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Characterization and Optimization of 1-Aminocyclopropane-1-Carboxylate Deaminase (ACCD) Activity in Different Rhizospheric PGPR along with *Microbacterium sp*. Strain ECI-12A

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Abstract

A total of nine strains of plant growth promoting rhizobacteria were analy[illegible] deaminase activity was found in *Klebsiella* sp strain ECI-10A (539.1 nmol α-keto butyrate/ mg [illegible]) and lowest in *Microbacterium* sp strain ECI-12A (122.0 nmol α-keto butyrate/ mg protein/ h). Although *Microbacterium* sp strain [illegible]A showed lowest level of ACC deaminase activity, but, the species of *Microbacterium* isolated from rhizosphere is the first report. *M[illegible]obacterium* sp strain ECI-12A was also analyzed under varying conditions of time, amount of 1-An[illegible]ACC), and temperature for optimization of the ACC deaminase activity. The optimum activity was recorded[illegible]M ACC at 30°C temperature after 24h of culture growth. All the nine strains showed *acdS* gene in the PCR a[illegible] rhizospheric *Microbacterium* species showing ACC deaminase activity have been reported earlier, therefo[illegible]se activity in *Microbacterium* sp ECI-12A isolated from rice rhizosphere is a novel finding.

3-6 key words, separated by semi colon (;)

Key words: ACC deaminase activity; rhizospheric bacteria; *Microbacterium* sp; PGPR.

References to the literature cited for the manuscript should be written as shown here. In case of multiple references, they should be separated by (;) sign

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Introduction

1-Aminocyclopropa[illegible]carboxylate (ACC) deaminase, an enzyme capable [illegible]g ACC, the immediate precursor of ethylene was firstly [illegible]ported in 1978, which was isolated from *Pseudomon*[illegible]in ACP (Honma and Shimomura, 1978). The ACC deamina[illegible]e has been detected in the fungus, *Penicillium citrinum*[illegible]n a number of bacteria (Ma *et al.*, 2003; Blaha *et al.*, 2006; Madhaiyan *et al.*, 2006; Belimov *et al.*, 2007). The gene re[illegible]nsible for ACC deaminase activity (*acdS*) has been recently found in *Azopirillum*., *Burkholderia cepacia* genomovars (which include PGPR, phytopathogens and opportunistic human pathogens), and *Agrobacterium* genomovars (Blaha *et al.*, 2006). These microorganisms were identified by their ability to grow on minimal medium containing ACC as its sole nitrogen source (Honma and Shimomura, 1978; Belimov *et al.*, 2007; Ma *et al.*, 2003).

The plant growth-promoting bacterium, *Pseudomonas putida* GR12-2, which contains the enzyme ACC deaminase, stimulates root elongation (Glick *et al.*, 1994) and significantly reduces the level of ACC in emerging roots and shoots. Three separate mutants of *Pseudomonas putida* GR12-2, deficient in ACC deaminase activity, were reported to lose the ability to promote canola root elongation under gnotobiotic conditions (Glick *et al.*, 1994)**.** The ACC in the exudates may be taken up by the bacteria and subsequently hydrolyzed by the enzyme, ACC deaminase, to ammonia and α-ketobutyrate. The uptake and cleavage of ACC by plant growth-promoting bacteria decrease the amount of ACC outside the plant. Increasing amounts of ACC are exuded by the plant in order to maintain the equilibrium between interna1 and external ACC levels. It is proposed that plant growth-promoting bacteria that possess the enzyme ACC deaminase and are bound to seeds or roots of seedlings can reduce the amount of plant ethylene and thus cause inhibition of root elongation. Thus, these plants should have longer roots and possibly longer shoots as well, in as much as stem elongation, except in flooding resistant plants, is also inhibited by ethylene (Abeles *et al.*, 1992). Soil bacteria that have ACC deaminase activity should then have a selective advantage over other soi1 bacteria in situations in which the main bacterial nutrients are from exudates of plants (Shah *et al.*, 1998). It should also be borne in mind that soil bacteria may acquire ACC deaminase genes by mechanisms other than fortuitous mutation-transfer of such a gene from another soil bacterium is another possible mechanism. The regulation of ethylene production in plants, especially to prevent increased ethylene production and accumulation, may reduce many of the inhibitory effects of this hormone (Jacobson *et al.*, l994). Many agricultural and horticultural crops are particularly sensitive to ethylene levels which regulate fruit ripening and control the deleterious effects of senescence in vegetables and flowers (Sisler and Serek, 1997). The bacterial enzyme, ACC deaminase, is potentially a valuable tool for controlling the levels, and hence the effects of ethylene in plants. ACC deaminase

