Influence of Cellulolytic Bacterial Augmentation on Organic Carbon and Available Phosphorus in Sandy Loam Soil under Cultivation

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Abstract

Microorganisms are major key players for sustaining the soil quality degraded by intensive use of synthetic chemicals for increasing crop production and therefore, use of them as inoculants or biofertilizers is an integral part of sustainable agriculture. An effort was, therefore, made to examine the effect of cellulose degrading bacterial isolates on legume (Chickpea) based cropping systems. No chemical/organic fertilizer was added during this study. The bacterial isolates viz., *Serratia* sp. (MSK1 and MSK24) and *Pseudomonas* sp. (MSK13) exhibiting cellulase activity of 3.83, 4.21 and 4.52 mM glucose ml^{-1} h^{-1} respectively were introduced as inoculants. The ERIC-PCR results showed the good survivability of introduced strains in soil, measured after crop harvest, respectively 40.2. 56.8 and 34.4 %. A significant enhancement in organic carbon and available phosphorus was observed in the inoculated plots over the control plot, indicating beneficial effect of the bioaugmentation of these inoculants.

Keywords: Agricultural soils, Phosphatase, Cellulase, Rhizobacteria, Survivability

1. Introduction

Soil is a dynamic, living matrix that is an essential part of the terrestrial ecosystem (Corstanje *et al*., 2007). It is a critical resource not only for agricultural production and food security but also towards maintenance of most life processes. The functions of soil biota are central to decomposition processes and nutrient cycling (Vikram *et al.,* 2007). However, soils under intensive crop production are prone to organic matter losses that may result in reduced enzyme activity and microbial biomass, which bring about deterioration of the physic-chemical and biological condition (Haynes and Tregurtha, 1999).

Agricultural residues are a rich source of cellulose (Hameeda, 2006). As the main component of plant fiber structures, cellulose is arranged in crystalline to amorphous forms and is a substrate to numerous species of both fungi and bacteria relying on extracellular enzymes. Up until now, the most studied group of cellulose-degrading microorganisms is the fungi, which are characterized by multicomponent, synergistic cellulolytic enzyme systems (Eriksson *et al.,* 1990; Berg and Laskowski, 2006). Although cellulose-decomposing bacteria are ubiquitous in soils, systematic studies on the structure and activities of cellulolytic communities are rare. Cellulases play an important role in carbon availability and so can be used to give a preliminary indication of some of the physical chemical properties of soil, thus, easing agricultural soil management strategies (Ndakidemi and Makoi, 2008). In

soil ecosystems, phosphatases play a critical role in plant growth by enhancing the availability of phosphorus due to enhanced solubilisation and remobilisation of phosphate (Speir and Ross, 1978). Studies have shown that activity of these enzymes in agricultural soils are affected by several factors such as temperature, soil pH, water and oxygen contents (abiotic conditions), the chemical characteristics of organic matter and its location in the soil profile horizon (Rubidge, 1977; Gomah, 1980; Tabatabai, 1982; Klein, 1989; Alf and Nannipieri, 1995). These findings suggest that activities of cellulases can be used to give a preliminary indication of some of the physical chemical properties of soil, thus, easing soil management strategies.

The present study, outlines: isolation, screening, characterization and identification of potential cellulose degrading bacteria and the use of their population dynamics as an index of organic fertility and nutritional status of soil.

2. Materials and Methods

2.1 Sampling and analysis of soil

Soil samples used for the isolation of cellulose degrading bacteria were collected from different chickpea (*Cicer arietinum L.*) agricultural fields of Patiala District, Punjab, India. Soil samples were collected randomly at from 0-30 cm of depth from nine different sites in one acre field by alcohol sterilized implements and stored in sterile containers. Three composites samples were prepared, air-dried and pulverized for organic carbon, nitrogen, available phosphorus, potassium, moisture level, texture and pH by following standard methods (Jackson, 1967).

2.2 Determination of Soil Enzyme Activities

The soil fertility was monitored by estimating two soil enzymes: cellulase (Bailey *et al.,* 1985) and phosphatase (Tabatabai and Bremner, 1969) in soil taken at planting (0 week) and harvest (16^{th} week) time. Soils were processed the same day for microbial and enzymatic activities using standard protocols and stored at 4 ^{o}C for soil analysis.

