Science doi: 10.3389/fpls.2016.01314.

Sutherland I (2001) Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 1473-1479.

Sugawara M, Okazaki S, Nukui N, Ezura H, Mitsui H, Minamisawa K (2006) Rhizobitoxine modulates plant–microbe interactions by ethylene inhibition. Biotechnology Advances 24: 382–388

Syrmis MW, O'Carroll MR, Sloots TP, Coulter C, Wainwright CE, Bell SC Nissen MD (2004) Rapid genotyping of *Pseudomonas aeruginosa* isolates harboured by adult and paediatric patients with cystic fibrosis using repetitive-element-based PCR assays. Journal of Medical Microbiology 53: 1089–1096.

Tadra-Sfeir M, Souza E, Faoro H, Müller-Santos M, Baura V, Tuleski T, Rigo L, Yates M, Wassem R, Pedrosa F (2011) Naringenin regulates expression of genes involved in cell wall synthesis in *Herbaspirillum seropedicae*. Applied and Environmental Microbiology 77: 2180-2183.

Taghavi S, Van der Lelie D, Hoffman A, Zhang YB, Walla MD, Vangronsveld J, Newman L, Monchy S (2010) Genome sequence of the plant growth promoting endophytic bacterium *Enterobacter* sp. 638. PLoS Genetics 6: e1000943.

Taiz, L, and Zeiger E (2006) Plant Physiology, Fourth Edition. Sinauer Associates. Sunderland, MA, 764.

Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt sress using *Arabidopsis* microarray. Plant Physiology 135: 1697–1709.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Molecular Biology Evolution 30: 2725–2729.

Tapia-Hernández A, Bustillos-Cristales MR, Jiménez-Salgado T, Caballero-Mellado J, Fuentes-Ramírez LE (2000) Natural endophytic occurrence of *Acetobacter* diazotrophicus in *pine apple* plants. Microbial Ecology 39: 49–55.

Tattini M, and Traversi ML (2008) Responses to changes in Ca^{++} supply in two mediterranean evergreens, *Phillyrea latifolia* and *Pistacia lentiscus*, during salinity stress and subsequent relief. Annals of Botany 102: 609-622.

Tittabutr P, Piromyou P, Longtonglang A, Noisa-Ngiam R, Boonkerd N, Teaumroong N (2013) Alleviation of the effect of environmental stresses using co-inoculation of mung bean by *Bradyrhizobium* and rhizobacteria containing stress-induced ACC deaminase enzyme. Soil Science and Plant Nutrition 59: 559‑571.

Torok Z, Horvath I, Goloubinoff P, Kovacs E, Glatz A, Balogh G, and Vigh L (1997) Evidence for a lipochaperonin: association of active protein-folding GroESL oligomers with lipids can stabilize membranes under heat shock conditions. Proceeding of Natural Academy of Science USA 94: 2192–2197.

Tucaliuc RB, Risca IM, Drochioiu G, Mangalagiu I (2008) Biological effect of some new pyridazine derivatives on wheat in germination experiments. Biotechnology Letters 13: 3837–3842.

Uchiumi T, Oowada T, Itakura M, Mitsui H, Nukui N, Dawadi P, Kaneko T, Tabatta S, Yokoyama T, Tejima T, Saeki K, Oomori H, Hayashi M, Maekawa T, Sriprang R, Murooka Y, Tajima S, Simomura K, Nomura M, Suzuki A, Shimoda S, Sioya K, Abe M, Minamisawa K (2004) Expression islands clustered on symbiosis island of *Mesorhizobium loti* genome. Journal of Bacteriology 186: 2439-2448.

Umezawa T, Fujita M, Fujita Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Engineering drought tolerance in plants: discovering and tailoring genes to unlock the future. Current Opinion in Biotechnology 17: 113–122.

Upadhyay SK, Singh JS, Saxena AK, Singh DP (2011) Impact of PGPR inoculation on growth and antioxidant status of wheat under saline conditions. Plant Biology 14: 605‑611.

Van Domselaar GH, Stothard P, Shrivastava S, Cruz JA, Guo A, Dong X, Lu P, Szafron D, Greine R, and Wishart DS (2005) BASys: a web server for automated bacterial genome annotation. Nucleic Acids Research 1: 33.

