Int J Pharm Bio Sci 2013 Oct; 4(4): (B) 1129 - 1138

Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

OPTIMIZATION OF PROCESS PARAMETERS FOR BIOSYNTHESIS OF CELLULASE BY *CLADOSPORIUM CLADOSPORIOIDES* USING AGRO WASTES.

ISAIE MUSHIMIYIMANA AND *PADMAVATHI TALLAPRAGADA

Department of Microbiology, Centre of PG Studies, Jain University,
18/ 3, 9th Main, Jayanagar 3rd Block, Bangalore, India

ABSTRACT

Cellulases play an important role in biomass utilization. Many fungi produce cellulases with high activities. The aim of this study is to describe the optimization of process parameters for the production of extracellular carboxymethyl cellulase (CMCase) by *Cladosporium cladosporioides*. Cellulase production was carried out using different agro wastes as substrates like potato peel, onion peel, carrot peel and sugar beet peel. The enzyme produced and its activity was determined under different process parameters like incubation period, pH, temperature and effect of carbon and nitrogen sources. A submerged type of fermentation was carried out for the enzyme production with the substrate concentration of 5.8 % (w/v) along with ammonium nitrate of 0.8% w/v as nitrogen source. The highest cellulase activity was observed with potato peel on 6th day and for onion peel, carrot peel and sugar beet peel was on 7th day of incubation. The highest cellulase activity was observed with sugar beet at the pH of 5.5 (1.56 U/ml) and at temperature 30ºC (1.65U/ml). With different carbon and nitrogen sources, the highest enzyme activity was observed with maltose and sugar beet as substrate (2.24U/ml) and with urea and onion peel as substrate (2.45U/ml).

KEY WORDS: **Agro wastes, *Cladosporium cladosporioides*, Cellulase, optimization.**

PADMAVATHI TALLAPRAGADA
Department of Microbiology, Centre of PG Studies, Jain University,
18/ 3, 9th Main, Jayanagar 3rd Block, Bangalore, India

INTRODUCTION

Cellulase is an important industrial enzyme which can be obtained from economical agro-wastes and could be used in food industries, animal feed industries, brewing and wine making industry, agriculture biomass refining, pulp and paper industries, textile and laundry industries and ethanol production. Researchers are interested in cellulases production because of their applications in various industries [1,2]. A cellulosic enzyme consists of three main components which can degrade cellulose by the synergistical action of these enzymes: endoglucanase also called as carboxymethyl cellulase (CMCase) (endo-1,4-β-Dglucanase, EG, EC 3.2.1.4); exoglucanase (also called ascellobiohydrolase) (exo-1,4- β -D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1,4- β -Dglucosidase, BG, EC 3.2.1.21) [3, 4]. India is an agriculture country, producing tons of agro-waste in the form of crop tubers and bulbs. Onion, potato, carrot and sugar beet are the most important vegetable used as daily food and grown in tropical and subtropical climate zone. Agro-wastes are one of the most important environmental pollutants in the world today. Agro-wastes when used as substrate can produce economical cellulase when compared to using pure form of cellulose[5]. Utilization of agro wastes for the production of enzymes from microorganisms is the one way to resolve the environmental pollution problems[6].

Cellulose is considered the most abundant and beneficial biopolymer in the world[7]. Soil, water, food wastes and decaying wood are the most natural environmental habitats of cellulose degrading microorganisms[8]. *Aspergillus* or *Trichoderma sps* are the best fungi that produce cellulases under submerged cultivation systems[9,10]. *Cladosporium cladosporioides* is one of the most common species with worldwide distribution, present in many different decaying plants, air, soil, textiles, food wastes, debris and numerous other substrates[11,12,13,]. Agro wastes and food processing wastes are available in mass quantities all over the world, which largely become a source of chronic disease and environment problem. These wastes contain cellulose (30-40%), hemicellulose (xylan 20-40%), and lignin (20-30%)[15].

Microbial enzymes can be produced by submerged fermentation technique to produce cellulases which are produced by different microorganisms. Many microorganisms, such as fungi and bacteria produce cellulose degrading enzyme [16.] Enzymes could be produced by submerged state fermentation (SmF) because it involves in the production of enzymes by microorganisms in a liquid nutrient media. Carboxy Methyl Cellulose (CMC) was used for isolation of cellulolytic fungi. Many reports described using different agriculture wastes for cellulase production such as rice bran[17], wheat straw [18], banana waste[19] and food process wastes like oil palm[20], and apple wastes[21] for cellulase production. Production of cellulase depends on the type of substrate, pretreatment, medium and strain of microorganism used[22]. In the present study we have reported the utilization of four agro wastes such as potato peel, onion peel, carrot peel and, sugar beet peel for cellulase production with *Cladosporium conidiosporoides* in submerged fermentation by optimizing various processes parameters.

