JOURNAL OF PURE AND APPLIED MICROBIOLOGY, April 2009. Vol. **3**(1), p. 143-150

Fingerprinting of Cellulose Degrading Bacteria from Agricultural Soils

Machiavelli[2,3], N. Tejoprakash[2] and Sunil Khanna[1,2]*

[1]Department of Biotechnology and Bioinformatics, NIIT Institute of Information Technology (TNI), Balaji Estate, Kalkaji New Delhi - 110 019, India.
[2]Department of Biotechnology & Environmental Sciences, Thapar University, Patiala - 147 004, India.
[3]Department of Biotechnology Engineering, Ambala College of Engineering and Applied Research, Mithapur, VPO: Sambalakha, Ambala – 133 101, India.

(Received: 10 September 2008; accepted: 25 October 2008)

Bacteria were isolated and screened for cellulase activities in agricultural soil of Patiala (India). Three most efficient cellulose degraders identified using 16S rDNA, as *Serratia sp., Pseudomonas sp.* and *Serratia marcescens* showed cellulase activity of 3.83, 4.21 and 4.52 mM glucose ml^{-1} h^{-1} respectively. . Enterobacteriaceae Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) was performed to obtain fingerprint and showed clear genetic variability among all three isolates. The bacteria were mass cultured and re-inoculated in soils to study the population dynamics under chickpea (*Cicer arietinum L.*) cultivation. The role of cellulose degrading bacteria was studied in sustainability of soil and organic carbon indicating good survival of 40.2, 34.4 and 56.8 % of the introduced strains respectively. The highly positive correlation (R^2=0.916) among the soil cellulase activity and soil organic carbon was observed during the chickpea plot trails towards inoculated bacterial strains.

Key words: Agricultural soils, Chickpea, Cellulase, *Pseudomonas*, *Serratia*, ERIC-PCR.

Intensification of agriculture and increases in population pressure has reduced the structural stability of soils. To cope with low productivity, inorganic fertilizers have been intensively used for the past two decades. This escalated the soil problems including structural degradation, reduction of organic matter and cellulose. Agricultural residues are a rich source of cellulose (Hameeda, 2006). This is the most abundant organic compound in the biosphere, comprising almost 50% of the biomass synthesised by photosynthetic fixation of CO_2 (Eriksson et al., 1990). Cellulose serves the most abundant carbon source for the growth and survival of agriculturally important microorganisms. However, cellulose in plant debris has to be degraded into glucose, cellobiose and high molecular weight oligosaccharides by cellulases enzymes (White, 1982). Cellulases are a group of enzymes that catalyse the degradation of cellulose, polysaccharides build up of α-1, 4 linked glucose units (Deng and Tabatabai, 1994). It has been reported that cellulases in soils are derived mainly from plant debris incorporated into the soil, and that a limited amount may also originate from fungi and bacteria in soils (Richmond, 1991). Studies have shown that activity of cellulases in agricultural soils are affected by several factors

* To whom all correspondence should be addressed.
Tel.: +91-9811605054;
E-mail: skhanna@tni.edu.in

such as temperature, soil pH, water and oxygen contents (abiotic conditions), the chemical structure of organic matter and its location in the soil profile horizon (Rubidge, 1977; Gomah, 1980; Tabatabai, 1982; Klein, 1989; Alf and Nannipieri, 1995), quality of organic matter/plant debris and soil mineral elements (Hope and Burns, 1987; Sinsabaugh and Linkins, 1989;) and the trace elements (Arinze and Yubedee 2000; Atlas *et al.,* 1978). Several mechanisms have been proposed in the degradation of cellulose by cellulases (Rees, 1975; Wood, 1991). All these findings suggest that activities of cellulases can be used to give preliminary indication of some of the physical chemical properties of soil, thus, easing agricultural soil management strategies. Research efforts now focused on discovering new enzymes from microbial diversity in the soil, the most appropriate practices that may positively influence their activities for improved plant growth as well as improving the biological environments in order to sustain other life types (Ndakidemi and Makoi, 2008).

The present study is on the isolation and screening of cellulose degrading bacteria from the agricultural fields of chickpea (*Cicer arietinum L.*) crop, which push us to identify and fingerprint new bacterial strains capable to utilize cellulose from local agricultural soils.