Van Loon LC, Rep M, Pieterse CMJ (2006) Significance of inducible defenserelatedproteins in infected plants. Annual Review of Phytopathology 44:135–162.

Van Loon LC (2007) Plant responses to plant growth-promoting rhizobacteria. Europian Journal of Plant Pathology 119: 243–254.

Van Loon LC (1984) Regulation of pathogenesis and symptom expression in diseased plants by ethylene. In Y. Fuchs, & E. Chalutz (Eds.), Ethylene: Biochemical, physiological and applied aspects (pp. 171–180). The Hague: Martinus Nijhoff/Dr W. Junk.

Velazhahan R, and Vidhyasekaran P (1994) Role of phenolic compounds, peroxidase and polyphenol oxidase in resistance of groundnut to rust. Acta Phytopathologica et Entomologica Hungarica 29: 23-29.

Venkateswarlu B, Desai S, Prasad YG (2009) Agriculturally important microorganisms for stressed ecosystems: Challenges in technology development and application. In: Agriculturally Important Microorganisms Vol.1. Kachatou rians GG, Arora DK, Rajendran TP, Srivastava AK, (Eds.). Academic World, Bhopal pp 225–46.

Vetrivel U, Subramanian G, Dorairaj S (2011) A novel in silico approach to identify potential therapeutic targets in human bacterial pathogens. Advance Research 5: 25–34.

Viterbo A, Landau U, Kim S, Chernin L, Chet I (2010) Characterization of ACC deaminase from the biocontrol and plant growth-promoting agent *Trichoderma asperellum* T203. FEMS Microbiology Letters 305: 42–48.

Walsh C, Pascal RA, Hohnston M, Raines R, Dikshit D, Krantz A, Honma M (1981) Mechanistic studies on the pyridoxal phosphate enzyme 1- aminocyclopropane-1-carboxylate deaminase form *Pseudomonas* sp.. Biochemistry 20: 7509-7519.

Wang C, Knill E, Glick BR, Defago G (2000) Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its gacA derivative CHA96 on their growth-promoting and disease-suppressive capacities. Canadian Journal of Microbiology 46: 898–907.

Wang WX, Barak T, Vinocur B, Shoseyov O, Altman A (2003) Abiotic resistance and chaperones: possible physiological role of SP1, a stable and stabilizing protein from *Populus*. Kluwer, Dordrecht, pp 439–443.

Wang Q, Li F, Zhang X, Zhang Y, Hou Y et al. (2011) Purification and characterization of a CkTLP protein from *Cynanchumkomarovii* seeds that confers antifungal activity. PLOS One 6: e16930.

Welbaum GE, Sturz AV, Dong Z, Nowak J (2004) Fertilizing soil microorganisms to improve productivity of agroecosystems. Critical Review in Plant Science 23: 175–193.

Wheatley RE (2002) The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie Van Leeuwenhoek 81: 357–364.

Wheeler TR, Ellis G, Prasad P (2000) Temperature variability and the yield of annual crops. Agriculture Ecosystem and Environment 82: 159-167.

Willey DL, Howe CJ, Auffret AD, Bowman CM, Dyer TA, Gray JC (1984) "Location and nucleotide sequence of the gene for cytochrome f in wheat chloroplast DNA. Molecular Genetics and Genomics 194: 416-422.

Wink J, Kroppenstedt RM, Seibert G, Stackebrandt E (2003) *Actinomadura namibiensis* sp. nov. International Journal of Systematic and Evolutionary Microbiology 53: 721–724.

Wu Y, Spollen WG, Sharp RE, Hetherington PR, Fry SC (1994) Root growth maintenance at low water potentials (increased activity of xyloglucan endotransglycosylase and its possible regulation by abscisic acid). Plant Physiology 106: 607–615.

Wu, Sharp RE, Durachko DM, Cosgrove DJ (1996) Growth maintenance of the maize primary root at low water potentials involves increases in cell-wall extension properties, expansin activity, and wall susceptibility to expansins. Plant Physiology 111: 765–772.