Estimation of soil phosphatase activity was carried out by taking 10g of soil in 4ml sterile universal buffer. 1ml of 0.115M disodium *p*-nitrophenyl phosphate was added and incubated in dark at 37^{0}C for 2h, followed by an addition of 5ml of 0.5N sodium hydroxide to stop the reaction. The reaction mixture was thoroughly mixed, filtered and filtrate was measured at 410nm. The phosphatase activity was expressed as *p*-nitrophenol (PNP) released per gram of soil i.e., 1μm of PNP/g dry wt. soi/h. Soils were processed the same day for microbial and enzymatic activities using standard protocols and stored at 4^{o}C for soil analysis. For estimating cellulose activity, 10gm of soil was suspended in 5ml of 0.05M phosphate buffer (pH 7.0). 0.1ml of filtered suspension was taken to which 0.9ml of 1.0% carboxymethyl cellulose (CMS) was added. The reaction mixture (1.0ml) was incubated at 37^{0}C for 1h, followed by addition of 2ml dinitro salicylic acid (DNSA) reagent and further incubation of 15min in boiling water bath. One unit of cellulase activity was defined as amount of enzyme necessary to release 1 μm of glucose equivalent/min.

2.3 Isolation and Enumeration of microbial population

For isolation and enumeration of cultivable bacteria, soil samples were diluted in saline (0.89% NaCl; w/v) solution and plated on soil extract agar for total microbial count, Pikovskaya agar (PA) for the isolation of phosphate solubilisers and Bushnell Hass Agar (BHA) supplemented with 1% carboxy methyl cellulose (w/v) as sole source of carbon for cellulose degraders. Plated triplicates of three different dilutions (10^{-5} to 10^{-7}) were incubated for 48 h at 37°C and colonies were counted (cfu g^{-1} dry wt. of soil).

2.4 Screening of Cellulolytic Isolates

Congo red was used as an indicator for the detection of cellulolytic activity on CM–cellulose–agar medium, as described by Teather and Wood (1982). Cellulolytic activity of isolates was evaluated according to the extent and intensity of the hydrolytic clearing zones. Bacterial isolates were grown in 50ml Bushnell Hass broth (BHB) with carboxymethyl cellulose (1% ; w/v) in 250-ml flask and incubated on rotary shaker at 37°C, 120 rpm. After 72 h, the cells were centrifuged (9000g, 10 min) and supernatant was collected for enzyme assay. Cellulase (CMCase) activity was measured by the 3,5-dinitrosalicylic acid (DNS) method (Miller, 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 *et al.,* 1992). Enzyme assay was carried out at 37 °C for 1h 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 μM glucose g^{-1} h^{-1}.

2.5 ERIC-PCR based fingerprinting of isolates

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') – ATGTAAGCTCCTGGGGAATCAC- and ERIC – 2 (5' – 3') - AAGTAAGTGACTGGGGTGAGCG- 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 (9000 x g, 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 were as follows: initial denaturation at 94°C (4 min) followed by 41 cycles at 94°C (1 min) then at 50°C (1 min) and finally at 72°C (2 min). The ultimate extension was done at 72°C (5 min). The reaction was terminated by using a loading dye (1 µl) containing Ficoll 15%, bromophenol blue 0.25%, and xylene cyanol 0.25%. Each PCR product was resolved by electrophoresis on a 1.5% agarose gel.

2.6 Plot studies

Plot studies were conducted under 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 ($4m^2$) sown with chickpea (*Cicer arietinum L.*) crop. Cultures were grown in 10 litre shake flasks containing Luria broth (LB) medium and incubated at 37°C with rotary shaking set at120 rpm. After 36 h, the cells were harvested and washed with 10mM phosphate buffer (pH 6.8). Pellets were resuspended in buffer and inoculated in plots at cell concentration of approximately 2 x 10^8 cfu/g soil. Control plot received only BHB without any bacterial cells. The prescribed package of practices (PAU, 2005) was followed and monitored during the cultivation of the chickpea. At 0 week (planting and inoculation time) and 16^{th} week after sowing (harvest time) soil samples were collected from different points, composite samples were prepared and taken in triplicate to evaluate the population dynamics and other soil parameters. One gram of soil was suspended in 9 ml of sterilized saline water (0.8% NaCl; w/v) by vigorous vortexing followed by serial dilution. An aliquot of 100 µl was plated on LA containing ampicillin (120 µg/ml). Plates were incubated for 36 h at 37 °C. Individual colonies obtained after this incubation were restreaked on LA plates containing ampicillin (120 µg/ml). Colonies grown were scored and their DNA was isolated for fingerprinting protocol based on ERIC-PCR.