MATERIAL AND METHODS

Soil sampling and analysis

Soil samples used for the isolation of cellulose degrading bacteria were collected from different chickpea (*Cicer arietinum L.*) agricultural fields in four blocks of Patiala District, Punjab. The samples were drawn with a hollow pipe (4 cm in diameter) from 25cm in depth. Air-dried and pulverized soil samples were analyzed for organic carbon, total nitrogen, available phosphorus, moisture level and pH by standard method. (Jackson MH, 1967).

solation of bacterial strains for cellulose degradation

Soil (approximately 10 g) was enriched for cellulose degrading bacteria using carboxymethyl cellulose (CMC) as the only added source of carbon in Bushnell Haas Broth (BHB) medium having composition of magnesium sulphate-0.20 g/L; calcium chloride-0.02 g/L; monopotassium phosphate-1 g/L; di-potassium phosphate-1 g/L; ammonium nitrate-1 g/L; ferric chloride-0.05 g/L. Bacterial strains were then isolated by dilution technique on Bushnell Haas Agar (BHA) plates containing carboxymethyl cellulose (1%; w/v) and incubated at 37 °C for 3 days.

Enzyme assay for screening of bacterial isolates for cellulose degradation

Bacterial isolates were grown in 50ml Bushnell Haas Broth (BHB) with carboxymethyl cellulose (1% ; w/v) in 250-ml flask and incubated on rotary shaker at 37°C, 120 rpm. After 3 days, the cells were centrifuged (9000 xg, 10 min) and supernatant was collected for enzyme assay. Cellulase (CMCase) activity was measured by the DNS (3,5-dinitrosalicylic acid) method (Miller, G.L., 1959), through the determination of the amount of reducing sugars liberated from carboxymethyl cellulose (CMC) solubilised in 50 mM Tris–HCl buffer (pH 7.0) (Baily, M.J., *et al.,* 1992). Enzyme assay was carried out at 37 °C for 1hr and the reaction was stopped by the addition of DNS solution. Samples were then boiled for 10 min, cooled on ice for color stabilization, and the optical density was measured at 540 nm. Cellulase activity was determined by using a calibration curve for glucose and expressed as ìM glucose g^{-1} h^{-1}.

Molecular characterization of cellulose degrading bacterial isolates

Chromosomal DNA was extracted by a modified ROSE (rapid one step extraction) method (Steiner *et al.*, 1995). Bacterial isolates were identified by amplification of 16S rDNA gene sequence with the following primers: Forward primer 5' -AGA GTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGGCGGTGTGTTC -3' (Weisberg et al. 1991). DNA amplification was performed with Genamp PCR system (Applied Biosystem, USA). Reaction mixture for the PCR contained 1X PCR buffer (Invitrogen, USA), each deoxynucleotide triphosphate at a concentation of 200µm, 1.5 mM $MgCl_2$, each primer at a concentration of 0.1 µm and 2.5U of Taq DNA polymerase (Invitrogen, USA) in a final volume of 100 µl. PCR conditions were as follows: Preheating at 92°C for 2min, 36 cycles of 92°C for 1min, 48°C 30sec and 72°C for 2min and final

extension 72°C for 6min. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5µl) in 1.0% agarose gel in 1X TAE buffer. PCR product was cloned into the pDrive-cloning vector and transformed into *E. Coli* DH5á (Hanahan, 1983). 16S rDNA sequence of the bacterial isolates and similar sequences were aligned using the Clustal W and dendrograms were generated by the distance neighbour-joining method using MEGA3 software (Kumar, et al., 2004). Numbers are percent gap values after 500 bootstrap replications. Only values above 75% were considered.