Yamaguchi-Shinozaki K, Shinozaki K (2006) Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annual Review of Plant Biology 57*:* 781–803.

Yang SF, and Hoffman NE (1984) Ethylene biosynthesis and its regulation in higher-plants. Annual Review of Plant Physiology 35: 155–189.

Yan J, Smith MD, Glick BR, Liang Y (2014) Effects of ACC deaminase containing rhizobacteria on plant growth and expression of Toc GTPases in tomato (*Solanum lycopersicum*) under salt stress. Botany 92: 775-781.

Yang J, Kloepper JW, Ryu CM (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends in Plant Science 14: 1-4.

Zahir ZA, Munir A, Asghar HN, Shaharoona B, Arshad M (2008) Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions. Journal of Microbiology Biotechnology 18: 958–963.

Zang X, and Komatsu S (2007) A proteomics approach for identifying osmotic-stress-related proteins in rice. Phytochemistry 68: 426–437.

Zhang H, Kim MS, Sun Y, Dowd SE, Shi H, Paré PW (2008) Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. Molecular Plant Microbe Interaction 21: 737–744.

Zhang HX, and Blumwald E (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. Nature Biotechnology 19: 765-768

Zhang HX, Hodson JN, Williams JP, Blumwald E (2001) Engineering salt-tolerant *Brassica* plants: characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proceeding of Natural Academy of Science USA 98: 12832-12836

Zhang JL, and Shi H (2013) Physiological and molecular mechanisms of plant salt tolerance. Photosynthetic Research 115: 1–22.

Zhou G, Wang Y, Wang S (2002) Responses of grassland ecosystems to precipitation and land use along the northeast China transect. Journal of Vegetarian Science 13: 361–368.

Zimmermann P, and Zentgraf U (2005) The correlation between oxidative stress and leaf senescence during plant development. Cellular and Molecular Biology Letters 10: 515‑534.

Zurdo-Pineiro JL, Rivas R, Trujillo ME, Vizcaı´no N, Carrasco JA et al. (2007) *Ochrobactrum cytisi* sp. nov., isolated from nodules of *Cytisus scoparius* in Spain. International Journal of Systematic and Evolutionary Microbiology 57: 784–788.

Research Publications

Published:

Singh RP, Jha PN (2016) A halotolerant bacterium *Bacillus licheniformis* HSW-16 augments induced systemic tolerance to salt stress in wheat plant (*Triticum aestivum*). *Front Plant Sci.* **7**:1890. doi: 10.3389/fpls.2016.01890.

Singh RP, Jha PN (2016) The multifarious PGPR *Serratia marcescens* CDP-13 augments induced systemic resistance and enhanced salinity tolerance of wheat (*Triticum aestivum* L.). *PLOS One* 11(6): e0155026. doi:10.1371/journal, pone.0155026.

Singh RP, Jha PN (2016) Alleviation of salinity-induced damage on wheat plant by an ACC deaminase-producing halophilic bacterium *Serratia* sp. SL- 12 isolated from salt lake. *Symbiosis* 69 (2): 101–111.

Baig N, **Singh RP**, Jha PN, Saha AK (2016) Synthesis of glucose derived glycoconjugates and studies of their antimicrobial activities: Mechanistic insight. *Chem select* DOI: 10.1002/slct.201600786.

Tantak MP, Gupta V, Nikhil K, Arun V, **Singh RP**, Jha PN, Shah K, Dalip Kumar (2016) Sequential one-pot synthesis of bis(indolyl)glyoxylamides: Evaluation of antibacterial and anticancer activities. *Bioorg Med Chem Lett*. 26: 3167.

Chander S, Ashok P, Chow LMC, Wong ILK, **Singh RP**, Jha PN, Murugesan S (2016) Synthesis and in-vitro anti-leishmanial activity of (4-arylpiperazin-1-yl) (1-(thiophen-2-yl)-9H-pyrido[3,4-b]indol-3-yl) methanone derivatives. *Bioorg chem.* (Accepted).

Singh RP, Jha PN (2016) Mitigation of salt stress in wheat plant (*Triticum aestivum*) by ACC deaminase bacterium *Enterobacter* sp. SBP-6 isolated from *Sorghum bicolor*. *Acta Physiol Plant*. 38: 110.

