Jr. of Industrial Pollution Control 21 (2)(2005) pp 361- 370

CHARACTERIZATION OF ACC DEAMINASE IN PLANT GROWTH PROMOTING PSEUDOMONAS FROM TANNERY SLUDGE

G. RENUGA

Department of Biochemistry, Madurai Kamarnj University
Madurai - 625021, India

Key words : Pseudomonas, Acc deaminase.

ABSTRACT

The plant growth promoting strains isolated from tannery sludge and charaeteri/ed is *Pseudomonas strain.* The plant growth promoting *Pseudomonas pittidu* can utilize 1-aniino-cyclopropanc-l-carboxylase (ACC) as a sole nitrogen source because it possess the unusual enzyme ACC dcaminase, which hydroly/es ACC to ammonia and a-ketobutyrate. This enzyme which is though to be intimately involved in the mechanism that the bacterium uses to promote root elongation in developing seedlings, under field study was purified and characterized. ACC deaminase activity is found in the cytoplasm of the bacterium, is induced by low levels (l00nM) of ACC, and has a temperature optimum at approximately 30'c and a pll optimum of 8.5. These properties are very similar to those reported for ACC" deaminase from another soil bacterium *Pseudomonas* strain. In heavily contaminated soil, sludge where the pollutant exceeds the limit of plant tolerance, it may be possible to treat plant with plant growth promoting bacteria, increasing plant growth on polluted soils.

INTRODUCTION

Many of the bacteria found in soil are bound to the surface of soil particles and are found in soil aggregates a number of soil bacteria and interact specifically with the roots of plants. The interaction between bacteria and the roots of plants may be beneficial, harmful or neutral for the plant and sometimes the

effect of a particular bacterium may vary as a consequence of soil conditions (Lynch 1990). Thus for example, a particular organism that facilitates plant growth by fixing nitrogen, which is usually present in the soil in limited amounts, is unlikely to provide benefit to a plant in a setting where exogenous fixed nitrogen is added to soil.

PGPR (Plant growth promoting rhizobacteria) can affect plant growth in two different ways, indirectly or directly. The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effect of one or more phytopathogenic organisms. The direct promotion of plant growth by PGPR for the most part entails either providing the plant with compound that is synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment.

It was recently reported that a small number of soil *Pseudomonas* possess the enzyme 1- aminocyclopropane 1 carboxylase (ACC) deaminasc (Klce *el al.*, 1991) this enzyme hydrolyzes ACC, the immediate biochemical precursor of ethylene in plants, to ammonia and a - ketobutyrate (Honma and Shimomura 1978). The use of the enzyme by Klec *el al.*, (1991) to alter gene expression in transgenic plants carrying a functional ACC deaminase gene by lowering ethylene levels prompted us to question whether plant growth promoting rhizobacteria (PGPR) such as *Pseudomonas putida* (Lifshitz *et al.*, 1987) might also posses ACC deaminase activity and if they did, whether this enzyme was somehow involved in the promotion of plant growth by these PGPR.

In this study, the subcellular localization of the enzymes in the colonizing Pseudomonas has been examined. Consequently, we compared enzyme activities in cells from two sources at different time during the colonization process.

MATERIAL AND METHOD

Bacterial strain

Wild type strains *Pseudomonas putida* and *Pseudomonas fluorescens* are plant growth promoting and disease-suppressing strains were isolated from the sediments of tannery effluent. In the laboratory this strain was stored in sterile 15 % glycerol at - 80°c and grown on solid tryptone - soybean (TSB) medium. Growth in liquid TSB medium with shaking (200-250 rpm on a Lab - line orbital shaker) was at room temperature (22 ± l°c).

