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DIVERSITY OF CELLULASE ACTIVE MICROMYCETES IN THE ANT-HILL SOIL ENVIRONMENT OF PARKS AND GARDENS OF JAMMU CITY (INDIA)

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ABSTRACT

One hundred and twenty soil samples collected from ant-hills of various parks and gardens of Jammu City (India) were screened for the cellulase active micromycetes. In all, forty species belonging to sixteen fungal genera were recovered from the samples by using dilution plate method. In the present study, Congo red test was performed for detecting the cellulase active micromycetes and twenty seven fungal species were detected to be positive. Quantification of cellulase activity of the positive fungal species was done by using dinitrosalicylic acid (DNSA) method and eight of them, namely *Trichoderma viride, T. harzianum, Penicillium chrysogenum, P. griseofulvum, Trichothecium roseum, Aspergillus niger, Fusarium pallidoroseum* and *Acremonium implicatum* were discovered to be highly cellulolytic in comparison to other positive micromycetes. Their cellulase activity ranged from 0.42 to 0.76 µmol/ml/min. Maximum cellulase activity was shown by *T. viride.*

KEYWORDS: Micromycetes, Ant-hill, Congo red, Cellulase, Dinitrosalicylic acid, Parks and Gardens.

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INTRODUCTION

The biological diversity of this planet comprises of innumerable varieties of living organisms with multitude activities but none is more marvellous than the fungi. Among the various groups of known fungi, micromycetes occupy a prime place within the microorganisms as they are ubiquitous in distribution and play a vital role in ecosystem function. Micromycetes are the fungi, which have microscopic sporocarps and belong to Zygomycetes, Ascomycetes, Hyphomycetidae and Coelomycetidae. They are major decomposers in certain ecosystems and essential associates of many organisms. One such microhabitat (niche) of soil, which is comparatively less explored and in which less information is available is an ant-hill. According to Stone[1], "an ant-hill is a pile of earth, sand, or clay or a composite of these and other materials that build up at the entrances of the subterranean dwellings of ant colonies". Different ant species create nests varying in structure and size due to the difference in their feeding strategies. Most of the ants are generalist predators, scavengers and indirect herbivores but some have evolved specialised ways of obtaining nutrition by engaging in mutualistic symbiosis with fungi[2]. The ants provide the fungi certain proteolytic enzymes, which are present in their faecal matter and are in turn used for compensating the metabolic deficiency of the fungi during their growth[3]. On the other hand, ants are dependent on mycobiont partners because their food is essentially cellulosic material and since ants do not produce cellulose dissolving enzymes, the digestion of cellulose is carried out with the aid of associated micromycetes. Cellulases are extracellular and inductive in nature and are thus synthesised in significant amount only when inducing substrate is present[4]. Since this inducing substrate is present in surplus amount in the soil of ant-hills, it may form one of the most suitable habitat for cellulase producing micromycetes. These cellulolytic active micromycetes degrade organic matter aerobically in their natural habitat and thus catalyse cellulolysis. The result of cellulolysis is the conversion of organic substrate into inorganic form, which is made available once again to the living organisms. Therefore, in natural biodegradation processes of lignocellulosic material, fungal cellulase plays an important role. In addition, fungal cellulases have found novel industrial applications in areas such as protoplast production and fermentation of biomass into biofuels[5], animal feed production[6], production of fermentable sugars and ethanol[7], production of detergents and other chemicals[8], food processing[9], textile production[10], cellophane processing as well as biotransformation of cellulose containing waste to fermentable sugar[11], pulp and paper processing involving de-inking fibre surfaces and improvement of pulp drainage[12]. Virtually, all the fungi that have been reported so far as producers of cellulases are mesophilic in nature and are distributed in diverse habitats like soil, dead organic matter, plant and animal residue[13]. The present work concentrates on the isolation of cellulase active micromycetes from the ant-hill soils of various parks and gardens of Jammu City (India). The climate and geographical diversity of Jammu makes it a potential interesting habitat for studying the cellulase active micromycetes associated with the soil of ant-hills.