Plot studies

Plot studies were conducted in sandy loam agricultural soil. Chemical composition of this soil was organic carbon, 0.78±0.03 %; total nitrogen, 0.0823±0.005 %; and available phosphorus 44.00±5.6 mg/kg. Randomized design was adopted to study the degradation of cellulose in the agriculture soil by inoculating the most efficient cellulose degraders in plots (2×2m) sown with chickpea (*Cicer arietinum L.*) crop. Cultures were grown in 10 litre shake flasks containing LB medium and incubated at (37°C, 120 rpm). After 36 h, the cells were harvested and washed with 10mM phosphate buffer (pH 6.8). Pellets were resuspended in buffer and inoculated to plots to achieve 2×10^8 cfu/g soil. Control plot received only BHB without any bacterial cells. The prescribed package of practices was followed for growth of the chickpea and monitored regularly. After regular intervals of time during different stages of crop growth, soil samples were collected from five different points, mixed together and then three samples were taken to evaluate the population dynamics and other soil parameters (microbial load, enzymatic activity, organic carbon, total nitrogen, available phosphorus and microelements). Equal amount of soil (1 g/ sample) was resuspended in 9 ml of sterilized saline water (0.8% NaCl; w/v) by vigorous vortexing and serially diluted and an aliquot of 100 ul was plated on Luria agar (LA) containing ampicillin (120 ug/ ml). Plates were incubated for 24–36 h at 37 °C. Individual colonies obtained after this incubation were patched on LA plates containing ampicillin (120 ug/ml). Colonies grown were scored and their DNA was isolated for DNA fingerprinting based on ERIC-PCR.

Development of DNA fingerprints and tracking the fate of introduced population

Enterobacteriaceae Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) was carried out to obtain DNA fingerprints of the cellulose degraders. PCR primer sequences (Versalovic, *et al.,* 1991), ERIC – IR (5' – 3') – ATG TAA GCT CCT GGG GAA TCA C- and ERIC – 2 (5' – 3') - AAG TAA GTG ACT GGG GTG AGC G- were obtained from Life Technologies, USA. Single isolated colonies were picked at random from the LA plates, suspended in 50 µl water, and lysed by heating for 10 min at 95°C. Cell lysate was centrifuged (9000Xg, 3 min) at 4°C, and 2 µl of the supernatant was used in the reaction mixture. The reaction mixture (25 µl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% gelatin (wt/vol), 0.25 mM each of dNTPs, 0.375 µM each of primers ERIC-1R and ERIC-2, and 2.0 units of Taq polymerase (Life Technologies, USA). The amplification was done on Genamp PCR system (Applied Biosystem, USA). The PCR conditions are as follows: initial denaturation at 94°C for 4 min followed by 41 cycles at 94°C for 1 min, at 50°C for 1 min and at 72°C for 2 min. The final extension was done at 72°C for 5 min. The reaction was terminated by using a loading dye (1 µl) containing 15% Ficoll, 0.25% bromophenol blue, and 0.25% xylene cyanol. Each PCR product was resolved by electrophoresis on a 1.5% agarose gel.

Data analysis

Statistical analysis was performed by ANOVA and special effects were tested by the Newman Keuls test with a critical range of 5% using COSTAT software. Data characterized by the same letter were not significantly different.

RESULTS AND DISCUSSION

Isolation and screening of cellulose degrading bacteria

Various bacterial isolates obtained from chickpea (*Cicer arietinum L.*) agricultural soils were screened for cellulase activity. Three isolates *MSK1, MSK13* and *MSK24* based on their high efficacy to degrade carboxy methyl cellulose (CMC) were chosen for further studies and were identified as *Serratia sp., Pseudomonas sp.* and

Serratia marcescens respectively by using 16S rDNA. *Serratia sp., Pseudomonas sp.* and *Serratia marcescens* showed the cellulase activity of 3.83, 4.21 and 4.52 mM glucose ml^{-1} h^{-1} respectively after hrs (Fig: 1). Sindhu et al, (2001) reported both aerobic cellulolytic organisms such as *Pseudomonas* & actinomycetes, facultative anaerobes such as *Bacillus* and *Cellulomonas*, and also strict anaerobes such as *Clostridium* while *Serratia marcescens* EB 67 and *Pseudomonas sp*. CDB 35 have been shown to possess cellulolytic activity in the presence of crop residues (Hameeda *et al,* 2006).

Bacterial strains can be differentiated by genomic DNA fingerprints based on ERIC-PCR gel electrophoresis, as some of the standard strains are shown in Fig. 2 (Lane 11-13). DNA fingerprinting based on the ERIC-PCR (enterobacterial repetitive intergenic consensus) of these isolates showed 10 to 14 amplified bands with sizes ranging from 11,000 bp to 100 bp of varying intensity. Genotypic relationships among microorganisms have been determined by analyzing the genomic DNA with PCR-based methods (Pooler, 1996). In the present study, unique ERIC PCR genotypic fingerprints of different strains isolated from agricultural soil samples were standardized and used for the enumeration of the introduced cellulolytic bacterial isolates. ERIC-PCR has been used successfully to generate DNA fingerprints to distinguish between genetically unrelated isolates and closely related bacterial strains (Dombek, 2000). Although 16S rDNA sequence analysis showed that *MSK1* and *MSK24* to have close homology with *Serratia sp.*, but ERIC-PCR based fingerprinting analysis enabled us to differentiate between both the isolates.

