World Journal of Agricultural Sciences 3 (5): 602-608, 2007
ISSN 1817-3047

Cellulase Activities in Nitrogen Fixing *Paenibacillus* Isolated from Soil in N-free Media

G. Emtiazi, M. Pooyan and M. Shamalnasab

Department of Biology, School of Sciences, University of Isfahan, P.O. Box 117, Isfahan 81744, Iran

Abstract: Nitrogen fixing *Paenibacillus* strains were isolated from different soils on N-free media by heat shock and grown on N-free media in anaerobic condition. The cellulase positive *Paenibacillus* were selected by reduction of congored color on CMC medium. Three of these isolates with CMCase activities were identified as *Paenibacillus* strain E, H and SH. This nitrogen fixing strains with cellulase activities *Paenibacillus*, grow well on N-free media with sucrose or mannitol as the only sources of carbon and produced 3.16 and 1.3 mM ammonium respectively. *Paenibacillus* strain E produced 4 u ml^{-1} CMCase when it was grown on CMC as the only sources of carbon.This phenomena might help to plant microbe-interaction for nitrogen fixation.

Key words: *Paenibacillus* % nitrogen fixing % FPase % CMCase

INTRODUCTION

Biomass resources, such as starchy and cellulosic materials of plant origin, are the most abundant renewable resources on earth. Utilization of these wastes for production of energy and chemicals has attracted considerable interest in recent years [1, 2]. However most yeast strain used as SCP can not biodegrade cellulose and there are few bacteria such as *Cellulomonas* [3], *Clostriduim* [4], *Cytophaga*[5], *Vibrio* [6], *Ruminococcus* [7] which are cellulytic. In recent years rhizoplane microorganism has attracted great interest because of the positive response of many plants to inoculation with suitable plant-growth-promoting bacterial strain [8] and their cellulase activities may help intraction of them for interaction [9]. Cellulase activities have seen in many nitrogen fixing bacteria such as *Sinorhizobium fredii* [10, 11], *Bacillus spharricus* [12], *Bacillus circulans* [13], *Paenibacillus azotofixans* [14], *Gluconacetobacter* [9], *Azospirillum* [15]. Although with the change of *Bacillus* classification, all nitrogen-fixing Bacillus strains *B. polymyxa, B. macerans, B. azotofixans*) are now assigned to *Paenibasillus* [14, 16-20] but there are other many spore forming that might fix nitrogen. In this work spore forming bacteria were isolated on N-free media under anaerobic condition and cellulase activities of them were studied.

MATERIALS AND METHODS

Microorganisms: Soil was heated in bath (80°C) for about 10 min and 1ml of heated suspension solution transfer to Mannitol Agar medium with the following composition:

20 gr l^{-1} Mannitol, 4 gr l^{-1} NaCl, 0.75 gr l^{-1} K_2HPO_4,
0.02 gr l^{-1} $Na_2MoO_4.2H_2O$, 0.25 gr l^{-1} KH_2PO_4,
0.3 gr l^{-1} $CaCO_3$, 0.4 gr l^{-1} $MgSO_4.7H_2O$,
0.4 gr l^{-1} $FeSO_4$, pH = 7.2.

They kept in anaerobic jar for about 24 hours.*Bacillus* genus was determined with gram staining and biochemical tests according to Bergey`s Manual [21]. They were maintained in Nutrient Agar medium and tested for confirming nitrogen fixation in aerobic and anaerobic condition and cellulase activities.

Nitrogen fixation: The growth in N-free medium with different carbon source and production of ammonium (mM) was assayed as nitrogen fixation. Amount of ammonia in these media measured with Nessler`s reagent and determined by, optical density measuring at 410 nm.

Enzyme assay: The strains were transfered to a cellulose liquid medium with the following composition:

Corresponding Author: Dr. G. Emtiazi, Department of Biology, School of Sciences, University of Isfahan, P.O. Box 117, Isfahan 81744, Iran

10 gr l^{-1} cellulose, 0.004 gr l^{-1} $FeCl_3$, 1 gr l^{-1} $(NH_4)_2SO_4$, 0.6 gr l^{-1} NaCl, 0.5 gr l^{-1} K_2HPO_4, 0.5 gr l^{-1} $MgSO_4.7H_2O$, 0.5 gr l^{-1} KH_2PO_4, 0.002 gr l^{-1} $CaCl_2.2H_2O$, pH = 5-7.