MATERIALS AND METHODS

Samples of ant-hill soil were collected from parks and gardens of Jammu city which is located at an altitude of 327 m above sea level and has a latitude and longitude of 32.73^{o}N and 74.87^{o} E. It has a subtropical type of climate, where summers are hot and humid (maximum temperature 45^{o}C), whereas winters are cold (minimum temperature 4^{o}C). The samples were collected with the help of properly sterilized spatula and were brought to the laboratory in pre-sterilised polythene bags for the isolation of micromycetes. Isolation of the micromycetes were made on Czapek Dox agar (CDA) medium following dilution pour plate technique[14]. The petriplates were incubated for 7 days at 28 ± 2^{o}C till the proper growth of the fungal colonies was obtained. The recovered fungal species were identified

by studying their cultural and morphological characters and using various keys and relevant literature.[15-21]

(***i) Screening cellulase active micromycetes from ant-hill soil***

Cellulase activity of the recovered micromycetes was determined by following the method of Pointing[22]. In this method, the micromycetes recovered from the soil of ant-hills were first grown in cellulolysis basal medium (CBM) for 5-6 days. Then agar discs were cut from the margin of the colony and put over CBM plated petriplate supplemented with carboxymethyl cellulose (CMC). Three replicates of each petriplate were incubated in darkness for 2-5 days till the colony attained a diameter of approximately 30mm. The petriplates were then flooded with 2% w/v aqueous Congo red (C.I 22120) and left for 15 minutes. The stain was then poured off and agar surface was rinsed with distilled water. The petriplates were again flooded with 1M NaCl to destain for about 15 minutes. Finally, NaCl solution was also poured off. The carboxymethyl cellulose degradation around the colony appeared as yellow opaque area against the red colour for undegraded carboxymethyl cellulase. Congo red stain binds with β-1-4 linked glycosidic bonds. Fungal strains producing cellulase will hydrolyse all cellulose around their colonies in CBM-CMC plate so that Congo red does not bind around these colonies and a clear yellow opaque zone appears. The diameter of yellow opaque area gives a direct qualitative estimate of the efficiency of cellulolytic activity of the test fungus.

(ii) Quantitative estimation of cellulase activity

Fungal species producing opaque zones were selected for the quantitative estimation of cellulase amount produced by them. First of all, production medium (CBM without agar) was prepared and about 100ml was put in each 250ml conical flasks. Then discs of 1cm diameter of the selected fungal cultures were cut and inoculated in the medium. The inoculated flasks were put on a rotary shaker for 5-6 days at 28ºC and then centrifuged at 10,000g for 10 minutes. The supernatant, which was enzyme equivalent was taken and assayed by dinitrosalicylic acid (DNSA) method as given by Miller *et al*.[23] Cellulase activity was determined by mixing 0.5ml of 1.0% (w/v) CMC (prepared in 0.5ml of 10mM phosphate buffer pH-7.0) with 0.5ml of crude extracellular enzyme source and incubating at 50ºC for 15 minutes[24]. The reaction was stopped by the addition of 1.5ml of 3,5-dinitrosalicylic acid (DNSA) and the contents were boiled for 10 minutes. The colour developed was read in a spectrophotometer (Analytik jena, Specord 200) at 575nm. The amount of reducing sugar liberated was quantified using glucose as standard. One unit of cellulase is defined as the amount of enzyme that liberates 1µmol of glucose equivalents per assay condition[25] and is calculated by 1 Enzyme Unit = µmol/ml/min.

RESULTS

Results presented in table 1 show that among the 40 recovered fungal species, 27 were positive for cellulase production. Such fungi primarily break the cellulose polymers with the aid of cellulase releasing glucose and nitrogenous compounds for their use. Among the 27 fungal species that were positive for cellulase production, large opaque zones were produced by *Trichoderma viride, T. harzianum, Trichothecium roseum, Penicillium chrysogenum, P. griseofulvum* and *Fusarium verticilloides* (Fig. 2). In addition to these, clear zones were also produced by *Aspergillus niger, A. japonicus, A. nidulans, Acremonium implicatum, Fusarium solani* and *F. pallidoroseum,* but the zones were comparatively smaller than those produced by the former group (Fig.3). As depicted in table 1, all the members of Zygomycetes (*Mucor hiemalis, M. microsporus* and *Rhizopus stolonifer*) and few Deuteromycetes (*A. fumigatus*, *A. ochraceous, A. versicolor, Cladosporium oxysporum, Paecilomyces lilacinus, P. victoriae, P. citrinum, P. oxalicum* and *Scopulariopsis brumptii*) did not show cellulolytic activity. The fungal species producing opaque zones were selected for quantitative estimation of cellulose by DNSA method. The addition of DNSA (3,5-dinitrosalicylic acid) is a redox reaction in