MATERIALS AND METHODS

Isolation

Agro wastes, such as Potato peel, carrot peel, onion peel and, sugar beet peel were collected from Bangalore Restaurants, which was used for isolation of fungi by serial dilution method. From the appropriate dilutions, 0.1 ml was spread on the Potato dextrose agar (PDA) plate[23]. The plates were incubated for 7 days at 28±2 °C. Different colonies of fungi obtained were identified and maintained on PDA slants.

Isolation of cellulolytic fungi using Carboxy methyl cellulose (CMC)

This assay was a good indicator to check the cellulolytic ability of fungi. Cellolysis basal medium (CBM) agar plates were prepared, supplemented with 1% w/v of CMC and 1. 7 % w/v agar. The isolated fungal cultures were spot inoculated and incubated at 28 ^{o}C for 7 days. After incubation, agar plates were flooded using Iodine solution for detection of cellolytic activity. If a zone of clearance was seen then the fungal culture is positive for cellolytic activity.

Identification

Isolated fungal culture was identified depending on its morphological, culture, biochemical and physiological characteristics as *Cladosporium cladosporioides*. The results were confirmed by National Fungal Culture Collection of India (NFCCI), Pune.

Pretreatment of Substrates and media composition.

Four different substrates such as potato peel, carrot peel, onion peel and sugar beet peel which were collected from different parts of Bangalore (India), were used as substrates for the cellulase production. The peels were first sun dried, oven dried, milled and sieved. These sieved substrates were used for submerged fermentation. The production medium consisted of 3.5 g (5.8%w/v) of the substrate and 0.48g of ammonium nitrate as nitrogen source with 60ml of water in a 150ml conical flask.

CMCase activity assay

Carboxyl methyl cellulase activity was determined following the method[24]. Reaction mixture comprised of 0.5 ml carboxyl methyl cellulose (1 % w/v) in 0.05 M citrate buffer of pH 4.8 and 0.5 ml crude enzyme solution in test tubes. Mixture was incubated at 28 °C for 15 min. After incubation 1ml of Dinitro-salicylic acid (DNS) reagent was added to stop the reaction. The reactants in test tubes were incubated for 10 min in a water bath at 100 ^{o}C and cooled. After cooling, 8ml of distilled water was added and absorbance of the samples was measured at 540 nm using a colorimeter. One unit of CMCase enzyme activity was expressed as 1 µ mole of glucose released per ml enzyme per minute[24].

Optimization of factors affecting enzyme activity

Different experimental set up were maintained to check the effect of incubation time, effect of pH, temperature and carbon and nitrogen source on the fungi for the production of cellulase[25].

Effect of incubation period

To evaluate the effect of different incubation period on cellulase production, the flasks with diferent subtrates were incubated at 28°C for different time periods ranging from 1 to 7 days intervals. Samples were taken periodically at every 24 hours and assayed for cellulase activity.

Effect of pH

The effect of pH on enzyme production was studied under various levels of pH (4.5, 5.5, 6.5 and 7.5) in production medium. The pH was adjusted using 1N HCl and1N NaOH solutions.

Effect of incubation temperatures

To study the effect of different incubation temperatures on cellulase production, the flasks were kept at temperatures ranging from 20°C to 50°C at every 10 ^{0}C intervals.

Effect of Carbon and Nitrogen sources on enzyme production

Experiments were carried out to investigate the effect of different nitrogen sources at 0.5% (w/v) such as urea, peptone, and yeast extract and ammonium sulphate. Carbon sources such as lactose, fructose and maltose on the production of cellulase enzyme. These carbon and nitrogen sources were supplemented to the production medium.The enzyme activity was determined by DNS method for all the above parameters [24].