2.7 Data analysis

Variance (ANOVA), regression and multiple comparison analysis were carried out at $p \leq 0.05$, using COSTAT software.

3. Results and Discussion

Thirty-one cellulose degrading bacterial isolates were isolated from agricultural soils cultivated with chickpea (Figure 1). Three isolates, namely MSK1, MSK13 and MSK24 showed their high efficacy to degrade carboxy methyl cellulose (CMC) in plate assay. The values were: 3.83, 4.21 and 4.52 mM glucose ml^{-1} h^{-1} respectively. These 3 strains were chosen for further morphological and biochemical characterisation (Table 1). MSK1, MSK13 and MSK24 were Gram negative, non-spore forming and positive to extracellular CMCase and xylanase activity. The optimum growth conditions for these isolates were 25 – 37 ^oC at pH 6.0-8.0. MSK1 and MSK24 showed catalase positive, oxidase and nitrate reduction negative reaction, but MSK13 was catalase negative, oxidase positive and nitrate reduction positive. Antibiotic screening showed that MSK1, MSK13 and MSK24 were sensitive to norfloxacin, gentamicin, chloramphenicol, cefuroxime, ciprofloxacin, resistant to ampicillin. These isolates were identified by partial sequencing of 16S rRNA, so homology search revealed that MSK1, MSK13 and MSK24 were 99% similar to *Serratia* sp., *Pseudomonas* sp. and *Serratia marcescens* respectively (data not shown).

Investigations by other researchers showed that aerobic bacteria belonging to species of *Pseudomonas*, *Bacillus* and *Cellulomonas*, and anaerobe such as *Clostridium* have cellulolytic potential (Sindhu *et al.,* 2001). *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). The residing microflora maintains the soil health and affects the agronomic parameters of the crops planted on those plots. Thus, farming management trials along with the inoculants enhance the growth and yield of the crops (Dickey *et al.,* 1994). Cellulolytic soil bacteria had been studied in various soils under different land use systems with respect to the effect of environmental conditions on the abundance and decomposing activity (Hiroki and Watanabe, 1996; Dilly *et al.,* 2001). All of these studies

were mostly based on the estimates of population densities using agar plate techniques; with population dynamics and characterisation being rarely analyzed (Ulrich *et al.,* 2008)

Survival of the microorganisms in the soil after their application is a deciding factor in the rate of degradation of carbon source (Ramos *et al.,* 1991) which is observably assessed with confidence mainly with PCR based molecular techniques (Versalovic *et al.,* 1991; Thiem *et al.,* 1994; Weidmann-Al-Ahmad *et al.,* 1994; Giovanni *et al.,* 1999). Repetitive DNA sequences have been characterized primarily from *Escherichia coli* and *Salmonella typhimurium*. These sequences, referred to as enterobacterial repetitive intergenic consensus sequence (ERIC) or intergenic repeat units (IRUs), are 126-bp elements containing a highly conserved central inverted repeat and are located in extragenic regions (Versalovic *et al.,* 1991). The analysis of this element by PCR-synthesized oligonucleotide has provided unique DNA fingerprints. It is reported that ERIC probes hybridized preferentially to genomic DNA from Gramnegative bacteria (Giovanni *et al.* 1999). In the present study, survival of the microorganisms was tracked with the ERIC-PCR based genomic DNA fingerprinting method. ERIC-PCR of the isolates in the present study showed 10 to 14 distinctly amplified bands with sizes ranging from 11,000 bp to 100 bp (Figure 2). Although 16S rDNA sequence analysis showed that *MSK1* and *MSK24* to have close homology with *Serratia* sp., ERIC-PCR enabled to differentiate between these isolates. The populations of *Serratia* sp. MSK1, *Pseudomonas* sp. MSK13 and *Serratia* sp. MSK24 showing a survival of 40.2, 34.4 and 56.8 % respectively after 16 weeks (Figure 3). The strains of *Serratia* sp. and *Pseudomonas* sp. were found to be stable at the end of 16 weeks in the treated plots which is presumed to be due to their adaptability to local soil environment from where these were isolated. Among all the three treatments tested, significantly high values of microbial count and soil enzyme activities were observed in plots inoculated with *Serratia* sp. compared to control (Table 2). It was observed that the conventional plate counting method resulted in lower rates of survival of these isolates (41%) as compared to the survival rate obtained from PCR fingerprinting (58%). In absence of proper control, mere decrease in antibiotic counts cannot be related to the survival of specific inoculated bacterial isolate in a complex environment like agricultural soil. This is expected since in natural environment several microorganisms have no specific-drug resistance (Nakaune *et al.* 1998; Mathew *et al.* 1999) and therefore are selected on ERIC-PCR fingerprinting. The results of investigation also support the hypothesis that indigenous isolates will results in greater adaptation to the system as well promote the microbial population and activities in the soil.