Media and culture condition

Psudomonas were also grown on DF salts minimal medium (containing 4g KH_2Po_4, 6g Na_2HPo_4, 0.2g $MgSo_4$, lµg $FeSo_4$, 10µ.g H_3Bo_3, 10µg $MnSo_4$. 70µg $ZnSo_4$, 50µg $CuSo_4$ and 10µg MoO_3 with 0.2% glucose, 0.2% gluconic acid and 0.2% citric acid /1 litre) supplemented with either 2g of $(NH_4)_2SO_4$ or 3mM ACC as a nitrogen source. The ACC solution was filter sterilized before being added to the autoclaved and cooled DF salts minimal medium. All of the bacteria were grown aerobically at room temperature (22 ± 1°c). The growth was monitored by measuring the O.D of the culture at 600nm.

ACC deaminase assay

ACC deaminase activity was determined by monitoring the amount of ammonia generated by the enzymatic hydrolysis of ACC using the protocol of Nagatsu and Yagi (1966). Cell extracts (0.5ml) were incubated at room temperature with 1ml of l0mM ACC. Aliquotes of 0.25ml were periodically withdrawn and assayed chemically for the production of NH_3. The amount of NH_3 in the assayed samples was quantified by $(NH_4)_2SO_4$ and gave a linear response from 5 to 100µg/ml ACC deaminase activity was expressed as the amount of NH_3 produced in units/mg of protein where 1 unit is equivalent to 1nmol/hr.

Optimum Temperature and pH of ACC deaminase activity

The ACC deaminase activity assays were carried out in triplicate as follows in: 0.1M MES (2 - N - Morphotino ethane sulfonic acid) at pH 5.5, 6.0 and 6.5. 0.1M phosphate buffer at pH 6.5, 7.0 and 7.5, 0.1M borate buffer at pH 8.0. 8.5 and 9.0 and l.0M CAPSO (3 - cyclohexylamino - 2 hydroxy - 1 - propane sulfonic acid) at pH9.5, 10.0 and 10.5.

Induction of ACC deaminase activity:

One litre culture of *Pseudomonas pulida* was grown on DF were minimal medium plus $(NH_4)_2SO_4$ for 48hr. The cells were pelleted by centrifugation in a sorval RC 5C centrifuge *at* 8300 Xg for l0min, at room temperature, washed with 500ml of 0.1M phosphate buffer, pll 7.0 and then centrifuged again under the same condition. The cell pellet was resuspended in 500ml of DF salts minimal medium and 90ml was distributed into 5 separate sterile 250ml conical flasks. 10ml of ACC in DF salts minimal medium was added to flask to give final ACC concentration of 1 .mM, l00µM. l0µM, lµM and l00nM. The cell suspensions were then incubated at room temperature on an orbital shaker at 200 - 250 rpm for 24h. The cells were subsequently pelleted by high speed centrifugation and dialyzed cell free extracts were prepared and assayed for ACC deaminase activity.

The time course of ACC deaminase induction in the presence of ImM ACC was monitored by with drawing aliquotes of a *Pseudomonas pulida* suspension at various times after the addition of the ACC, preparing cell free extracts from these aliquotes, and assaying for ACC deaminase activity.

Cell - free extracts

Bacteria were grown for 48h in 500ml of DF salts minimal medium containing either 3 mM ACC or 2g/l $(NH_4)_2SO_4$ at room temperature on an orbital shaker at 200 - 250rpm. The cell were harvested by centrifugation in a Sorval RC 5C centrifuge using a GS - 3 rotor at 8300 X g for 10 min at room temperature. The liquid was discarded and the cell pellet in each tube was resuspended in 100ml of 0.1M phosphate buffer, pH 7.0 and then centrifuged again. The

cell pellet in each tube was resuspended in 10ml of 0.1 M Po4 buffer, pH 7.0. Cell suspension usually 20 - 40 ml maintained at 0°c were disrupted with a sonic 2000 sonicator by using three alternating 60 - 3s periods of sonication (at 140 - 160W) and cooling. The disrupted samples were eetrifuged at 5°c in a Beckman L8 -70 ultra centrifuge for 30 min at approximately 10,000 X g. the supernatant was filtered through a 0.2µm filter (crude enzyme extract) and stored at 4°c.