has already been used to substantially reduce ethylene levels in transgenic tomato plants which have exhibited a prolonged ripening phase (Klee *et al.*, 1991) and to lower stress ethylene levels following infection by bacterial and fungal pathogens (Lund *et al.*, 1998). Strains of plant growth-promoting bacteria that contain ACC deaminase are known to reduce ACC, and hence ethylene, levels in canola seedlings (Penrose and Glick, 2003), promote root elongation in a variety of plants (Hall *et al.*, 1996), decrease the deleterious effects of flooding on tomato plants, and prolong the shelf-life of ethylene sensitive cut flowers (Klee *et al.*, 1991). Therefore, plant growth promoting bacteria containing ACC deaminase activity can be utilized for the improvement of crop yields. Based on our knowledge, there is no any report of *Microbacterium* having ACC deaminase activity present in the rhizosphere of plants, whereas, only a few endophytes has been reported for this activity. The objective of the present study was to evaluate the activity of the ACC deaminase enzyme in rhizospheric plant growth promoting rhizobacteria with special reference to *Microbacterium* in the various conditions. In addition to above objective, we attempted to find out the presence of *acdS* gene in all the strains used in this study.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains and their culture conditions of rhizospheric plant growth promoting bacteria showing nitrogen fixation, IAA production, siderophore production and P-solubilization properties were previously reported (Shrivastava, 2012). With an objective to optimize of various growth conditions for the estimation of ACC deaminase activity and amplification of *acdS* gene, nine most efficient strains isolated from rice rhizosphere of Indo-Nepal border region (1 *Microbacterium* sp strain ECI-12A; 3 *Klebsiella* sp strains ECI-10A, AF-4C and BN-4A; 2 *Agrobacterium* sp strains AF-1D and BN-2A; 2 *Pseudomonas* sp strains AF-4B and PN-4D and 1 *Serratia* sp strain AF-5A) were taken.

Estimation of 1-Aminocyclopropane-1-Carboxylate Deaminase (ACC Deaminase) Activity

For the measurement of ACC deaminase activity, selected isolates were grown overnight in 10 mL of NB medium and thereafter harvested by centrifugation. The pellet was washed with normal saline and suspended in 7.5 mL of JNFb$^-$ medium containing 5 mM of 1-aminocyclopropane-1-carboxylate (ACC). Tubes were incubated at 28^{o}C with shaking (120 rpm) for growth. ACC served as the sole source of nitrogen in the medium. After 24h of growth, cells were centrifuged at 8000 rpm at 4^{o}C for 10 min. The pellet was suspended in 1 mL of 0.1M Tris-HCl (pH 7.6) and again centrifuged at 15000 rpm for 15 min. Pellet was collected and supernatant discarded. The pellet was re-suspended in 600 µL of 0.1 M Tris-HCl (pH 8.5). 30 µL of toluene was added to the cell suspension and vortexed at higher setting for 30 s. Tube was kept at 4^{o}C for 1h and then centrifuged at 1200 rpm for 10 min at 4^{o}C. The thin layer of toluene was aspirated by micro-pipette gently. Now, the toluenized cells were equally distributed in two eppendroff tubes. First part was stored at 4^{o}C for protein assay and other part was used for ACC deaminase assay immediately. 200 µL of toluenized cells was transferred in a fresh 1.5 mL microcentrifuge tube and 20 µL of 0.5 M ACC was added to the suspension. It was briefly vortexed and incubated at 30^{o}C for 15 min. 1.0 mL of 0.56 M HCl was added, vortexed and centrifuged for 10 min at 12000 rpm. Now, 1mL of the supernatant was taken in another tube and 800 µL of 0.56 M HCl was added and vortexed briefly. Thereafter 300 µL of 2,4, dinitrophenylhydrazine (2 % w/v) was added to the tube. It was mixed properly by vortexing and incubated at 30^{o}C for 30 min. 2µL of 2M NaOH was added and after mixing absorbance was recorded at 540 nm. The amount of µmol of α-ketobutyrate produced by this reaction was determined and compared with a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 µmol. For the purpose of standard curve generation a sock solution of 100 mM α-ketobutyrate (Sigma-Aldrich Co., USA) was prepared in 0.1M Tris-HCl pH 8.5 and stored at 4^{o}C. Enzyme activity was expressed as µ mol/mg protein/h.