RESULTS AND DISCUSSION

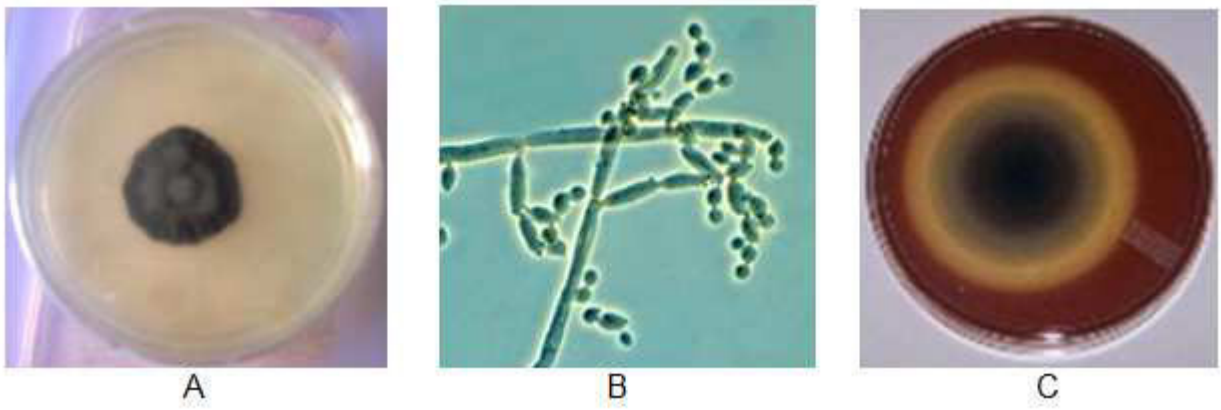
Isolation and Identification

The isolated fungal culture was identified as *Cladosporium cladosporioides* by preparing a wet mount using lactophenol cotton blue. And it was confirmed as Cladosporium cladosporioides by NFCCI, Pune. *Cladosporium cladosporioides* produced colonies off white felt with brownish to dark-green color asexual spores; Conidia of *Cladosporium cladosporioides* in general are elliptical to cylindrical in shape, pale to dark brown in color and have dark hila. They occur in branching chains that readily disarticulate. *Cladosporium cladosporioides* produces unicellular conidia. (Fig 1A&1B)

Screening of Fungi for Cellulase Activity

Cladosporium cladosporioides was screened for cellulase activity using CMC agar (selective agar) and showed a zone of clearance after flooding and destaining with iodine solution. *Cladosporium cladosporioides* was positive for CMCase. The isolated fungi showed the hallow zone of 100mm on the CM-cellulose agar media.(Fig 1C).

Figure 1



A: Morphology of Cladosporium cladosporioides
B: Microscopic view.
C: Zone of clearance with Iodine solution

Optimization of culture conditions

Incubation time

The production of cellulase enzyme increased with increase in incubation time. The highest amount of cellulase was recorded on 6th day for potato peel with maximum cellulase activity (8.9 U/ml) (Figure-1); and carrot peels, onion peels and sugar beet peels showed maximum cellulase axctivity on 7th day with maximum cellulase activity of (32.3U/ml); (25.1 U/ml) and (23.3 U/ml) respectively (Figure-2). The production of enzyme was related to the incubation period. Carrot peel produced large amount of cellulase enzyme upon 7 days of incubation, whereas potato peel produced small amount of cellulase enzyme upon 6 days of incubation. S.W. Kang.;et al (2004) [26] reported that *A.niger* and *Trichoderma* produced favorable commercial enzyme on 4th and 6th days of fermentation. Guatam et al (2010) [27] reported the production of CMcase (endoglucanase) was (1.84U/mL) after 4 days of incubation with A.niger.

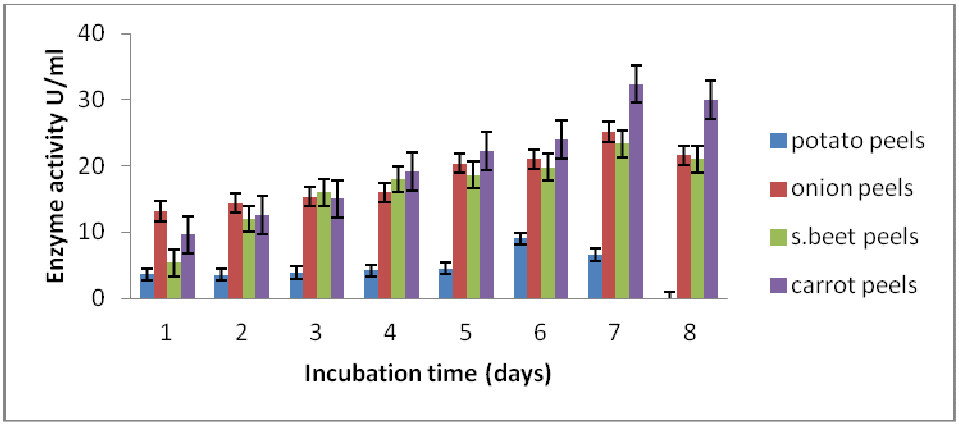


Figure 2
Effect of incubation time on enzyme activity by using different substrates