which DNSA reacts with reducing sugars at 90ºC in alkaline condition. Reducing sugars get converted into corresponding acids of sugars and 3,5-dinitrosalicylic acid gets converted into 3-amino,5-nitro-salicylic acid. 3,5-dinitrosalicylic acid, which has yellow colour that changes to orange red on reduction into 3-amino, 5-nitro salicylic acid (Fig. 1). The red colour was read spectrophotometrically and amount of cellulase was quantified. It was found that the amount of cellulase produced by 27 positive fungal species ranged from 0.08 to 0.76 µmol/ml/min (Table 1). *T. viride* showed the maximum cellulolytic activity (0.76 µmol/ml/min), followed in decreasing order by *T. harzianum* (0.68 µmol/ml/min), *P. chrysogenum* (0.54 µmol/ml/min), *Trichothecium roseum* (0.52 µmol/ml/min), *P. griseofulvum* (0.48 µmol/ml/min), *A. niger* (0.46 µmol/ml/min), *F. pallidoroseum* (0.44 µmol/ml/min) and *Acremonium implicatum* (0.42 µmol/ml/min). Among the fungal species studied for quantitative assessment of cellulase activity, *Cladosporium cladosporioides* showed the lowest activity (0.08 µmol/ml/min).

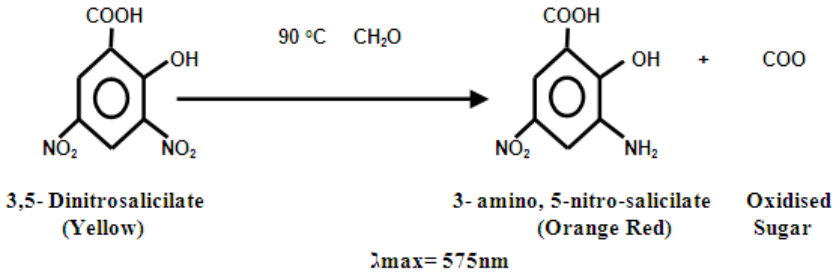


Figure 1
Redox reaction involving DNSA and reducing sugar.

Table 1
Production of cellulase by micromycetes recovered from soil of ant-hills.

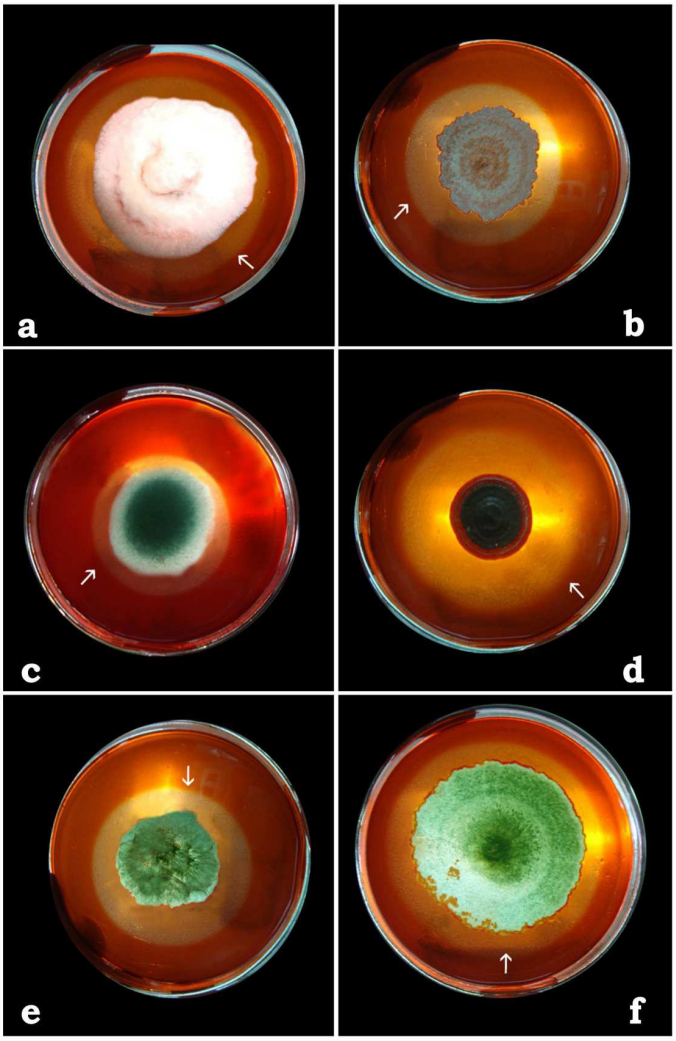
Micromycetes	Cellulase activity	Amount of cellulose produced (µmol/ml/min)
Acremonium implicatum	+	0.42
Alternaria alternata	+	0.30
Aspergillus candidus	+	0.31
A flavus	+	0.32
A. fumigates	-	-
A. japonicus	+	0.38
A. nidulans	+	0.36
A. niger	+	0.46
A. ochraceous	-	-
A. parasiticus	+	0.28
A. sydowii	+	0.22
A. terreus	+	0.24
A. versicolor	-	-
A. wentii	+	0.30
Cladosporium cladosporioides	+	0.08
C. oxysporum	-	-
Curvularia lunata	+	0.21
C. pallescens	+	0.18
Doratomyces purpureofuscus	+	0.19
Drechslera australiensis	+	0.26
Emericella nidulans var. *echinulatus*	+	0.26
Fusarium pallidoroseum	+	0.44
F. solani	+	0.36
F. verticilloides	+	0.31