(CMC liquid medium is similar to Cellulose liquid medium but instead of 10 gr l^{-1} Cellulose there is 10 gr l^{-1} CMC). FPase and CMCase activity was studied in supernatant with different carbon sources. Cellulose medium was used as basal medium with changing carbon sources to test cellulase activity in different carbon sources. 1ml of the bacterial isolates (OD = 0.5) were inoculated in 100ml cellulose medium in 250ml conical flasks and enzyme activity were analyzed for 5-6 days.

CMCase activity: 1ml of culture filtrate and 1ml of culture was added to 0.05gr CMC, 1ml 0.05M citrate buffer pH 4.8 in test tube and incubated at 50°C for 1h after 2 ml DNS were added to the test tube and incubated at 100°C for 15 min after that 1ml tartarate sodium potassium and 5ml water were added to the test tube then released glucose was measured by optical density method, at 575 nm.

Fpase activity : 1ml culture filtrate and 1ml culture were added to test tube contains 0.05gr Whatman No.1 filter paper strip (1x 6 cm) and 1ml 0.05M citrate buffer pH 4.8 and incubated at 50°C for 1h. After that 2 ml DNS were added to test tube and incubated at 100°C for 15 min. Then 1ml Tartarate sodium potassium and 5ml water were added to test tube then released glucose was measured by optical density method, at 575 nm.

Enzyme activity was expressed as U ml^{-1} {the amount of reducing sugars (mM) released ml^{-1} filtrate/hour}

Evaluation of bacterial growth: Bacterial biomass in CMC substrate media was determined by optical density method, measuring the absorbance at 600 nm. Bacterial biomass in Cellulose substrate media was determined by colony count.

Productiuon of sugar in cellulose media: 2ml DNS was added to 1ml of Cellulose media and CMC media in test tube and incubated at 100°C for 15 min then 1ml Tartarate sodium potassium and 5ml water were added to the test tube and the changes in color was measured by optical density at 575 nm.

RESULTS AND DISCUSSION

Isolation and identification: Aliquots of the different soil samples were heated for 10 min at 80°C and were

Table 1: Biochemical test for identification of isolated nitrogen fixing *Paenibacillus* strains

Tests	*Paenibacillus E*	*Paenibacillus* H	*Paenibacillus* SH
Gram reaction	+	+	+
Pigment	-	-	-
Morpholog cells	Bacillus-spore	Bacillus-spore	Bacillus-spore
Oxidase	-	-	-
Catalase	+	+	+
Thioglycolate	+	+	+
Starch	-	+	+
TSI	+	+	+
Citrate	-	-	-
Glucose (A)	+	+	+
Sucrose	+	+	-
Xylose	+	+	+
Sorbitol	+	+	+
Adonitol	-	-	+
Trehalose	-	-	+
Lactose	-	+	+
Arabinose	-	-	-
Manose	+	+	+
MR	-	-	+
VP	+	+	-
Licethinase	-	-	+
BHI	+	+	+
SIM	-/-/+	-/-/+	-/-/+
NO3 reduction	+	+	+
CMCase activity	+	+	+
Fpase activity	+	+	+
Amylase activity	-	+	+

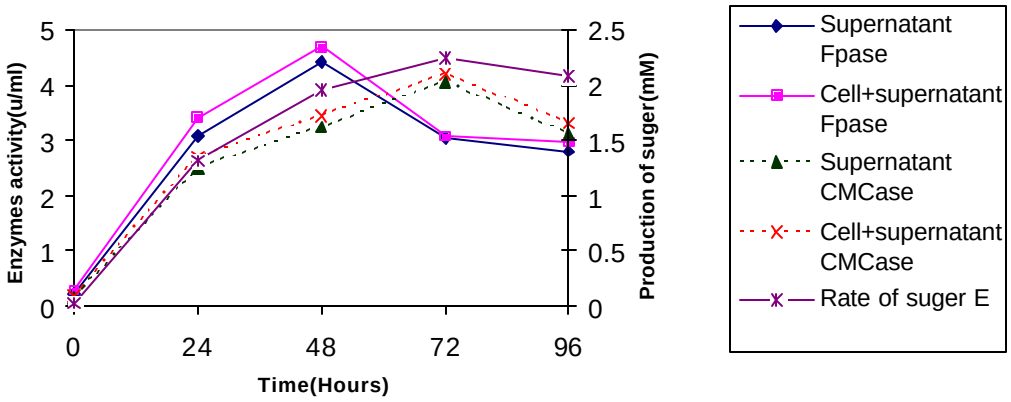

Fig. 1: Enzymes activities of *Paenibacillus E* grown on CMC as the only sources of carbon (pH = 7, T = 30)