Cell - free extracts were dialyzed against 1 litre of 0.1 M Po_4 buffer, pH 7.0 with stirring at 4°c for 3-5 with hourly replacement of the buffer. Dialyzed samples were stored for upto 1 week at 4°c until used. There was no measurable decline in enzyme activity under these conditions. Protein concentrations were determined using the procedure of Bradford (1976) with bovine serum albumin as a standard.

Isolation of periplasmic proteins

Pseudomonas putida cells grown at room temperature on DF salts minimal medium containing 3mM ACC were pelleted and transferred to a modified DF salts minimal medium containing (per litre) 0.22g Kcl, 0.25g Nacl, 0.87g Tris, 0.2g $MgSo_4$, 1mg $FeSo_4$, 10µg H_3Bo_3, l0µg $MnSo_4$, 70µg $ZnSo_4$, 50µg $CuSo_4$ and 10µg $MoSO_4$ containing 0.2% glucose, 0.2% gluconic acid, 0.2% citric acid & 3mM ACC. Following a 24h incubation in modified DF salts minimal medium the cells were pelleted, washed with 10ml of l0mM Tris, pH 8.0 and then resuspcnded in 5ml of 25% sucrose in l0mM Tris buffer pH 8.0 and gently mixed for l0min. The suspension was then centrifuged in a Sorval RC 5C centrifuge at 4400 X g in an for 10 min at 4°c. The supernatant was discarded, and 5ml of sterile ice - cold distilled water was added to the pellet and then mixed for l0min. The protein from the periplasm of the suspended cells was isolated as the supernatant after an additional spin at 4400 X g (Taylor *et al.,* 1987). Cytoplasmic proteins were isolated from the pellet following all disruption by sonication using the procedure for preparation of cell free extracts.

Alkaline Phosphatase assay

Alkaline Phosphatase activity was determined using the method of Garcn and Levinthal (1960). Samples (100µl) of either periplasmic or cytoplasmic protein extracts were mixed with 2.0ml of 2.0mg / ml p- nitrophenyl phosphate and incubated at room temperature for Ihr after which the absorbance was read at 410nm. Alkaline Phosphatase activity was expressed as nanomolcs of p-nitrophenol produced per h for each mg of protein.

Glucose — 6 — Phosphate dehydrogenase assay

Glucose - 6 - Phosphate dehydrogenase activity was measured in a coupled assay in which the conversion of NADP to NADPH was monitored at 340nm, (Olive and Levy 1967). Briefly, 2.7ml of 0.1M phosphate buffer pH 7.0, 0.1ml of 6.0mM NADP and 0.lml of 0.1M glucose - 6 - phosphate were mixed in a 3ml quart/ cuvette at room temperature. To this solution was added 0.lml of either periplasmic or cytoplasmic protein extract and the change in the absorbance read at 340nm as a function of time was recorded. The specific activity of Glucose-6-phosphate dehydrogenase was expressed as units/ mg of protein, where 1 unit was calculated by dividing the changes in the absorbance at 340nm / minute owing to the enzymatic conversion of NADP to NAPPH by 6.2.

RESULTS AND DISCUSSION

Growth of Pseudomonas strains on minimal medium

Pseudomonas strains *(Pseudomonas putida* and *Pseudomonas fluorescent)* were tested for the affinity to utilize ACC as a sole nitrogen source. Two strains grow well on DF salts minimal medium with ammonium sulfate can not shown and showed limited growth on DF salts minimal medium with no added nitrogen source (Fig 1). This limited growth may be due to utilization of residual nutrients within the cells and catabolism of some cellular metabolites. The limited growth of *Pseudomonas fluorescens* and *Pseudomonas putida* under these conditions could also be due to the ability of both of these stains fix atmospheric nitrogen (Hong *et al.,* 1991) where as the virtual absence of growth *Pseiidomonas fluorescens* may be because this strain is not diazotrophic. With ACC as the sole source of nitrogen *Pseiidomonas fluorescens* was unable to grow, where as *Pseiidomonas putida* grow well on this DF salts minimal medium. The substantial growth of *Pseudomonas putida* on DF salts minimal medium with ACC suggested that this strain has an enzyme, such as ACC deaminase that can hydrolyze ACC.