The modulations in the soil enzyme activity and nutritional properties influenced by the microbial inoculation were studied by examining the cellulase activity, phosphatase activity, organic carbon, total nitrogen content and available phosphorus in the experimental plots. Most of these parameters were observed and found to be enhanced with augmentation of inoculants in the plots in comparison to the control plot. The present study also indicated increase in availability of carbon and other nutrients assumed to be facilitated through enhanced enzyme activity. The increased phosphate activity in the soil drawn from the plots amended with *Pseudomonas* sp. MSK 13 could be related to the metabolic versatility of this microbial group that facilitates increasing activity of phosphatases when P is a limiting nutrient (Tadano *et al.,* 1993). The plots treated with *Serratia* sp. MSK1, *Pseudomonas* sp. *MSK13* and *Serratia* sp., *MSK24* showed significantly enhanced soil cellulase activity of 7.51, 9.02 and 10.25 (μM glucose g^{-1} dry wt. soil h^{-1}) respectively, in comparison to control plots (Table 2), which also correlated with the increase in population of the cellulose degrading inoculants (r^2 : 0.730). Similarly, an increase in soil phosphatase activity was also observed ranging from 0.858 μM PNP g^{-1} dry wt. soil h^{-1} in plots inoculated with *Serratia* sp. (MSK13) to 1.497 μM PNP g^{-1} dry wt. soil h^{-1} in plots augmented with *Serratia* sp. (MSK24) (Table 4) correlating with the levels of available phosphorus (Table 5). A significantly high value of total nitrogen was observed in the plots inoculated with *Serratia* sp. MSK1, *Pseudomonas* sp. MSK13 and *Serratia* sp. MSK24 (0.234 %, 0.245 % and 0.272% respectively) when compared to control (Table 3). The increase in organic carbon also correlated with increase in cellulase activity (r^2: 0.916). The increased population of phosphate solubilisers showed the increased availability of available phosphorus significantly high as compared to the control plot, to 98.66 mg/kg, 96.66 mg/kg and 99.66 mg/kg in the *Serratia* sp. MSK1, *Pseudomonas* sp. MSK13 and *Serratia* sp. MSK24 inoculated plots respectively. Similar investigations have earlier shown that legumes secrete more phosphatise enzymes than cereal (Yadav and Tarafdar, 2001). This may probably be due to a higher requirement of P by legumes in the symbiotic nitrogen fixation process as compared to cereals. The maximum available potassium of 115.0 mg/kg and significant enhanced nitrogen of 0.27 % was observed in the *Serratia* sp. MSK24 plot (Table 4). As reported by Sivaramaiah *et al.* (2007) the efficacy of *Bacillus* sp. to enhance nodulation, plant dry matter and grain yield on co-inoculation with rhizobia has also been reported for other legumes Bacterial strains isolated from the maize rhizosphere including *Bacillus, Pseudomonas* and *Serratia* were reported to improve the yield by 9–14% (Lalande *et. al.,* 1989). Significant increase in the root dry mass of rapeseed was observed due to inoculation with *Proteus*, *Klebsiella* and *Bacillus* (Bertrand *et. al.,* 2001). Considering the results

of this study in addition to the previous observation, it may be suggested that mass inoculation of aerobic cellulolytic bacteria used in present investigation can be a useful tool to maintain soil fertility by increasing microbial activity to decompose organic matter in leguminous ecosystem.