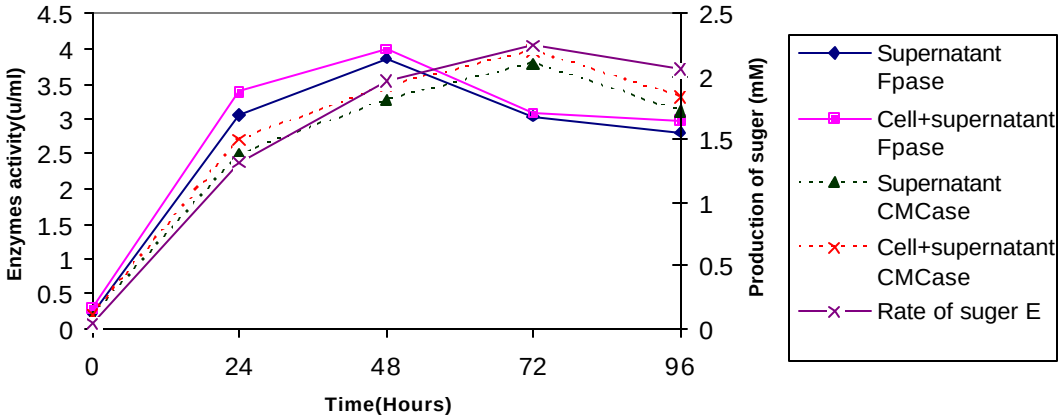

Fig. 2: Enzymes activities of *Paenibacillus H* grown on CMC as the only sources of carbon (pH = 7, T = 30)

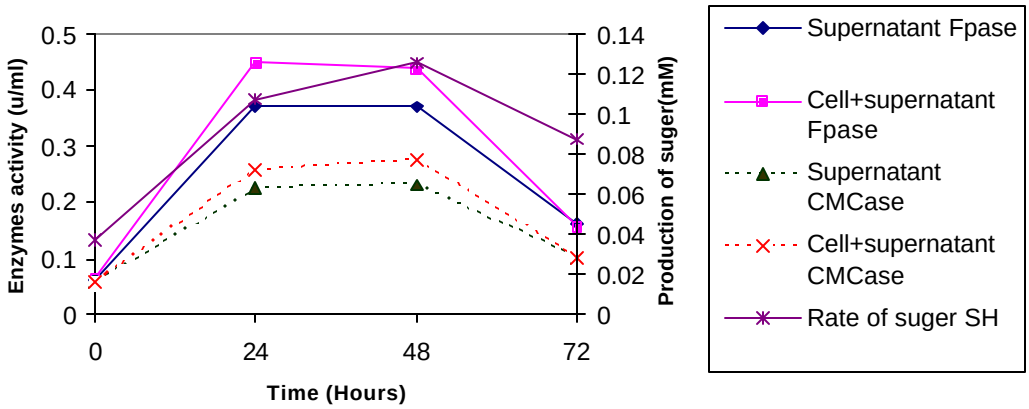

Fig. 3: Enzymes activities of *Paenibacillus SH* grown on CMC as the only sources of carbon (pH = 7, T = 30)

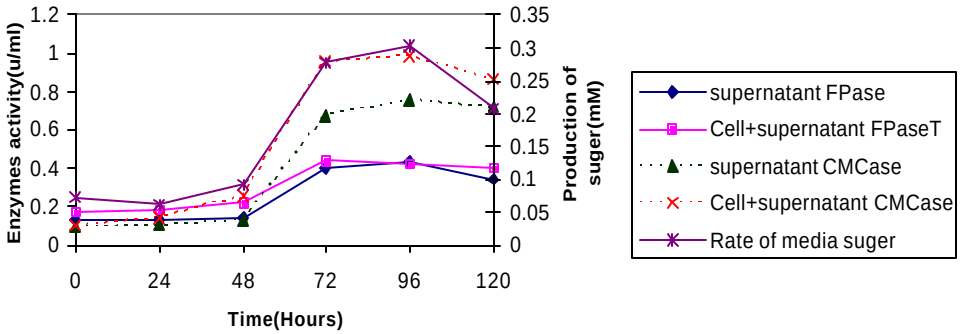

Fig. 4: Enzymes activities of *Paenibacillus E* grown on cellulose as the only sources of carbon (pH = 7, T = 30)