Amplification of acdS gene

The primers 5'- GGCAAGGTCGACATCTATGC-3' and 5'-GGCTTGCCATTCAGCTATG-3' (Duan et al., 2009) were used to amplify acdS gene. The thermal profile for amplification was 2-min initial denaturation at 94^{o}C, 35 cycles of 1-min denaturation at 92^{o}C, 50-s primer annealing at 58^{o}C, and 1 min of elongation at 72^{o}C. The amplified products were visualised with ethidium bromide stained agarose gel electrophoresis.

Result and Discussions

Estimation of ACCD activity in selected isolates

ACCD activity is indirectly responsible for growth promotion in plants; therefore, its activity was measured. Out of nine isolates tested highest activity was found in *Klebsiella* sp strain ECI-10A followed by *Pseudomonas* sp strain AF-4B. All the isolates showed activity in the range of 122 – 539.1 nmol α-ketobutyrate/mg protein /h (Table 1).

Time course assay of ACCD activity

Since all the isolates tested showed appreciable level of ACCD activity, it was desirable to test time course appearance of activity. Accordingly the *Microbacterium* sp strain ECI-12A was incubated in JNFb$^-$ medium containing 5mM ACC and ACCD activity was measured at desired time intervals (0, 4, 8, 12, 24 and 48h). It is evident from the data of Fig.1 that there was presence of some basal activity at 0 h which increased with time of incubation. Activity showed linear increase from 8 h, the maximum level was attained at 24 h (316.0 nmol α-keto butyrate/mg protein/h). Beyond 24 h there was no significant increase; it was almost constant after 24 h (Fig. 1)

Do varying concentrations of ACC affect activity?

Effect of varying concentrations of ACC was tested so as to find out the optimal concentration required for the ACCD activity. It is evident from the result of Fig. 2 that there was negligible activity without ACC addition to culture, but activity started appearing with the addition of as low as 0.1mM ACC in the culture medium. The level of enzyme activity increased with the increasing concentrations of ACC and maximum increase was attained with 5mM ACC. Further increase in ACC concentration did not show any increase in activity (data not shown).

Effect of varying temperature on ACCD activity

Since temperature plays important role in the regulation of plant growth promoting features of any bacteria, it was desirable to test ACCD activity at varying temperature. Keeping this objective in mind, ACCD activity at varying temperature was tested in *Microbacterium* sp strain ECI-12A. It is evident from the data of Fig. 3 that maximum ACCD activity was observed at 30ºC and thereafter there was decrease. Only 33.2% activity was left at 35ºC (Fig. 3).

Amplification of acdS gene

The PCR amplification of *acdS* gene responsible for ACCD activity was done in all the nine isolates. It is evident from the gel photograph (Fig. 4) that all the isolates showed amplicon of *acdS* gene (~ 1.0 kb).

Amelioration of salt stress has been reported in canola (*Brassica napus* L.) growth by the ACCD containing fluorescent pseudomonads (Jalili *et al.*, 2009). Prevalence of ACC deaminase activity in various PGPR including *Enteobacter cloacae* (Belimov *et al.*, 2005), *Pseudomonas* sp ACP (Sheehy *et al.*, 1991), *Serratia quinivorans* SUD165 (Belimov *et al.*, 2005), *Pseudomonas putida* strain UW4 (Shah *et al.*, 1998) and *Klebsiella pneumonie* strain Kp 342 (Iniguez *et al.*, 2005) as well as certain yeast and fungi (Yao *et al.*, 1995) has been reported. Results of the present finding are in agreement with above reports since isolates reported here in also belong to the genus *Klebsiella* sp (AF-4C and BN-4A), *Pseudomonas* sp (AF-4B and PN-4D), *Serratia* sp (AF-5A) and *Agrobacterium* sp (AF-1D and BN-2A). However presence of ACC deaminase activity in the *Microbacterium* species has not been reported from rhizosphere of any plant except a few reports from endophytic bacteria. Endophytic *Microbacterium* sp G16 isolated from rape (*Brassica napus*) roots showed ACC deaminase activity (Sheng *et al.*, 2009), whereas ACC deaminase activity showing *Microbacterium* sp ECI-12A isolated from rice rhizosphere is a first report. Time course study of the activity suggests that the presence of ACC in the medium is prerequisite for optimal activity of the enzyme. ACC deaminase activity plays vital role in maintaining ethylene level in any plants. ACC released exudates by roots of plants may be utilized as nitrogen source by root associated bacteria if they possess ACC deaminase enzyme. It has been reported that plants treated with ACC deaminase containing bacteria have longer roots and can show resistance to inhibitory effects of ethylene stress on plant growth. Several stresses such as flooding, heavy metals, salinity as well as pathogens are known to induce ethylene stress. Under such stresses PGPR possessing ACC deaminase activity would be useful to counteract the inhibitory effects imposed by ethylene. Furthermore, results of this study clearly show that all the nine isolates could be exploited to manage the ethylene stress if they establish colonization in root region of any plants.