Chander S, Ashok P, **Singh RP**, Jha PN, Murugesan S (2016) Rational design, synthesis, anti-HIV-1 RT and anti-microbial activity of novel 2-(6, 7-Dimethoxy-3,4-dihydroisoquinolin-2(1H)-yl)-N-phenylpropanamide derivatives. *Anti-Infective Agents*, Volume 14 (E-pub ahead of print).

Singh RP, Shelke GM, kumar A, Jha PN (2015) Biochemistry and genetics of ACC deaminase: A weapon to 'stress ethylene' produced in plants. *Front Microbiol*. 6: 937 doi: 10.3389/fmicb.2015.00937.

Singh RP, Jha P, Jha PN (2015) The plant growth promoting bacterium *Klebsiella* sp. SBP-8 confers induced systemic tolerance in wheat (*Triticum aestivum*) plant under salt stress condition. *J Plant Physiol*. 184: 57-67.

Singh RP, Jha PN (2015) Plant growth promoting potential of ACC deaminase rhizospheric bacteria isolated from *Aerva javanica*: A plant adapted to saline environments. *Intl J Curr Microbiol Appl Sci*. 4: 142-152.

Baig N, **Singh RP**, Chander S, Jha PN, Murugesan S, Saha AK (2015) Synthesis, evaluation and molecular docking studies of amino acid derived N-glycoconjugates as antibacterial agents. *Bioorg Chem*. 63: 110-115.

Mishra SM, **Singh RP**, Raghuvanshi S, Jha PN (2015) Deducing the Bio-perspective capabilities of Fe(II) oxidizing bacterium isolated from extreme environment. *Biochem Anal Biochem.* 4: 166.

Chander S, Ashok P, **Singh RP**, Jha PN Murugesan S (2015) A rapid, green, efficient microwave-assisted synthesis and antimicrobial activity of novel glycinamide of 6,7-dimethoxy-1, 2, 3, 4-tetrahydroisoquinolines. *Curr Microwave Chem*. 9: 33.

Khedar P, Pericherla K, **Singh RP**, Jha PN, Kumar A (2015) Click chemistry inspired synthesis of piperazine-triazole derivatives and evaluation of their antimicrobial activities. *Med Chem Res*. 24: 3117–3126.

Pallavi B, Sharma A, Jha PN, **Singh RP**, Shukla P (2015) Novel grinding synthesis of pyranopyrazole analogues and their evaluation as antimicrobial agents. *Heterocycles* 91: 1615-1627.

Manuscripts Communicated/Under Revision:

Singh RP, Jha PN. GC-MS analysis of fatty acid composition of a PGPR *Klebsiella* sp. SBP-8 and its ability to protect wheat plant under salt stress. *Symbiosis* (under revision).

Singh RP, Mishra S, Jha, P, Raghuvanshi S, Jha PN. Exploring the potential of *Enterobacter ludwigii* CDP-14 on phytoextraction of Zn by wheat (*Triticum aestivum* L). *Ecol Eng*. (under revision).

Singh RP, Jha PN. Bio-inoculation of Plant growth-promoting rhizobacterium *Enterobacter cloacae* ZNP-3 increased stress tolerance in wheat (*Triticum aestivum* L.) plants. *J Plant Growth Regul.* (under revision).

Book chapter

Singh RP, Jha PN (2016) Potential of plant growth promoting rhizobacteria in stress agriculture. (In press).

Abstracts at Conference/Symposium:

Singh RP, Sharma N, Sandhu M, Jha PN (2016) Exploring the potential of ACC deaminase bacteria for enhanced salinity tolerance of wheat (*Triticum aestivum* L.). In: BIOSANGAM 2016 - International Conference on Translational Biotechnology held at MNNIT, Allahabad, Feb. 2rd - 6th. Abstract no. ABTPR 16, Pg. No. 60 **(Best Poster Award)**.