Mucor hiemalis	-	-
M. microspores	-	-
Paecilomyces lilacinus	-	-
P. victoriae	-	-
Penicillium chrysogenum	+	0.54
P. citrinum	-	-
P. fellutanum	+	0.24
P. griseofulvum	+	0.48
P. oxalicum	-	-
P. waksmanii	+	0.20
Rhizopus stolonifer	-	-
Scopulariopsis brumptii	-	-
Trichoderma harzianum	+	0.68
T. viride	+	0.76
Trichothecium roseum	+	0.52
Sterile colony	-	-

+ , indicates presence of cellulase activity - , indicates absence of cellulase activity

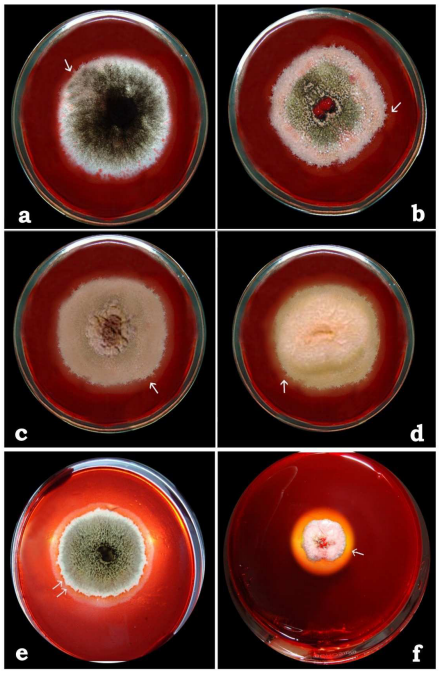
Figure 2

Cellulolytic active fungal species producing large opaque zones



a. Fusarium verticilloides ***b. Trichothecium roseum***
c. Penicillium chrysogenum ***d. P. griseofulvum***
e. Trichoderma harzianum ***f. T. viride***

Figure 3
Cellulolytic active fungal species producing small opaque zones



a. Aspergillus niger ***b. A. nidulans***
c. Acremonium implicatum ***d. Fusarium solani***
e. A. japonicus ***f. F. pallidoroseum***