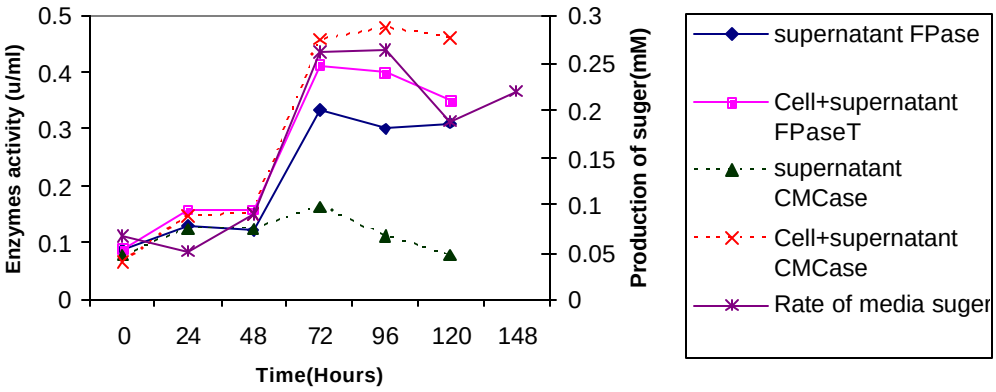

Fig. 5: Enzymes activities of *Paenibacillus SH* grown on cellulose as the only sources of carbon (pH = 7, T = 30)

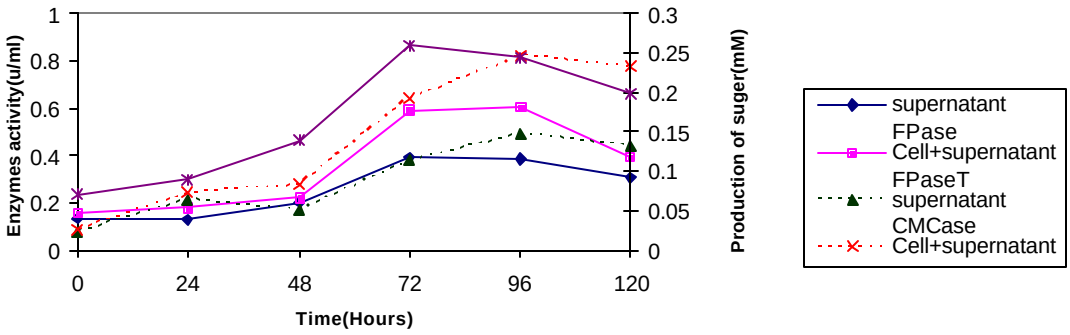

Fig. 6: Enzymes activities of *Paenibacillus H* grown on cellulose as the only sources of carbon by isolated strains (pH = 7, T = 30)

Table 2: Aerobic and anaerobic growth of the isolated on different nitrogen fixing media

		Bacteria		
	Carbon sources	*Paenibacillus SH*	*Paenibacillus H*	*Paenibacillus E*
Nitrogen fixation in anaerobic condition	Glucose	+	+	+
	Sucrose	+++	+++	+++
	Monitol	++	++	++
	Malic acid	+	+	+
Nitrogen fixation in aerobic condition	Glucose	-	-	-
	Sucrose	+	+	+
	Mannitol	-	-	-
	Malic acid	-	-	-

transfered to N-free media and incubated at 30°C in aerobic and anaerobic condition. *Paenibacillus* determined with biochemical test and nitrogen fixing condition. Biochemical test are shown in Table 1 [13]. As it is shown in this Table all three isolates have NO_3 reduction, CMCase, FPase activities and two of them has amylase activity. All isolates fix nitrogen under anaerobic condition when glucose, sucrose, mannitol and malic acid were used as the only sources of carbon, however under aerobic condition when sucrose was used as the only source of carbon all the three isolates gave positive growth without addition of any nitrogen sources (Table 2).