Sandhu M, Jha P, Paul A, **Singh RP**, Jha PN (2016) Characterization of biphenyl metabolozing gram positive bacterium *Rhodococcus* sp. MAPN-1 isolated from rhizosphere of *Morus alba* (Mulberry plant). International Conference on Strategies for Environment Protection and Management (ICSEPM-2016) at JNU, New Delhi, Dec. 11th-13th. Abstract No. EB 115, Pg. No. 110 **(Best Poster Award)**.

Singh RP, Jha PN (2016) Biochemical and molecular characterization of ACC deaminase halobacterium *Klebsiella* sp. SBP-8 isolated from *Sorghum bicolor*. (BCGGR- 2016), BITS Pilani, Dept. of Biological Sciences, Abstract no. P-46, Pg. No. 83.

Khan S, **Singh RP**, Jha PN (2016) Unveiling the potential of multifarious PGPR *Serratia marcescens* CDP-13 for enhanced salinity tolerance of wheat (*Triticum aestivum*). ASM-2016, Gauhati University, Assam, Abstract no. EM-76, Pg. No. 169.

Sandhu M, Sharma N, Jha P, Paul AT, **Singh RP**, Jha PN (2016) PCB degrading potential of bacterial isolates from the rhizosphere of Morus alba. BCGGR-2016, BITS Pilani, Dept. of Biological Sciences, Abstract no. P-44, Pg. No. 81

Singh RP, Agarwal K, Jha PN (2016) Bacterial ACC deamianse: Regulation, genetics and reaction mechanism. (OCSD-2016), BITS Pilani, Dept of Chemistry, Aug. 29th -30th, Abstract no. PP-42, Pg. No. 68.

Singh RP, Jha PN (2016) Mitigation of salt stress in wheat plant by ACC deamianse bacterium *Enterobacter* sp. SBP-6 isolated from *Sorghum bicolor*. (OCSD-2016), BITS Pilani, Dept of Chemistry, Aug. 29th -30th, Abstract no. PP-51, Pg. No. 77.

Singh RP, Nanwani R, Jha PN (2016) The plant growth promoting bacterium *Klebsiella* sp. SBP-8 confers salt tolerance to wheat plants. (OCSD-2016), BITS Pilani, Dept of Chemistry, Aug. 29th -30th, Abstract no. PP-51, Pg. No. 77.

Singh RP, Khan S, Jha PN (2016) The multifarious PGPR *Serratia marcescens* CDP-13 augments induced systemic resistance and enhanced salinity tolerance of wheat (*Triticum aestivum*). (OCSD-2016), BITS Pilani, Dept of Chemistry, Aug. 29th -30th, Abstract no. PP-58, Pg. No. 84.

Singh RP, Nalwaya S, Jha PN (2016) Induced systemic tolerance mediated by a halotolerant plant growth promoting bacterium *Bacillus licheniformis* HSW-16 isolated from salt lake. (OCSD-2016), BITS Pilani, Dept of Chemistry, Aug. 29th -30th, Abstract no. PP-60, Pg. No. 86.

Nahata S, **Singh RP**, Jha PN (2015) Mediation of induced systemic tolerance (IST) by ACC Deaminase bacteria isolated from desert region of Rajasthan. (WAICEE-2015), BITS Pilani, Dept of Chemical Engineering, Feb. 27th -28th, Pg. No. 30 **(Best Poster Award).**

Nehra P, **Singh RP**, Sivasubramanian SC, Jha PN, Khungar B (2015) Synthesis, characterization and applications of an ionic liquid-supported Zinc complex. National conference on "Frontiers at the chemistry-allied sciences interface", Department of Chemistry, University of Rajasthan, Jaipur, Mar. 13th -14th, Abstract no. PP-43, Pg. No. 99.

Reddy POV, Tantak MP, **Singh RP**, Singh OM, Sadna R, Kumar D (2015) Synthesis and biological evaluation of novel carbazolyl glyoxamides as anticancer and antibacterial agents. NDSC (National developments in chemical science), Dept. of chemistry, BITS Pilani. Abstract No. PP-135, Pg. No. 214.

Khedar P, Pericherla K, **Singh RP**, Jha PN, Kumar A (2015) Synthesis of nove 1,2,3-triazole derivatives through click chemistry and evaluation of their antibacterial and antifungal activities. NDSC (National developments in chemical science), Dept. of chemistry, BITS Pilani. Abstract no. PP-150, Pg. No. 229.