4. Conclusion and Recommendations

- Thirty-one strains were isolated from chickpea cultivated soils.
- Three of the isolates: MSK1, MSK13 and MSK24, showed higher efficacy to degrade carboxy methyl cellulose in plates.
- 16S rDNA sequence analysis and fingerprint method (ERIC-PCR) allowed identifying three species: *Serratia* sp. (MSK1), *Pseudomonas* sp. (MSK13) and *Serratia* sp. (MSK24), 99% close to *Serratia marcescens*.
- A significant enrichment in organic carbon and available phosphorus was observed in the inoculated plots over the control plot indicating beneficial effect of the bioaugmentation of these inoculants.

The local isolates of both *Serratia* sp. and *Pseudomonas* sp. could be mass produced as a biological agent towards magnification of microbial diversity and soil organic carbon. Moreover, the introduction of such bacteria in chickpea soils, or cultural practices aims to increase the activity of native strains of these bacteria and they could greatly contribute to the efficiency of soil organic matter and the cellulolytic potential of soil bacterial communities as major contributors towards sustainable agriculture. Also, the rhizobacteria from the local rhizosphere soil could be exploited for use as microbial inoculants to improve nodulation (in legumes) and crop productivity of both cereals as well as legumes. Although potential clearly exists for developing such inoculants, their widespread application remains limited by a poor understanding of microbial ecology and population dynamics in soil; these studies are needed.

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Table 1. Biochemical characterization of the screened bacterial isolates from agricultural soils

Character	MSK1	MSK13	MSK24
Colony morphology	White	Creamy Yellow	Pinkish White
Gram stain	-	-	-
Spore	-	-	-
Catalase	+	-	+
Oxidase	-	+	-
Nitrate reduction	-	+	-
Growth at 25-37^{o}C	+	+	+
Growth at pH 6-8	+	+	+
CMCase	+	+	+
Xylanase	+	+	+
Ampicillin	R	R	R
Tetracyclin	S	S	S
Gentamycin	S	S	S
Kanamycin	S	S	S

+: positive reaction; -: negative reaction; R: Resistant at 120µg; S: Sensitive

Table 2. Microbial populations and soil enzyme activities in soils of chickpea plot. Cellulose degraders count, Phosphate solublisers count, Cellulase and phosphatase activity were determined at 0 week (Chickpea sowing stage) and 16 weeks (Chickpea harvest stage) in soils

Treatment	cellulose degraders (cfu x10^6 / g dry wt soil)		phosphate solubilisers (cfu x10^6 / g dry wt soil)		cellulase activity (um glucose / g dry wt soil/ hr)		phosphatase activity (um pnp / g dry wt soil/ hr)	
	0 WEEK	16 WEEK	0 WEEK	16 WEEK	0 WEEK	16 WEEK	0 WEEK	16 WEEK
Control	10.04±0.93a	32.61±2.57b	13.00±0.96a	26.76±1.43b	0.784±0.048b	4.489±0.322d	0.167±0.004a	0.554±0.016c
Serratia sp. MSK1	9.64±1.56a	56.11±6.64a	17.09±1.28a	40.37±5.41ab	1.404±0.102a	7.518±0.392c	0.171±0.004a	0.662±0.019b
Pseudomonas sp MSK13	10.88±0.88a	58.64±6.83a	14.09±2.10a	44.99±4.38ab	1.287±0.013a	9.026±0.233b	0.161±0.013a	0.711±0.041ab
Serratia sp. MSK24	12.87±1.41a	73.58±6.35a	14.37±0.71a	53.14±6.28b	1.279±0.080a	10.251±0.391a	0.157±0.009a	0.836±0.045a

Data characterized by the same letter are not significantly different ($p \leq 0.05$) in the rows. Above table represents Mean± Standard error of mean (SEM).