ACC deaminase activity of Pseudomonas putida cell free extracts

Cell free extracts *of Pseudomonas putida* grown on DF salts minimal medium plus either ACC or ammonium sulfate were assayed for ACC deaminase activity by incubating the extract with ACC and monitoring the production of ammonia with time. Cell free extracts of *Pseudomonas putida* cells grown on DF salts minimal medium with ACC hydrolyzed an increasing amount of ACC with increasing times of incubation of the extract with the substrate, to produce ammonia (**Fig. 2**). By comparision when dialyzed extracts *of Pseudomonas putida* cells grown on DF salts minimal medium with ammonium sulfate were incubated with ACC only very low levels of ammonia were produced.

Cellular localization of ACC deaminase activity

Table 1. Summarizes the ACC deaminase activity found in the cytoplasmic and periplasmic compartments and compares it with the activity of two marker en/ymes i.e. alkaline phosphatase which was found to be 91.8% localized in the periplasm and glucose - 6 - phosphate dehydrogenase, which was 88.6% localized in the cytoplasm. When cytoplasmic and periplasmic protein extractsof Psudomonas putida cells were assayed for ACC deaminase activity all of the observed activity was found to be associated with the cytoplasmic fraction. In addition AC deaminase a ctivity was never found in the cell growth medium.

Table - 1
Localization of ACC deaminase activity in *Pseudomonas putida.*

Enzyme	Alkaline Phosphatase		Gluconse-6-phosphate dehydrogenase		ACC deaminase	
Cell compartment	% activity	Specific activity	% activity	Specific activity	%activity	Specific activity
Periplasm	92.4	302	10.4	1.5	0	0
Cytoplasm	8.3	26	88.6	10.9	100	20.2

Specific activity is expressed in the follwoing units: nmol, h^{-1} mf $protein^{-1}$ (alkaline phosphatase), nmol, h^{-1}, mg $protein^{-1}$ (glucose- 6- phosphate dehydrogenase) and nmol h^{-1} (ACC deamibase).

The fact that ACC deaminase activity occurred exclusively in the cytoplasmic compartment of the cells is consistent with the absence of a leader peptidc, or any intracellular transport signals proceeding the amino acid sequence for the mature enzyme in the two studies in which the gene for this enzyme from two different *Pseudomonas* was isolated and sequcnced (Klee *et al.,* 1991). This localization results also suggests that if ACC from plant seeds or root is degraded by ACC deaminase from *Pseudomonas putida* then it must first be transported inside the bacterium before it is hydrolyzed.

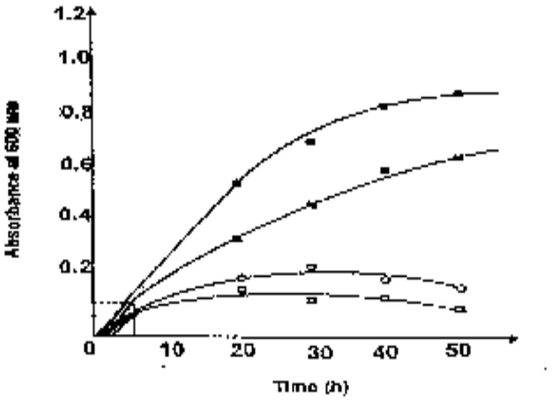
Induction of ACC deaminase activity

In the present study a low level of ACC deaminase activity was routinely found in extracts of cells cultured on DF salts minimal medium with ammonium sulfate, but this activity never exceeded 5% of the total activity observed in extracts from DF salts minimal medium with ACC. This low level of ACC deaminase activity probably represents a basal constitutively expressed enzyme activity. On the other hand, the increase in the amount of ACC deaminase activity in the presence of ACC suggests that ACC deaminase is part of an inducible system. The extent of induction of ACC deaminase activity as a function of the concentration of ACC was examined (Fig. 3).