DISCUSSIONS

The study clearly indicates that isolates of *Trichoderma viride, T. harzianum, Penicillium chrysogenum, P griseofulvum, Trichothecium roseum, Aspergillus niger, Fusarium pallidoroseum* and *Acremonium implicatum,* which were recovered from ant-hill soil were highly cellulolytic. Such fungi primarily break the cellulose polymers with the aid of cellulase and intricate exo-enzyme complex, releasing glucose and nitrogenous compounds for their use[26]. So far, cellulase activity of *Penicillium griseofulvum* that has been recovered from soil of ant-hills has not been reported but the isolate recovered from ant-hill soil possessed good activity. The role of some species of *Acremonium*, *Chaetomium*, *Trichoderma*, *Penicillium*, *Phanerochaete*, *Fusarium* and *Aspergillus* in the cellulose degradation process of various environments has been well documented[27]. Kader *et al*.[28] carried out scientific expedition to Bario highlands (Malaysia) and found two isolates of *Trichoderma* and *Aspergillus* that were highly cellulolytic in comparison to other fungal species. Similarly, Saxena *et al.*[29] found isolate of *Aspergillus niger* to be highly cellulolytic active and carried out a kinetic study on cellulase enzyme produced by it. Few other workers like Duncan *et al.*[30] screened cellulolytic fungi from Antarctica, whereas Makut and Godiya[31] carried a survey of cellulolytic mesophilic fungi from Nigeria soils. However, quantitative estimation of cellulase activity was not made by these workers. Recently, diversity of cellulolytic microfungi like *Alternaria alternata*, *Aspergillus nidulans*, *Trichoderma viride, Penicillium chrysogenum, Drechslera* sp., *Fusarium solani, Curvularia lunata* and *Acremonium implicatum* have been reported by Gautam *et al.*[32]. Incidentally, many of these fungal species have been detected to be cellulolytic during the present investigation also. While investigating the micromycetes of ant-hills, all the species of *Rhizopus* and *Mucor* were found to be negative for cellulase production but elsewhere cellulolytic activity of a soil isolate of *R. stolonifer* has been reported by

Lotfi *et al.* [33]. Similarly, during the present investigation, species of *Paecilomyces* (*P. lilacinus* and *P. victoriae*) were found to be negative for cellulase production but few other species such as *P. farinosus* and *P. inflatus* have been reported to be cellulolytic by Tribak *et al*. [34].

CONCLUSION

Through this investigation, it can be inferred that the strongly cellulolytic strains of *Trichoderma viride, T. harzianum, Penicillium chrysogenum, P griseofulvum, Trichothecium roseum, Aspergillus niger, Fusarium pallidoroseum* and *Acremonium implicatum,* which have been recovered from ant-hill soil can be used for the local management of solid wastes. In addition, these organisms can also be harnessed for the industrial production of enzyme cellulase that has utmost importance in textile, laundry, detergent, pulp and paper industries. However, further studies need to be carried on these eight organisms for determining the parameters required for their maximum enzyme activity.

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REFERENCES

1. Stone RH and Ndu FOC, New Biology for West African Schools, New York, Longmans Group Limited (1985).
2. Solomon SE, Mueller UG, Schultz, TR, Currie CR, Price SL, Oliveira da Silva-Pinhati AC, Bacci B and Vasconcelos, Nesting biology of the fungus growing ant *Mycetarotes emery* (Attine, Formicidae). Insectes Sociaux 51: 333-338 (2004).
3. Martin JS, and Martin MM, The presence of protease activity in the rectal fluid of attine ants. Journal Insect Physiology 16: 227-232 (1970).
4. Enari TM, Microbial cellulases. In Fogarty, W.F (ed) Microbial enzymes and biotechnology. Applied science Publishers. London pp. 183-223, (1983).
5. Mandels ML, Hontz L and Nistron J, Enzymatic hydrolysis of waste cellulose. Biotechnol Bioengineer 16: 471-473, (1974).
6. Ishikuro E, Free additives. Modern Media 46: 289-296, (1993).
7. Olsson L and Hagerdalu B, Fermentation of lignocellulose hydrolisates for ethanol production. Enzyme and Microbial Technology 18: 312- 331, (1997).
8. Oksonen T, Pere J, Paavilainen I, Buchert J and Vii Kari L, Treatment of recycled craft pulps with *Trichoderma reesei* cellulates and cellulases. Journal of Biotechnology 78: 39-48, (2000).
9. Penttila M, Limon C, Nevalainen H, Molecular Biology of *Trichoderma* and biotechnological applications. In: D. Arora (ed.). Handbook of Fungal Biotechnology. Inc, London. 20: 413-427, (2001).
10. Cavaco-Paulo A and Guibitz G, Catalysis and Processing In: Cavaco-Paulo A and Guibitz G (ed.) Textile Processing with enzymes. Woodhead Publishing Limited. pp. 86-119, (2003).
11. Van Wyk JPH and Mohulatsi M, Biodegradation of waste paper by cellulose from *Trichoderma viride*. Bioresource Technology 86: 21-23, (2003).
12. Suurnakki A, Niku-Paavola ML, Buchert J and Viikari L, Enzymes in pulp and paper processing. In: Achle W (ed.), Enzymes in Industry. Weinheim, Wiley-Vclt. pp. 437-439, (2004).
13. Makut MD and Godiya EM, A survey of cellulolytic fungi in the soil environment of Keffi Metropolis, Nasarawa State, Nigeria. African Journal of Microbiology Research 4: 2191-2195 (2010).