Table 3. Soil nutritional status of chickpea plot. Organic carbon (OC), Nitrogen (N), Carbon/Nitrogen ratio (C/N), Available phosphorus (P) and Available potassium (K) were determined at 0 week (Chickpea sowing stage) and 16 weeks (Chickpea harvest stage) in soils

Treatment	OC (%)		N (%)		C/N ratio		P (mg/kg)		K (mg/kg)	
	0 WEEK	16 WEEK	0 WEEK	16 WEEK	0 WEEK	16 WEEK	0 WEEK	16 WEEK	0 WEEK	16 WEEK
Control	0.340±0.030a	0.554±0.016c	0.0823±0.0056a	0.1695±0.0089b	4.13±0.8	3.26±0.4	44.0±5.68a	70.3±6.11a	52.3±3.17a	86.7±7.21
Serratia sp. MSK1	0.388±0.030a	0.662±0.019b	0.0818±0.0063a	0.2340±0.0204ab	4.74±1.2	2.82±0.4	47.0±6.24a	98.7±7.05a	50.7±8.98a	96.7±6.48
Pseudomonas sp. MSK13	0.360±0.036a	0.711±0.041ab	0.0851±0.0059a	0.2456±0.0219ab	4.23±0.2	2.89±0.5	48.7±4.97a	96.7±14.31a	54.3±7.62a	94.0±4.93
Serratia sp. MSK24	0.368±0.028a	0.836±0.045a	0.0867±0.0078a	0.2728±0.0237a	4.24±0.4	3.07±0.8	48.0±8.50a	99.8±11.89a	54.3±9.26a	115.0±11.37

Data characterized by the same letter are not significantly different in the rows. Above table represents Mean± Standard error of mean (SEM)

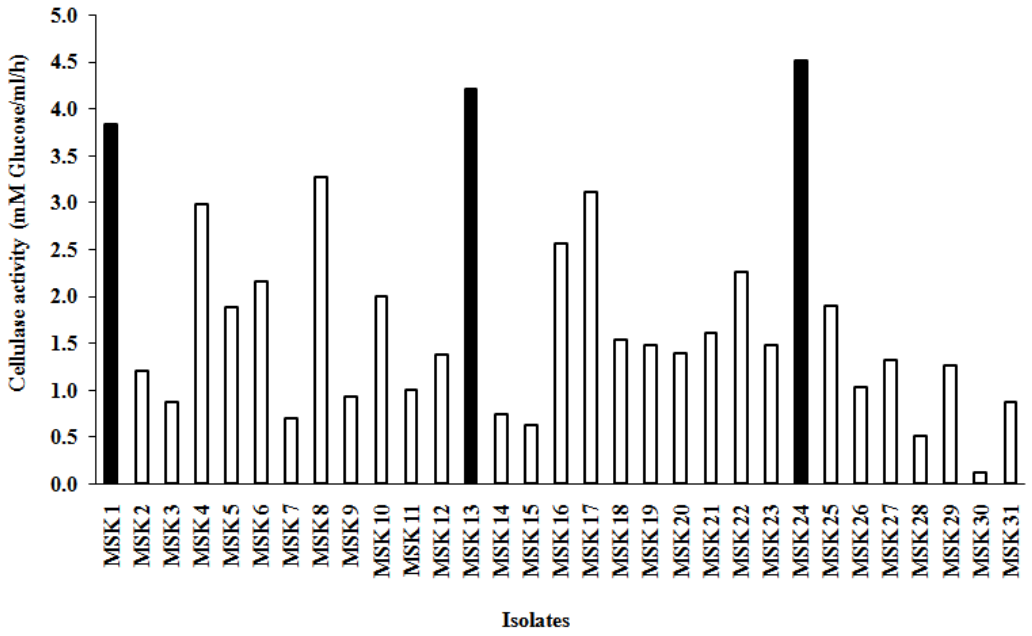

Figure 1. Screening of isolates for Cellulase (CMCase) activity from agricultural soils