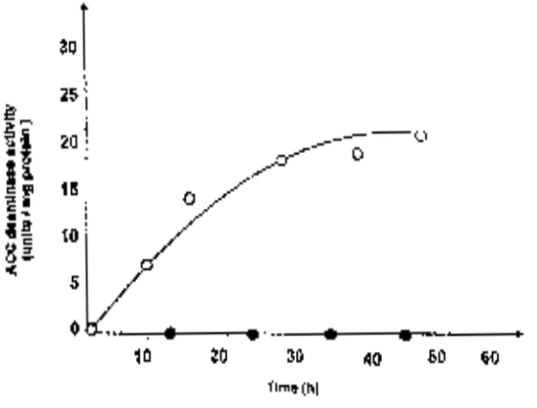
Temperature and pH optima for ACC deaminase activity

The temperature optimum for ACC deaminase activity was determined by incubating aliquotes of dialyzed cell free extracts from *Pseudomonas piilidu* cell grown on DF salts minimal medium plus ACC with ACC at different temperature between 5°c and 60°c and then assaying for the production of ammonia. ACC" deaminase activity increased from a low of 2.7 units mg/ protein at 5°c to 61.2 units/mg protein at 30°c. At temperature above 30°c the specific activity decreased until approximately 53°c was reached at which point no activity was observed (Fig. 4). The enzyme activity profile displayed by cell of *Pseudomonas pulida* which has an optimum of between 25°c and 30°c (Flong *et al.,* 1991)

Cell free extracts of *Pseudomonas putida* cells were grown on DF salts minimal medium plus ACC were assayed for ACC deaminase activity at pH values from 5.5 to 10.5. The results indicated that ACC deaminase activity occurred as a relatively sharp peak with an optimum at pH 8.5 and pKa values of ap-



Growth of *Pseudomonas putida* (●), *Pseudomonas fluorescens* (■) on DF salts medium with either ACC as the sole nitrogen source (○) or no nitrogen source (□).
Fig. 1- Comparative growth patteren of *Pseudomonas species*



Cell growth on DF salts minimal medium and ACC (○) or DF salts minimal medium and ammonium sulfate (●).
Fig. 2- ACC deaminase activity of dialyzed extracts of P. putida.

proximately 7.7 and 9.2 (Fig. 5). The pH optimum for ACC deaminase activity from *Pseudomonas putida* is identical to the value determined by Honma and Shimomura (1978) for ACC deaminase from a *Pseudemonas sp* strain. However, The overall shape of the two pH rate profile differs somewhat (Honma and Shimomura, 1978) found a broader pJI rate profile that was observed in the present study. The difference between their data and ours may reflect either intrinsic difference between in the two enzymes, which were isolated from separate and quite different organisms, or the different levels of purity of the the two enzymes. The pKa values observed for ACC deaminase from *Pseudomonas putida* suggest the possible involvement of histidinc and cysteine residues respectively in the catalytic functioning of the enzyme. Consistent

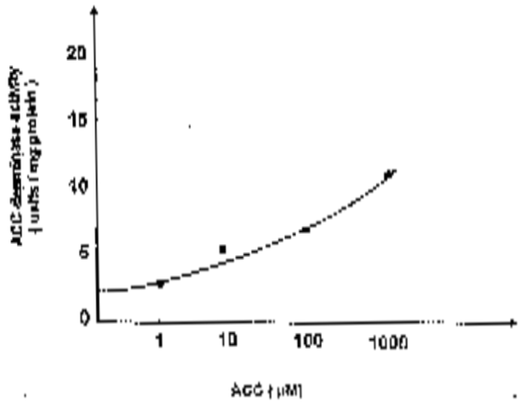


Fig. 3- ACC deaminase activities of Pseudomonas putida.