Acknowledgement

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References

Abeles FB, Morgan PW and Saltveit ME (1992) Ethylene in Plant Biology. Academic press, San Diego.

Belimov AA, Dodd IC, Safronova VI, Hontzeas N and Davies WJ (2007) *Pseudomonas brassicacearum* strain AM3 containing 1-aminocyclopropane-1-carboxylate deaminase can show both pathogenic and growth-promoting properties in its interaction with tomato. *J Exp Bot* **58**:1485-1495. DOI: 10.1093/jxb/erm010

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S and Glick BR (2005) Cadmium-tolerant plant growth-

promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). *Soil Biol Biochem* **37**:241-250. DOI: 10.1016/j.soilbio.2004.07.033

Blaha D, Prigent-Combaret C, Mirza MS and 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 Microbiol Ecol* **56**: 455-470. DOI: 10.1111/j.1574-6941.2006.00082.x

Duan J, Muller KM, Charles TC, Vesely S and Glick BR (2009) 1-aminocyclopropane-1-carboxylic acid deaminase genes in Rhizobia from southeren Saskatchewan. *Microb Ecol* **57**: 423-436. DOI: 10.1007/s00248-009-9493-0

Fallik E, Okon Y, Epstein E, Goldman A and Fischer M (1989) Identification and quantification of IAA and IBA in *Azospirillum-brasilense*-inoculated maize roots. *Soil Biol Biochem* **21:** 147-153. DOI: 10.1016/0038-0717(89)90024-2

Glick BR, Jacobson CB, Schwarze MMK and Pasternak JJ (1994) 1-aminocyclopropane-1-carboxylic acid deaminase mutants of the plant-growth promoting rhizobacterium *Pseudomonas putida* GR12-2 do not stimulate canola root elongation. *Can J Microbiol* **40:** 911-915. DOI: 10.1139/m94-146

Glick BR, Liu CP, Ghosh S and Dumbroff EB (1997) Early development of canola seedlings in the presence of the plant growth-promoting rhizobacterium *Pseudomonas putida* GR12-2. *Soil Biol Biochem* **29**: 1233-1239. DOI: 10.1016/S0038-0717(97)00026-6

Hall JA, Peirson D, Ghosh S and Glick BR (1996) Root elongation in various agronomic crops by the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2. *Israel J Plant Sci* **44:** 37-42. DOI: 10.1080/07929978.1996.10676631

Honma M and Shimomura
10.1271/bbb1961.4

Iniguez AL, Dong YM, Carte
by plant defenses. *M*

Jacobson CB, Pasternak JJ a
from the plant-grow
162

Jalili F, Khavazi K, Pazira E,
producing fluoresce
674. DOI: 10.1016/

Klee HJ, Hayford MB, Kretzr
transgenic tomato p

Lund ST, Stall RE and Klee
DOI: 10.1105/tpc.1

Ma WB, Sebestianova SB, Se
in *Rhizobium* spp. *A*

Madhaiyan M, Poonguzhali S
1-carboxylate deam

Patten CL and Glick BR (199

Penrose DM and Glick BR (2
Physiologia Plantar

Shah S, Li JP, Moffatt BA a
promoting rhizobacteria. *Can J Microbiol* 44: 833-843. DOI: 10.1139/w98-074

Sheehy RE, Honma M, Yamada M, Sasaki T, [illegible] (1991) Isolation, sequence, and expression in *Escherichia-coli* of the *Pseudomonas* sp strain ACP gene [illegible] clopropane-1-carboxylate deaminase. *J Bacteriol* **173**: 5260-5265.