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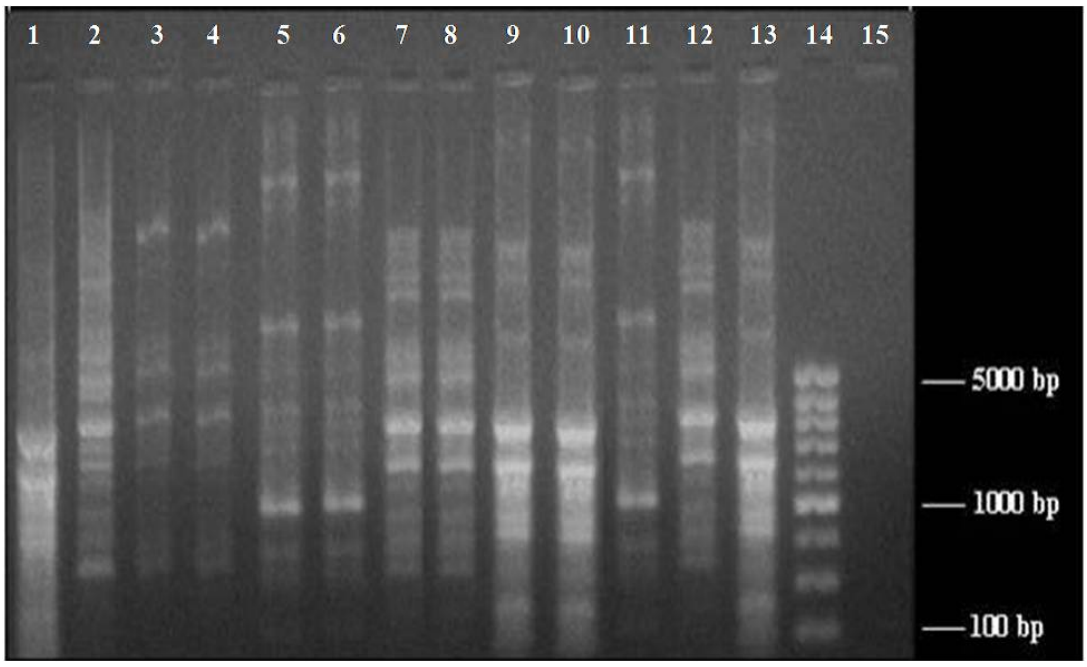

Figure 2. ERIC-PCR fingerprint of unknown bacterial isolates on 1.5% agarose gel. Bacterial colonies were isolated from soil in the plots. Pure colonies were picked, heat lysed, and whole genomic DNA was subjected to ERIC-PCR to get the fingerprint.

Lanes 1-10: fingerprints of different isolates obtained from selected plates containing 120µg/ml of Ampicillin
Lane 11: Standard bacterial strain of *Pseudomonas* sp. MSK13
Lane 12: Standard bacterial strain of *Serratia* sp. MSK1
Lane 13: Standard bacterial strain of *Serratia* sp. MSK24
Lane 14: 500bp ladder
Lane 15: Negative Control.

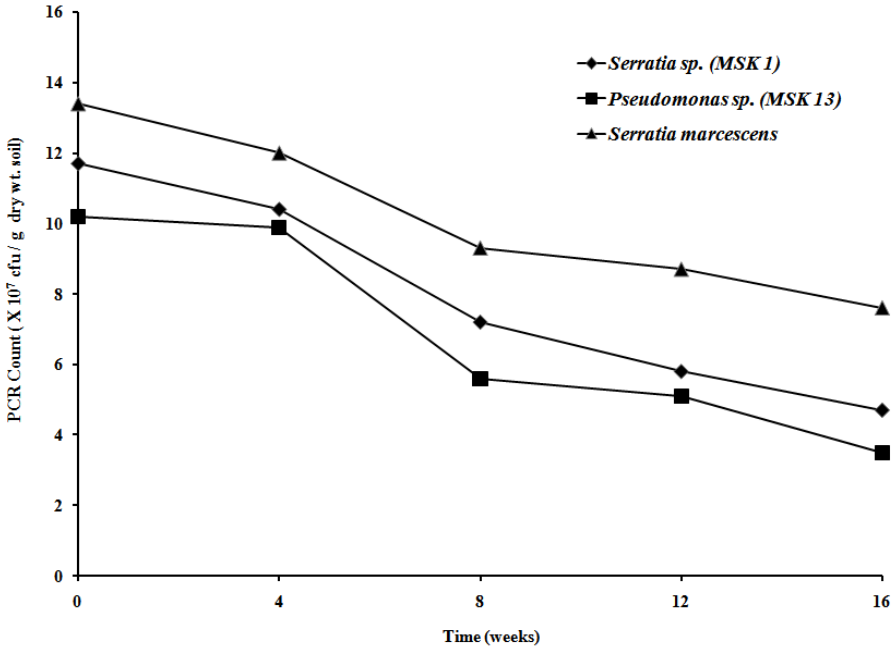

Figure 3. Survival of introduced bacterial strains during growing chickpea crop in plots