Effect of pH on enzyme production

The enzyme activity varied with the pH (Figure-3). For the substrate potato peel, the activity of the enzyme increased up to pH 6.5, which showed the highest activity (0.88U/ml) and decreased at the pH of 7.5. Similarly the enzyme activity for the onion peel substrate increased up to the pH 6.5 with the highest activity (0.84U/ml) and decreased at the pH of 7.5. With the carrot peel as the substrate the enzyme activity showed a gradually increase till the pH 7.5 which had the highest activity (1.36U/ml). With the sugar beet peel as substrate the enzyme activity was highest with the pH of 5.5 (1.56U/ml) and after which the activity decreased with the increase in pH. In comparison with the other substrates the sugar beet peel with pH 5.5 had the highest activity of the enzyme cellulase. This result suggests that the cellulase activity depends on the types of microorganisms, fermentation, different substrate and the pH conditions [22]. In the present study, *Cladosporium cladosporioides* isolates were able to grow at a wider pH ranges between 5.5-7.5. This result is considerably similar to reports of Xing-hua et.; al. (2010) [28] ,the optimum pH for cellulase production from *T. viride* was at pH 5. Optimum pH for fungal cellulase varies from species to species and with different substrates. This might be due to the fact that fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis [29]. [30]Tolan et.; al.(1999), reported that Cellulases which are active in the acidic pH range (4.8-6) are considered to be suitable for industrial application such as stone washing denim (pH 4-7), paper industry (pH 5), animal feed supplement (acidic pH) and textile industry.

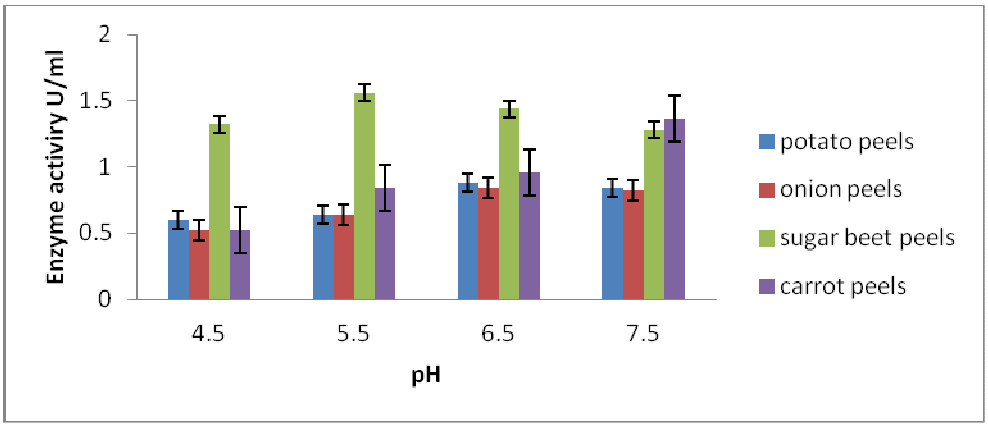


Figure 3
Effect of pH on enzyme activity by using different substrates

Effect of temperature

The effect of temperature on cellulase activity (Figure-4) is another important factor. For the substrate potato peel and onion peel, the enzyme activity increased at 40 ^{0}C, which showed the highest activity of (0.98U/ml) and (1.12U/ml) respectively and decreased for both at the 50 ^{0}C. Similarly for carrot peel and sugar beet peel, the enzyme activity was (0.85U/ml) and (1.65 U/ml) at 30 ^{0}C respectively and the activity decreased with increase in temperature. In comparison with other substrates, sugar beet peel exhibited highest cellulase activity at 30^{0}C.