Sheng XF, He LY, Zhou L, and Shen YY [illegible] terization of *Microbacterium* sp. F10a and its role in polycyclic aromatic hydrocarbon removal in low-temperatur [illegible] *Microbiol* **55**: 529–535. DOI: 10.1139/W09-005

Shrivastava UP (2012) Molecula [illegible] dy of Rice Plant Rhizobacteria of Indo-Nepal Border. LAP LAMBERT Academic Publishing GmbH & Co. KG, Heinrich-Böcking-Str. 6-8, 66121 Saarbrücken, Germany

Sisler EC and Serek M (1997) Inhibitors of ethylene responses in plants at the receptor level: Recent developments. *Physiologia Plantarum* **100:** 577-582. DOI: 10.1111/j.1399-3054.1997.tb03063.x

Xie H, Pasternak JJ and Glick BR (1996) Isolation and characterization of mutants of the plant growth-promoting rhizobacterium *Pseudomonas putida* CR12-2 that overproduce indoleacetic acid. *Curr Microbiol* **32**: 67-71. DOI: 10.1007/s002849900012

Yao M, Horiuchi A, Tanaka I and Honma M (1995) Crystallization of 1-aminocyclopropane-1-carboxylic acid deaminase from yeast. *Protein Peptide Lett* **2**: 305-30.

References should be Times New Roman, font size- 9, Indentation: special hanging -0.5", single spacing, 0 pt before and 6 pt after paragraph. Each reference should be written with hyperlinked DOI (If available) at last underlined & font color automatic.

For Journal Reference

Shrivastava UP and Kumar A (2011) A Simple and Rapid Plate Assay for the Screening of Indole-3-acetic Acid (IAA) Producing Microorganisms. *Int J Appl Biol Pharma Technol* **2**: 120-12

Jha PN and Kumar A (2009) Characterization of novel plant growth promoting endophytic bacterium *Achromobacter xylosoxidans* from wheat plant. *Microbial Ecol* **58**: 179-188.

For Chapter in Book Reference

Kumar A, Rai AK, Shrivastava UP**,** Tyagi MB and Kumar A (2011) Microcystins, a Novel Class of Toxins from Cyanobacteria. *In*: Roy BK and Chandary BR (Eds) *Plant Genome; Conservation, Manipulation and Diversity*. ***Chapter 11***, Narosa, New Delhi, 129-139.

For Book Reference

Benson HJ (1998) Microbiological applications: A laboratory manual in general microbiology. McGraw-Hill College.

For Patent Reference

Aviv H, Friedman D, Bar-Ilan A and Vered M (1996) Submicron emulsions as ocular

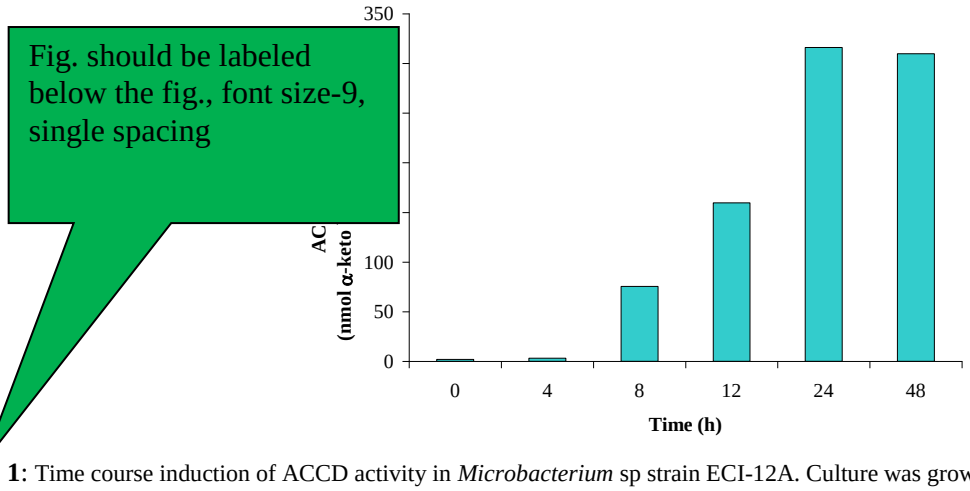


Fig. 1: Time course induction of ACCD activity in *Microbacterium* sp strain ECI-12A. Culture was grown with 5 mM ACC and activity was measured at desired time intervals. Data shown is the average of two independent experiments performed separately in identical condition.