Singh RP, Jha PN (2015) A halotolerant plant growth promoting bacterium *Bacillus licheniformis* HSW-16 confers induced systemic tolerance in wheat under salinity stress. NDSC (National developments in chemical science), Dept. of chemistry, BITS Pilani. Abstract no. PP-175, Pg. No. 254.

Singh RP, Jha PN (2014) Characterization of ACC deaminase bacterial isolated from plants of desert of Rajasthan". (ICETB-2014), International conference on emerging trends in biotechnology (XI convention of BRSI). JNU University, Nov. 6th –9th, Abstract No. F21.

Singh RP, Jha PN (2014) Mitigation of temperature stress by ACC deaminase bacteria isolated from desert region of Rajasthan. National conference on recent trends and future prospects of microbiology and biotechnology. Shri J J T University, March 3rd – 4th 2014. Abstract book OP-07 **(Best oral award).**

Singh RP, Jha PN (2014) Role of ACC deaminase in stress agriculture. National seminar on "Next generation sciences: Vision 2020 & Beyond". Held at M D University, Rohtak, Haryana, March 8th 2014 **(Best oral award).**

Singh RP, Jha PN (2013) Amelioration of salt stress by ACC deaminase bacteria isolated from desert region of Rajasthan. In: International Conference on Advances in Biotechnology and Bioinformatics - 2013 held in Pune, India, Nov. 25th - 27th 2013, Abstract book, PC-150.

Biography of Dr. P. N. Jha

Dr. P. N. Jha is working as Associate Professor in Department of Biological Sciences, Birla Institute of Technology and Sciences Pilani, Pilani campus, Rajsthan. He obtained his master's degree in Biotechnology from Lalit Narayan Mithila University, Darbhanga (Bihar). He completed his Ph.D in the area of Microbial Biotechnology from Banaras Hindu University, Varanasi (U.P.). He did his post-doctoralresearch at National Botanical Research Institute, Lucknow in the area of plant molecular biology. He has been engaged in teaching and research since 2007. He worked at Michigan State University with Frank Dazzo (2013-14) to study microbial ecology of plant growth promoting bacteria in rice using metagenomic approach. His broad area ofresearch interests are: Plant-Microbe interaction and Microbial Ecology. Dr. Jha has complete two research projects on endophytic bacteria, funded by Department of Science andTechnology (DST) and Department of Biotechnology (DBT) as principal investigator and one as Co-PI. He has published 34 research articles in journal of international repute, four chapters in edited books and, editedtwo books. He is currently guiding four students for Ph.D study and guided more than thirty undergradate and post-graduate students for their research studies. One student has already been awarded Ph.D under his supervision.

He is a recipient of Raman post-doctoral fellowship under India-US knowledge initiative 2013 programme (University Grant Commission, New Delhi), DST travel grant to attend international conference at Czeck-Republic, DBT- Postdoctoral fellowship, CSIR-SRF and K.C. Bose Young Scientist award (Gold medal). He was a Visiting Fellow in Department of Botany and Zoology, Institute of Life Sciences, Rajiv Gandhi University, Arunachal Pradesh, India to teach Molecular Biology and Plant Biotechnology to post-graduate student. He has delivered motivational talk to students of Navodaya Vidhyalaya, and NTSE scholars and scientific talks at research and academic institutes.

Biography of Rajnish Prakash Singh

Mr. Rajnish Prakash Singh completed M.Tech (Biotechnology) from VIT University, Vellore, (Chennai, India) and M.Sc (Biochemistry) from Lucknow University, Lucknow (Uttar Pradesh, India). He enrolled in the PhD programme of BITS Pilani, Pilani campus, Rajasthan in January, 2012. During his PhD programme, he received financial assistance from Department of Biotechnology, Ministry of Science and Technology, Govt. of India and research scholar fellowship from BITS Pilani. He qualified the GATE exam in 2008. During his research, he was involved in teaching of various courses of Department of Biological Sciences, BITS Pilani. He was awarded best poster and oral presentation in various national/international conferences. He has published several research articles in renowned international journals.