Enzyme activities: CMCase and FPase activities of the isolated spore forming bacteria grown on CMC and Cellulose as the only sources of carbon are illustrated in Fig. 1-6. As it is shown in Fig. 1, *Paenibacillus* E had maximum FPase activities on CMC as the only sources of carbon compared to cellulose. As it is shown the most enzyme are extra cellular and maximum production of enzyme is after 48h with maximum growth. As it is shown in Fig. 3 and 6, *Panebacillus* SH had low activities of CMCase or FPase (when it was grown on CMC or Cellulose). As it is shown in Fig. 4-6 all three isolated had maximum FPase activities when it was grown on CMC as the only source of carbon. Enzyme activities of the isolates, when they were cultured on Cellulose were shown in Fig. 1-6. As it is shown the activities of cellulase in this media was low, however the growth rate of three isolates showed that *Paenibacillus* E produced the maximum growth, however the enzyme activities is very low. Here also *Paenibacillus* E had maximum 1 u ml^{-1} CMCase activity and 0.5 u ml^{-1} cell membrane bound CMCase activity when it was grown on cellulose. In cells grown on CMC there was no cell membrane activities in *Paenibacillus* E. CMCase and cellulase activities of

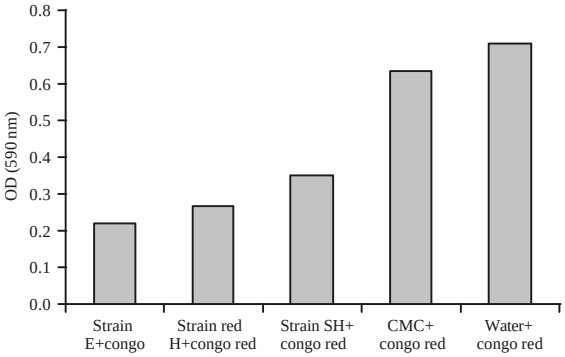

Fig. 7: The reduction of congored color as result of bacterial growth in CMC medium (7 days, pH = 7, T = 30°C)

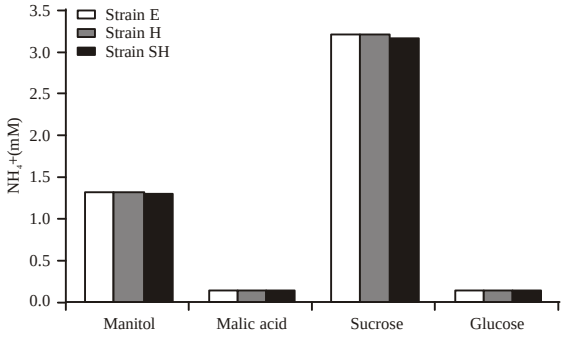

Fig. 8: The production of ammonium in N free medium with different carbon sources by isolated strains (7 days)

Paenibacillus SH is very low (Fig. 3). Here it was shown *Paenibasillus* E had the best CMCase activities maximum 4.1 u ml^{-1} and 4.8 u ml^{-1} FPase activities.The strain E did not have amylase activities.

The results were the same with reduction of congored when it was grown on CMC + congored. The results of reduction of congored color in CMC media for the three isolates are shown in Fig. 7.

Production of ammonium: The results of nitrogen fixing under aerobic condition and anaerobic are shown in Table 2. However nitrogen fixing microorganism fixes nitrogen to glutamine. Here in nitrogen free media the production of ammonium was measured by Nessler`s reagent and it was shown that *Paenibacillus* E, H, SH produce 3.16 mM ammonium when sucrose was the only sources of carbon and 1.3 mM ammonium when mannitol was the only sources of carbon (Fig. 8). All the three strains produced low ammonium when they were grown on glucose or malic acid as carbon sources.

CMCase activities is also shown in *Bacillus pumilus* [22], *Bacillus sphaericas* [12], *Bacillus circulans*[13], which some of them have ability to fix nitrogen. Also CMCase were purified from *Sinorhizobium fredii* with specific activity of 3.822 u mg^{-1} [10]. This study showed that most plant associated microorganism might have cellulase activity for adoption or establishment of a plant microbe interaction.