14. Tauro P, Kapoor KK and Yadav KS, Introduction to microbiology, New Age International (P) Limited, New Delhi pp. 1-413 (1986).
15. Rapper EB and Fennel DI, The genus *Aspergillus*. The Williams and Wilkins Company, Baltimore, USA. pp. 132-575 (1965).
16. Booth C, The genus *Fusarium*. Commonwealth Mycological Institute Kew, Surrey, England. pp. 1-237 (1971).
17. Ellis MB, Dematiaceous Hyphomycetes. Commonwealth Mycological Institute Kew, Surrey, England. pp. 1-608 (1971).
18. Barron GL, The genera of Hyphomycetes from soil. Robert E. Krieger Publishing Company, Huntington, New York. pp. 1-364 (1972).
19. Ellis MB, More Dematiaceous Hyphomycetes. Commonwealth Mycological Institute Kew, Surrey, England. pp. 1-507 (1976).
20. Pitt JI, The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces.* Academic Press, London. pp. 1-634 (1979).
21. Domsch KH and Gams W and Anderson TH, Compendium of soil fungi. Academic Press, London. pp. 1-859 (1980).
22. Pointing SB, Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi. Fungal Diversity 2: 17-33, (1999).
23. Miller GL, Blum R, Glennon WE and Burton AL, Measurement of carboxymethyl cellulase activity. Analytical Biochemistry 2: 127-132, (1960).
24. Casimir SJ, Davis S, Flechter A, Gysin B, Murray E, Perrolaz JJ and Zimmerman WS, Pulp bleaching with thermostable xylanase of *Thermomonospora fusca*. US patent (1996). (*http://www.google.com/patents/US5407827*).
25. Mandels M, Medeiros JE, Andreotti RE and Bissett FH, Enzymatic hydrolysis of cellulose: evaluation of cellulase culture filtrates under use conditions. Biotechnology and Bioengineering 23: 2009-2026, (1981).
26. Caneva G, Nugari MP and Salvadori O, Biology in the conservation of works of art Rome : 1 CC ROM pp. 26-43, (1991).
27. Bhat M and Bhat S, Cellulose degrading enzymes and potential industrial applications. Biotechnology Advances 15: 583-620, (1997).
28. Kader AJ, Omar O and Feng LS, Isolation of cellulolytic fungi from the Bario Highland, Sarewak. ASEAN Review of Biodiversity and Environmental Conservation. pp. 1-3, (1999).
29. Saxena A, Saxena A and Sarika Y, a kinetic study on cellulase enzymes from *Aspergillus niger*. International Journal of Pharma and Bio Sciences 2: 36-40, (2011).
30. Duncan SM, Farrell RL, Thwaites JM, Held BW, Arenz BE, Jurgens JA and Blanchette RA, Endoglucanase-producing fungi isolated from Cape Evans historic expedition hut on Ross Island, Antarctica. Environmental Microbiology 8: 1212-1219, (2006).
31. Makut MD and Godiya EM, A survey of cellulolytic fungi in the soil environment of Keffi Metropolis, Nasarawa State, Nigeria. African Journal of Microbiology Research 4: 2191-2195, (2010).
32. Gautam SP, Bundela PS, Pandey AK, Jamaluddin, Awasthi MK and Sarsaiya S, Diversity of cellulolytic microbes and the biodegradation of municipal solid waste by a potential strain. International Journal of Microbiology (doi:10.1155/2012/325907), (2012).
33. Lotfi A, Ghanbary MAT, Ranjbar GA and Asgharzadeh A, Screening of some Zygomycetes for cellulase activity. African Journal of Biotechnology 9: 4211-4216, (2010).
34. Tribak M, Ocampo JA and Romera IG, Production of xyloglucanolytic enzymes by *Trichoderma viride*, *Paecilomyces farinosus*, *Wardomyces inflatus*, and *Pleurotus ostreatus.* Mycologia 94: 404-410, (2002).