Survival of bacterial strains inoculated in plot trails

The cellulolytic bacterial *Serratia sp., Pseudomonas sp.* and *Serratia marcescens* were resistant to ampicillin, thus ampicillin was selected as marker for population survival along with ERIC-

Table 1. Survival of introduced population during the growth of chickpea crop in plots. Soil suspension diluted in physiological saline was plated on LA-ampicillin plate and genomic DNA of the ampicillin resistant bacteria was amplified by ERIC-PCR

Time (weeks)	Colony forming units (X 10^7cfu/g of dry wt. soil)	0	4	8	12	16
Control	Ampicillin count	2.2	1.8	1.5	1.7	1.3
	PCR count	0	0	0	0	0
Serratia sp.	Ampicillin count	15.0	13.0	10.0	8.5	8.0
	PCR count	11.7	10.4	7.2	5.8	4.7
Pseudomonas sp.	Ampicillin count	13.0	12.0	8.8	7.0	6.0
	PCR count	10.2	9.9	5.6	5.1	3.5
Serratia marcescens	Ampicillin count	15.0	14.0	11.5	10.5	9.0
	PCR count	13.4	12.0	9.3	8.7	7.6

Table 2. Cellulase activity and organic carbon in soils of chickpea plot. Cellulase activity and organic carbon were determined at 0 week (Chickpea sowing stage) and 16 weeks (Chickpea harvest stage) in soils

Treatmentplot	Cellulase activity (uM Glucose / g dry wt soil/ hr))		Organic carbon(%)	
	0 Week	16 Week	0 Week	16 Week
Control	0.784±0.048b	4.489±0.322d	0.340±0.030a	0.554±0.016c
Serratia sp.	1.404±0.102a	7.518±0.392c	0.388±0.030a	0.662±0.019b
Pseudomonas sp.	1.287±0.013a	9.026±0.233b	0.360±0.036a	0.711±0.041ab
Serratia marcescens	1.279±0.080a	10.251±0.391a	0.368±0.028a	0.836±0.045a

Tested by the Student Newman Keuls test with a critical range of 5%. Data characterized by the same letter are not significantly different in the rows. Above table represents Mean± Standard error of mean (SEM).

PCR method. In plot studies, ampicillin resistance plate count of *Serratia sp., Pseudomonas sp.* and *Serratia marcescens* showed 15, 13 and15 (x10^7cfu g dry wt. $soil^{-1}$) respectively at the time of inoculation , The population decreased gradually to 10, 8.8 and 11.5 (x10^7cfu g dry wt. $soil^{-1}$) during 8^{th} week or midtime of crop growth for *Serratia sp., Pseudomonas sp.* and *Serratia marcescens* respectively which further decreased to 8.0, 6.0 and 9.0 (x10^7cfu g dry wt. $soil^{-1}$) at the time of crop harvest (Table 1). Microbial population based on LA + ampicillin plates in control plot decreased