REFERENCES

1. Mielenz, J.R., 2001. Ethanol production from biomass:technology and commercialization status. Current Opinion in Microbiology, 4: 324-329.
2. Vasey, R.B. and K.A. Powell, 1984. Single cell protein biotechnology and genetic engineering review, 2: 285-311.
3. Rajoka, M.I., 2004. Influence of various fermentation variables on exo-glucanase production in *Cellulomonas flavigena*. Electronic. J. Biotechnol., 7: 259-266.
4. Schwarz, W.H., K. Bronnenmeier, B. Landmann, G. Wanner, W.L. Staudenbauer, N. Kurose and T. Takayama, 1995. Molecular characterization of four strains of the cellulolytic thermophile *Clostridium stercorarium*. Biosci. Biotech. Biochem., 59: 1661-1665.
5. Chang, W.T. and D.W. Thayer, 1977. The cellulase system of a *Cytophaga* species. Can. J. Microbiol., 23: 1285-1292.
6. Sanchez, C.R., C.S. Peres and H.R. Barbosa, 1999. Growth and endoglucanase activity of *Acetivibrio cellulolyticus* grown in three different cellulosic substrates. J. Rev. Microbiol., 30: 1-8.
7. Leatherwood, J.M., 1965. Cellulase from *Ruminococcus albus* and Mixed Rumen Microorganisms. Appl. Microbiol., 13: 771-775.
8. Kotchoni, O.S., O.O. Shonukan and W.E. Gachomo, 2003. *Bacillus pumilus* BpCRI 6, a promising candidate for cellulose production under conditions of catabolite repression. J. Biotechnol., 2: 140-146.
9. Emtiazi, G., Z. Etemadifar and M. Tavassoli, 2003. Anovel nitrogen-fixing cellulytic bacterium associated with root of corn is a candidate for production of single cell protein. Biomass. Bioenergy, 25: 423-426.
10. Chen, P.J., T.C. Wei, Y.T. Chang and L.P. Lin, 2004. Purification and characterization of carboxymethyl cellulase from *Sinorhizobium fredii*. Bot. Bull. Acad. Sin., 45: 111-118.
11. Mateos, P.F., J.I. Jimenez-Zurdo, J. Chen, A.S. Squartini, S.K. Haack, E. Martinez-Molina, D.H. Hubbell and F.B. Dazzo, 1992. Cell-associated pectinolytic and cellulolytic enzymes in Rhizobium leguminosarum *biovar trifolii*. 58: 1816-1822.
12. Singh, J., N. Batra and R.C. Sobti, 2004. Purification and characterization of alkaline cellulose produced by a novel isolate, *Bacillus sphaericus* JS1.J. Ind. Microbiol. Biotechnol., 31: 51-59.
13. Baird, D.S., D.A. Johnson and V.L. Seligy, 1990. Molecular coloning, expression and characterization of endo-$-1,4-glucanase genes from *Bacillus polymyxa* and *Bacillus circulans*. J. Bacteriol., 172: 1576-1586.
14. Rosado, A.S., F.S. de Azevedo, D.W. da Cruz, J.D. van Elsas and l. Seldin, 1998. Phenotypic and genetic diversity of *Paenibacillus azotofixans* strains isolated from the rhizolpane or rhizosphere soil of different grasses. J. App. Microbiol., 84: 216-226.
15. Steenhoudt, O. and J. Vanderleyden, 2000. *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: Genetic, biochemical and ecological aspects. FEMS Microbiol. Rev., 24: 705-787.
16. Gorska, E., B. Tudek and S. Russel, 2001. Degradation of cellulose by nitrogen-fixing strain of *Bacillus polymyxa*. J. Acta.-Microbiol. Pol., 50: 129-137.
17. Hossain, F.M.M., N.C. Rahman and M.A. Malek, 1999. Production of extracellular carboxymehtyl cellulose and cellobiase by a thermophilic *Bacillus* sp. Bangeladesh J. Microbiol., 16: 115-125.
18. Kashem, M.A., M.A. Manchur, M.S. Rahman and M.N. Anvar, 2004. Effect of carbon and nitrogen sources on the production of reducing sugars, extra-cellular protein and cellulolytic enzymes by two cellulolytic bacteria isolates. Pak. J. Biol. Sci., 7: 1660-1663.

19. Mawadza, C., R. Hatti-Kaul, R. Zvauya and B.O. Mattiasson, 2000. Purification and characterization of cellulases produced by two *bacillus* strains. J. Biotechnol., 83: 177-187.
20. Shawky, B.T., 1983. Growth and nitrogen fixation by cultures of *Bacillus* sp. S77g, *pseudomonas* sp. S44b and *Klebsiella* sp. S145 m, isolated from Egyption Soils. J. Zentralbl Mikrobiol., 138: 9-15.
21. Krieg, N.R. and J.G. Holt, 1984. Bergey's manual of systematic bacteriology,1st Edn. Williams and Wiilkins, Balimore, Md.
22. Grau, F.H. and P.W. Wilson, 1961. Physiology of nitrogen fixation by*Bacillus polymyxa*. Department of Bacteriology, University of Wisconsin. Madison. Wisconsin, 83: 490-496.