The dialysed cell extracts that were treated with different concentration of ACC Cells were first grown on DF salts minimal medium plus ammonium sulfate for 48 h and then on DF salts minimal medium plus ACC for 24 h before harvesting.

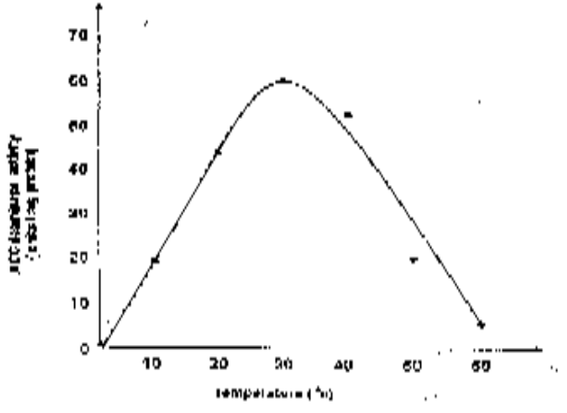


Fig. 4- The temperature- rate profile of P. Putida ACCdeaminase activity.

Dialyzed extracts of P. putida cells grown on DF salts minimal medium plus 3 mM. ACC were assayed for enzyme activity at different tempreature. Each point shown reporestns the mean of four measurements.

with the possible involvement of a cysteine residue in the active site of the enzyme (Honma, 1985) previously reported that the ACC deaminase from *Pseudomonas sp* strain is inhibited by several different sulfhydryl modifiying reagents.

ACC deaminase and plant growth promotion

The ability of *Pseudomonas putida* to hydrolyze ACC may in the soil provide

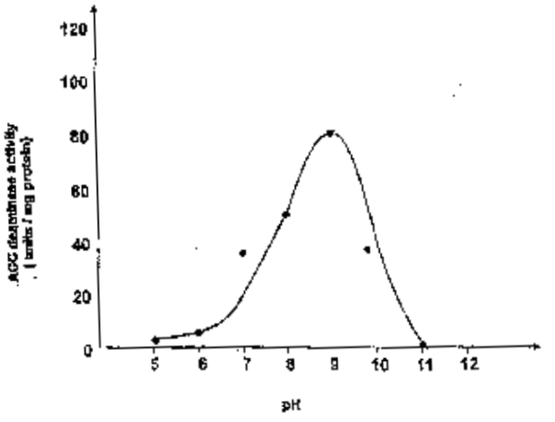


Fig. 5- The pH- rate profile of P. putida ACC deaminase

Dialysed extracts of P. putida cell grown on DF salts minimal medium plus 3mm ACC were incbated at different pH and the enzyme activity was determined . Each point shown represents the mean of six measurements.

it with a competitive advantage over other rhizosphcre micro organisms because it can use ACC as a nitrogen source. The enzymatic breakdown of ACC by *Pseudomonas putida* may provide the bacterium with a novel source of nitrogen and carbon and at the same time effect.

A significant induction in the pool of free ACC and therefore ethylene, within the host plant. This reduction in the ethylene level would then use to stimulate root elongation or, rather to prevent ethylene inhibition of root elongation. In agreement with the postulated for ACC deaminase in the promotion of early root development in tomato seedlings. It has been demonstrated that *Pseudomonas putida* to be an effective plant growth promotion strain binding to seeds prior to their germinatio is sufficient to observe a root elongation effect (Renuga, 1995). In addition, mutants of *Pseudomonas putida* that lacking in ACC deaminasc are no longer able to promote root elongation of canola seedlings (Glick *et al.*, 1994). If this postulated role of ACC deaminasc is correct, then it is likely that this enzyme activity is largely limited to microorganisms that either interact specifically with plants or produce ACC themselves and is not a general property of sludge soil microorganisms.