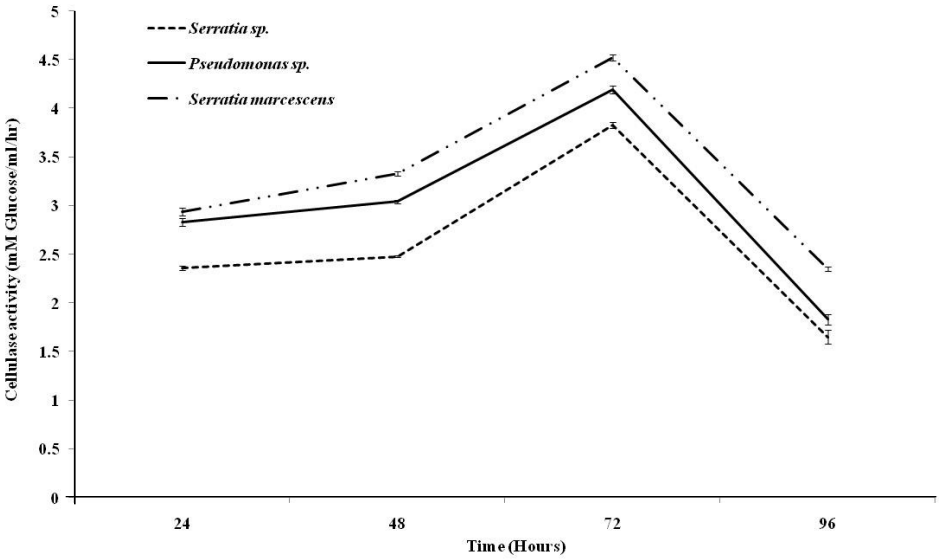

Fig. 1. Cellulase (CMCase) activity as a function of time of the three selected bacterial isolates (*Serratia sp., Pseudomonas sp.* and *Serratia marcescens*) in 1%CMC in BHB media by incubation at 37 for 120rpm.

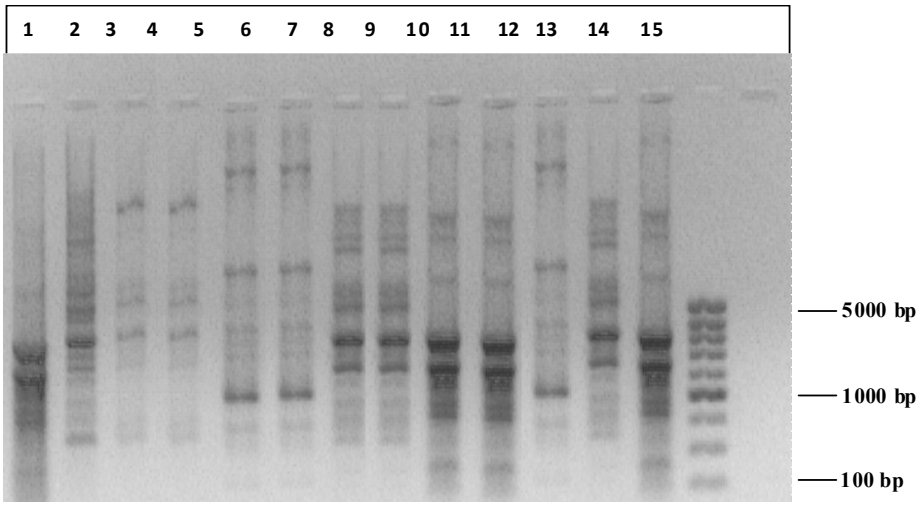

Fig. 2. ERIC-PCR fingerprint of unknown bacterial isolates on 1.5% agarose gel. Bacterial colonies were isolated from soil in the plots. Pure colonies were picked, heat lysed, and whole genomic DNA was subjected to ERIC-PCR to get the fingerprint. Lanes 1-10: fingerprints of different isolates obtained from selected plates containing 120ug/ml of Ampicillin, Lane 11: Standard bacterial strain of *Pseudomonas sp.*, Lane 12: Standard bacterial strain of *Serratia sp.*, Lane 13: Standard bacterial strain of *Serratia marcescens*, Lane 14: 500bp ladder and Lane 15: Negative Control.

from 2.2 to 1.3 (x10^7cfu g dry wt. $soil^{-1}$) after 16 week of chickpea harvest. Population dynamic studies based on ERIC-PCR showed that population of *Serratia sp., Pseudomonas sp.* and *Serratia marcescens* were 11.7, 10.2 and 13.4 (x10^7cfu g dry wt. $soil^{-1}$) at the time of inoculation during germinating or 2-3 leaflet stage of chickpea, and then decreased to 4.7, 3.5 and 7.6 (x10^7cfu g dry wt. $soil^{-1}$) at the end of 16th week. (Table.1) thus showing a survival of 40.2, 34.4 and 56.8 % respectively after 16 weeks (Table. 1). *Serratia sp., Pseudomonas sp.*