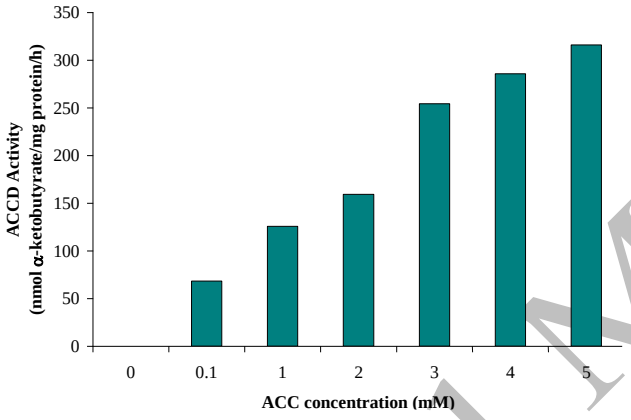


Fig. 2: ACCD activity in *Microbacterium* sp strain ECI-12A in the presence of varying concentrations of ACC. Data shown is the average of two independent experiments performed separately in identical condition.

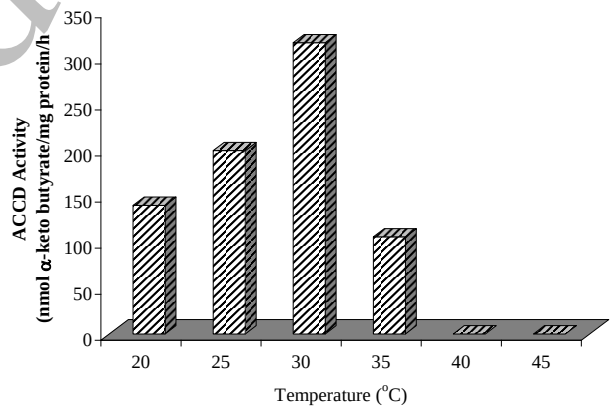
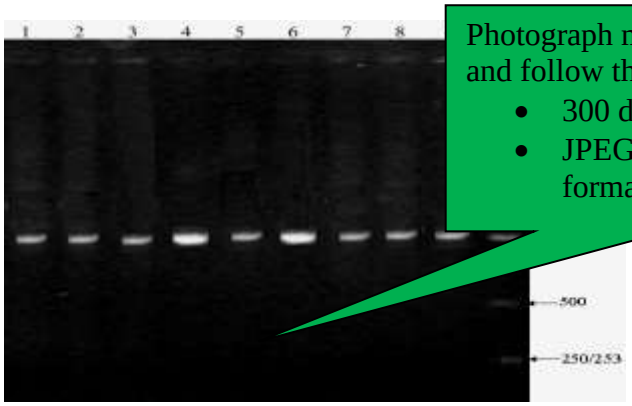


Fig. 3: ACCD activities at varying temperature in the Microbacterium sp strain ECI-12A.



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- 300 dpi or higher size
- JPEG, GIF, TIFF and PDF formats are preferred

Fig. 4: Agarose gel photograph showing acdS gene fragment (1.0 kb) in nine selected isolates. Lane 1 to 9: *acdS* gene fragment of various strains (*Klebsiella* sp strain ECI-10A, *Microbacterium* sp strain ECI-12A, *Agrobacterium* sp strain AF-1D, *Pseudomonas* sp strain AF-4B, *Serratia* sp strain AF-5A, *Klebsiella* sp strain AF-4C, *Pseudomonas* sp strain PN-4D, *Agrobacterium* sp strain BN-2A, and *Klebsiella* sp strain BN-4A); M: 1.0 kb DNA marker.

Table 1: ACC deaminase activity in PGP strains

Bacterial strains	**ACC deaminase activity (nmol α-keto butyrate/ mg protein/ h)**
Klebsiella sp strain ECI-10A	539.1
Microbacterium sp strain ECI-12A	122.0
Agrobacterium sp strain AF-1D	237.3
Pseudomonas sp strain AF-4B	435.2
Klebsiella sp strain AF-4C	171.9
Serratia sp strain AF-5A	305.7
Pseudomonas sp strain PN-4D	358.4
Agrobacterium sp strain BN-2A	316.0
Klebsiella sp strain BN-4A	261.9

Data shown is the average of two independent experiments performed in identical conditions. The induction of ACCD activity was tested with 5mM ACC

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