CONCLUSION

In this study, we have demonstratedthat the genus *Pseudomonas* has ability to metabolize a wide variety of both natural and synthetic organic compounds as sole carbon and energy sources. *Pseudomonas* comprises a vast group of bacteria that are found in a variety of natural environments (soils of polluted water) and in many different associations with plants and animals. Their ecological diversity is a reflection of their simple nutritional requirements and ability to metabolize a wide range of organic compounds. The fluorescent

Pseudomonas including the species *Pseudomonas putida, Pseudomonas jluorescens* constitute a major group among rhizosphere micro organisms. They produce a variety of secondary metabolites, some of which are antibiotics others may act as phytotoxins or as plant growth hormones (Gross and Cody 1985). Beneficial *Pseudomonas* strains have been isolated from tannery sludge and also it has plasmid. *Pseudomonas* play an important role in the growth of plant in the polluted soil

ACKNOWLEDGEMENT

The author is greateful to the Council for Scientific and industrial research, Government of India, New Delhi, for financial assistnace.

REFERENCES

Bradford, M. 1976. A rapid and sensitive method for the quantitation of micro gram quantities of protein utilizing the principle of protein - dye binding. *Anal Biochem*. 72 : 248-258.

Garen, A., and Levinthal, C. 1960. A fine structure genetic and chemical study of the enzyme alkaline phospharate of *E.coli*. 1.Purification and characterization of alkaline phosphatase. *Biochem. Biophys Acta* 38 : 470 - 483.

Click, B.R., Tacobson, C.B., Schwarze, M.M.K. and Pasternak, J. 1994. 1 - amino cyclopropane - 1 - carboxylic acid deaminase mutants of the plant growth promoting rhizobacterium *Pseudomonas putida* do not stimulate canola root elongation *Can J. Microbiol.* 40 : 911 - 915.

Honma, M. 1985. Chemically reactive sulfhydryl groups of 1 - amino cyclopropane - 1 - carboxylic acid deaminase. *Agric Biol Chem.* 49 : 567 - 571.

Honma.M., and Shimomura,T. 1978. Metabolism of 1-animocyclo propane -1-carboxylic acid, *Agri Biol chem.* 42 : 1825-1831.

Hong Y., Pasternak, J.J. and Click, B.R. 1991. Biological consequences of plasmid transformation of the plant growth promoting rhizobacterium *Pseudomonas putida Can J. Microbio.* 37 : 796 - 799.

Klee, H.J., Hayford, M.B., Kretzmer, K.A ., Barry, G.F.. and Kishore, G.M.1991. Control of ethylene synthesis by expression of bacterial enzyme in transgenic tomato plants. *Plants cell*. 3 : 1187 : 1193.

Lifshitz, R., Kloepper, J.W., Scher, P.M., Tipping, E.M. and Laliberte 1987. Growth promotion of canola seedlings by a strain of *Pseudomonas puitda* under genotobiotic conditions. *Can J. Microboil.* 33 : 390-395.

Lynch, J.M. 1990. The rhizosphere : Wiley-interscience, Chichester, England.

Nagatsu, T. and Yagi. K. 1966. A simple assay of monoamine oxidase and D-amino oxidase by measuring ammonia. *J. Biochem.* 60 : 219-221.

Olive, C., and Levy, H.R. 1967. The preparation and some properties of crystalline glucose 6 - phosphate dehydrogenase from leuconostoc mesenteroidcs. *Biochemistry.* 6 : 730 - 736.

Renuga, G. 2005. A plant growth promoting bacterium decreases chromium toxicity in *Lycopericon esculentum. AJMBES* . 7 : 2 (in press)

Taylor, K.A., Crosby, B., Me Gavin, M., Forsberg, C.W. and Thomus, D.Y. 1987. Characteristics of the endoglucanase encoded by a celgen from Bacteroids succinogenes expressed in *E. coli. Appl. Environ. Microbiol*. 53 : 41- 46.