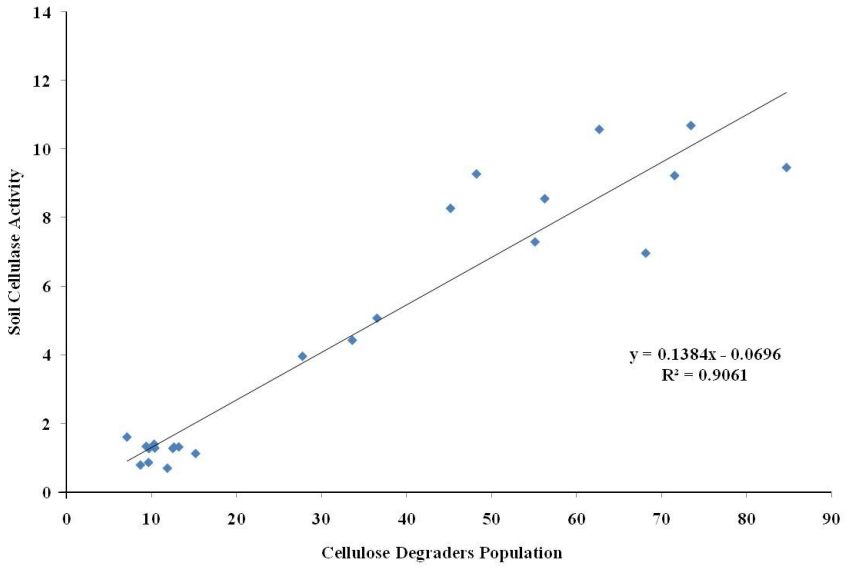

Fig. 3. Correlation between the cellulase activity & cellulose degraders population in soil during the chickpea plot experiments.

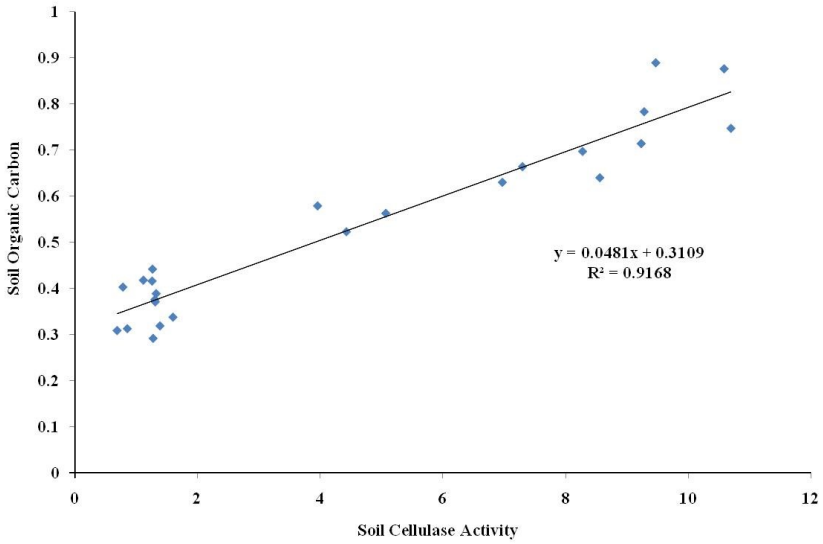

Fig. 4. Correlation between the cellulase activity & organic carbon in soil during the chickpea plot experiments.

and *Serratia marcescens* could not be detected in the soil at zero time (before application of the bacterial inoculums) and in control as well as test plots. These results clearly demonstrated that enumeration of the introduced cellulolytic bacterial isolates by antibiotic resistance method always shoed higher level of survival as compared to that by ERIC-PCR. Since ERIC-PCR enumerates the population of the introduced bacterial isolates without any interference from other bacteria, this method presents a very accurate estimation of the survival of the introduced bacteria.

Cellulase enzymes play a major role in degradation of carbohydrates in soils and the hydrolysis by these enzymes are believed to be important energy sources for the growth of soil microorganisms (Deng, 1996). The soil cellulase activity as well as organic carbon in the experimental plots was also observed and it found to be enhanced by the introduction of the inoculants in the plots in comparison to the control plot (Table.2). Soil cellulase activity in control plot increased from 0.784 (at the time of inoculation) to 4.489 µM glucose g dry wt. $soil^{-1}$ h^{-1}, 1.404 to 7.518 µM glucose g dry wt. $soil^{-1}$ h^{-1} in the *Serratia sp.*inoculated plot, 1.287 to 9.026 µM glucose g dry wt. $soil^{-1}$ h^{-1} in the *Pseudomonas sp.* inoculated plot and 1.279 to 10.251 µM glucose g dry wt. $soil^{-1}$ h^{-1} in the *Serratia marcescens* inoculated plot. Organic carbon also increased from 0.340 to 0.554 % in the control plot, 0.388 to 0.602 % in the *Serratia sp.* plot, 0.360 to 0.711% in the *Pseudomonas sp.* plot and 0.368 to 0.836% in the *Serratia marcescens* plot.

During plot experiments, a significantly high value of soil cellulase activity as well as organic carbon was shown in *Serratia marcescens* inoculated plots as compared to the plots inoculated with the other two isolates after 16 weeks of crop harvest (Table 2). Mass inoculations of bacterial culture in the plots resulted in a significantly enhancement of cellulose degraders population which could be positively correlated (R^2=0.906) to the higher soil cellulase activity (Fig.3). The overall scenario in the experiment results showed an inclined organic carbon in the treated plots (Table 2), which was observed as a positive correlation (R^2=0.916) among the soil cellulase activity and soil organic carbon during the chickpea plot trails (Fig. 4).

Since cellulases enzymes play an important role in global recycling of the most abundant polymer, cellulose in nature, it would be of critical importance to understand this enzyme as a predictive tool in soil fertility programmes. Understanding the dynamics of microbial populations and enzyme activities in these systems is crucial for predicting their interactions as their activities may, in turn, regulate nutrient uptake and plant growth. These bacterial populations or enzymes may have significant effects on soil biology, environmental management, growth and nutrient uptake in plants growing in ecosystems. Their activities may, however, be influenced by unknown cultural management practices.

ACKNOWLEDGMENTS

We thank the UNDP-TIFAC, Govt. of India for financial support and The Director, Thapar University, Patiala-Punjab for providing infrastructure as well as The Coordinator, TIFAC-CORE, for constant lab support and necessary facilities. We are also grateful to Dr. S. Rana & Dr. S. Chauhan for arranging plot trails during the present study.

REFERENCES

1. Alf, K., Nannipieri, P. Enzyme Activities, In: *Methods in Applied Soil Microbiology and Biochemistry* (Alf, K., Nannipieri, P. eds.). London: Academic Press, 1995; 311-373.
2. Arinze, A.E., Yubedee, A.G. Effect of fungicides on *Fusarium grainrot* and enzyme production in maize (*Zea mays* L.). *Glob. J. Appl. Sci.*, 2000; **6**(4): 629-634.
3. Atlas, R.M., Pramer, D., Bartha, R. Assessment of pesticide effects on non-target soil microorganisms. *Soil Biol. Biochem*., 1978; **10**: 231-239.
4. Hameeda, B., Reddy, H.K.Y., Rupela, O.P., Kumar, G.N., Reddy, G. Effect of Carbon Substrates on Rock Phosphate Solubilization by Bacteria from Composts and Macrofauna. *Current Microbiology*. 2006; **53**: 298–302.
5. Bailey, M.J., Biely, P., Poutanen, K. Inter-laboratory testing of methods for assay of xylanase activity. *J. Biotech.* 1992; **23**: 257-270.
6. Deng, S.P., Tabatabai, M.A. Cellulase activity

of soils. *Soil Biol. Biochem*. 1994; **26**: 1347-1354.

7. Deng, S.P., Tabatabai, M.A. Effect of tillage and residue management on enzyme activities in soils: II. Glycosidases. *Biol. Fertil. Soils* 1996; **22**: 208-213.
8. Dombek, P.E. , Johnson, L.A.K., Zimmerley, S.T.,Sadowsky, M.J. Use of repetitive DNA sequences and PCR to differentiate *Escherichia coli* isolates from human and animal sources. *Appl. Environ. Microbiol.* 2000; **66**: 2572-2577.
9. Eriksson, K.E.L., Blancbette, R.A., Ander, P. Biodegradation of cellulose. In: *Microbial and Enzymatic Degradation of Wood and Wood Components*, (Eriksson KEL, Blanchette RA, Ander P eds). New York Springer-Verlag, 1990; 89-180.
10. Gomah, A.M. CM-Cellulase activity in soil as affected by addition of organic material, temperature, storage and drying and wetting cycles. *Zeitschrift fuer Pflanzenernaehrung und Bodenkunde* 1980; **143**: 349-356.
11. Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 1983; **166**: 557-580.
12. Hope, C.F.A., Burns, R.G. Activity, origins and location of cellulases in a silt loam soil. *Biol. Fert. Soils*. 1987; **5**: 164-170.
13. Jackson, M.H. Soil chemical analysis: Prentice-Hall of India, New Delhi, India. 1967.
14. Jensen, M.A., Webster, J.A., Straus, N. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphism. *Appl. Environ. Microbiol.* 1993; **59**: 945–952.
15. Klein, D.A. Cellulose functions in arid soil development. *Arid. Soil. Res. Rehab*. 1989; **3**: 185-198.
16. Kumar, S., Tamura, K., Nei, M. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief. Bioinfo*. 2004; **5**: 150–163.
17. Miller, G.L. Use of dinitrosalycilic acid reagent for determination of reducing sugar. *Anal. Chem*. 1959; **31**: 426-428.
18. Ndakidemi, P.A., Makoi, J.R. Selected soil enzymes: Examples of their potential roles in the ecosystem. *Afr. J. Biotechnol*. 2008; **7**(3): 181-191.
19. Norman, P. A molecular view of microbial diversity and the biosphere. *Science* 1997; **276**: 734–740.
20. Pooler, M.R., Ritchie, D.F. Hartung, J.S. Genetic relationships among strains of *Xanthomonas fragariae* based on random amplified polymorphic DNA PCR, repetitive extragenic palindromic PCR and enterobacterial repetitive intergenic consensus PCR data and generation of multiplexed PCR primers useful for identification of this phytopathogen. *Appl. Environ. Microbiol.* 1996; **62**: 3121–3127.
21. Reese, E.T. Polysaccharides and the hydrolysis of insoluble substrates. In: *Biological Transformation of Wood by Microorganisms* (Leise W. Ed.). New York Springer-Verlag, 1975; 165-181.
22. Richmond, P.A. Occurrence and functions of native cellulose. In: *Biosynthesis and Biodegradation of Cellulose* (Haigler CH and Weimer PJ eds). New York: Marcel Dekker, 1991; 5-23.
23. Rubidge, T. The effect of moisture content and incubation temperature upon the potential cellulase activity of John Innes no. 1 soil. *Int. Biodeterior. Bul*. 1977; **13**: 39-44.
24. Sindhu, S.S., Dadarwal, K.R., Chitinolytic and cellulolytic *Pseudomonas sp*. antagonistic to fungal pathogens enhances nodulation by *Mesorhizobium sp*. *Cicer* in chickpea. *Microbiol. Res*. 2001; **156**: 353–358.
25. Sinsabaugh, R.L., Linkins, A.E. Natural disturbance and the activity of *Trichoderma viride* cellulase complex. *Soil. Biol. Biochem*. 1989; **21**: 835-839.
26. Steiner, J.J., Poklemba, C.J., Fjellstrom, R.G., Elliott, L.F. A rapid one tube genomic DNA extraction process for PCR and RAPD analyses. *Nuc. Acids. Res.* 1995; **23**: 2569-2570.
27. Tabatabai, M.A. Soil enzyme In: *Methods of Soil Analysis, Part 2* (Page AL Ed.). Wisconsin: Am. Soc. Agron. Madison, 1982; pp 903-948.
28. Versalovic, J., Koeuth, T., Lupski, J.R. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic. Acids. Res*. 1991; **19**: 6823-6831.
29. Weisberg, W.A., Barns, S.M., Pelletier, D.A., Lane, D.J. 16S rDNA amplification for phylogenetic study. *J. Bacteriol.* 1991; **173**: 697-703.
30. White, A.R. Visualization of cellulases and cellulose degradation. In: *Cellulose and Other Natural Polymer Systems: Biogenesis, Structure, and Degradation* (Brown RM Jr, ed.). Plenum Press, New York. 1982; 489-509.
31. Wood, T.M. Fungal cellulases. In: *Biosynthesis and Biodegradation of Cellulose* (Haigler CH, Weimer PJ. eds). New York: Marcel Dekker, 1991; 491-533.