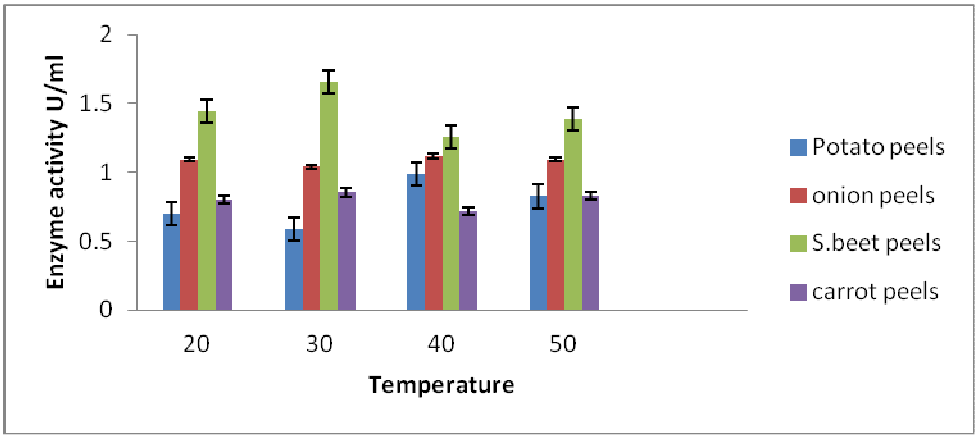


Figure 4
Effect of temperature on enzyme activity using different substrates

With *Cladosporium cladosporioides* maximum cellulase production was obtained at 30^o and 40 ^{0}C. This might be due to better growth of the isolates at this temperature. This result is considerably analogous to what was reported by Shafique and Bajwa. (2009)[31]who indicated that the optimum temperature for maximum cellulase production for *T. reesei* was 30^{o}C. However, the results appeared to be contradicting with results reported by Gautam et.; al. (2010) [27] who observed that the optimum temperature for cellulase production under SmF with municipal waste compost is between 40-50^{o}C for *T. viride*. S. Murao et.; al (1988)[32], W.Lu et al.(2004)[33], reported that the cellulase production by temperature depends on the strain of microorganism. Here in the present study, it is evident that 30°C is

optimum for the production of cellulase enzyme with sugar beet peel used as substrate.

Effect of Nitrogen sources on enzyme production.

The potato peel, sugar beet and onion peel were observed to have the highest enzyme activity with urea (1.47U/ml), (1.73U/ml) and (2.45U/ml) respectively and lowest enzyme activity with potato peel, onion peel and sugar beet peel were (1.12 U/ml) and 1.3 U/ml), and (1.5 U/ml) with ammonium sulphate. Carrot peel presented highest enzyme activity with ammonium sulphate at (1.25 U/ml) and the lowest enzyme activity found at (1.04 U/ml) with Yeast extract. In comparison with others substrates onion peel was the highest enzyme activity with urea (Fig-5). [27]Gautam et.; al. (2010), reported that the highest cellulase production with A. niger was (1.44 U/ml) with peptone. The enzyme production is affected significantly by different organic nitrogen sources. The production of cellulase is able to catalyze the nitrogen sources and nitrogen level in the medium[34]. The result of the present study showed that the sources have different effect on the enzyme activity. Among the nitrogen sources tested, the enzyme activity was high with Urea in onion peels.

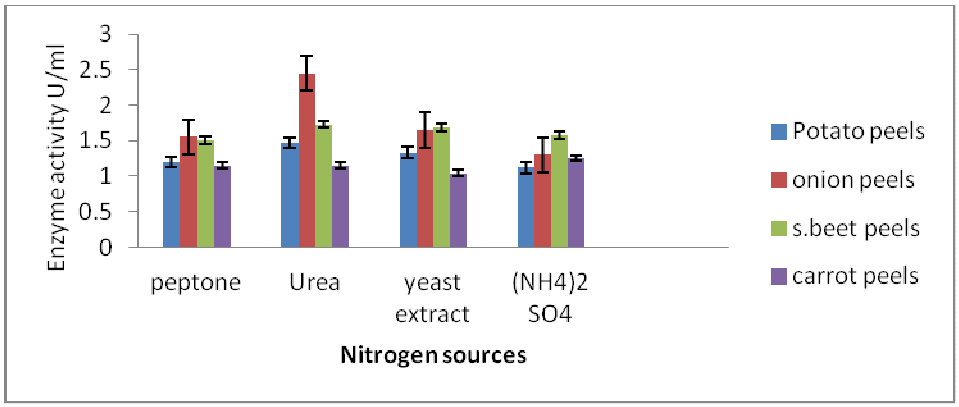


Figure 5

Effect of nitrogen sources on enzyme productivity by using different substrates

Effect of Carbon sources on enzyme production

Potato peel displayed highest enzyme activity (1.28 U/ml) with fructose, and the lowest enzyme activity found with maltose at (0.98U/ml). Onion peel, sugar beet peel showed enzyme activity with maltose (1.81 U/ml), (2.24U/ml) respectively. The lowest enzyme activity with onion peel and sugar beet peel found at (1.76 U/ml) with fructose and (1.41U/ml) with lactose for sugar beet peel. Sugar beet peel was the highest in enzyme activity with maltose compared to all the other substrates (Fig 6). Szakacs et.; al (2006)[35], Baig (2005)[19] have reported that glucose and fructose represses the production of cellulase activity and lactose, avicel and CMC induced the production of cellulase by *Trichoderma* spp.

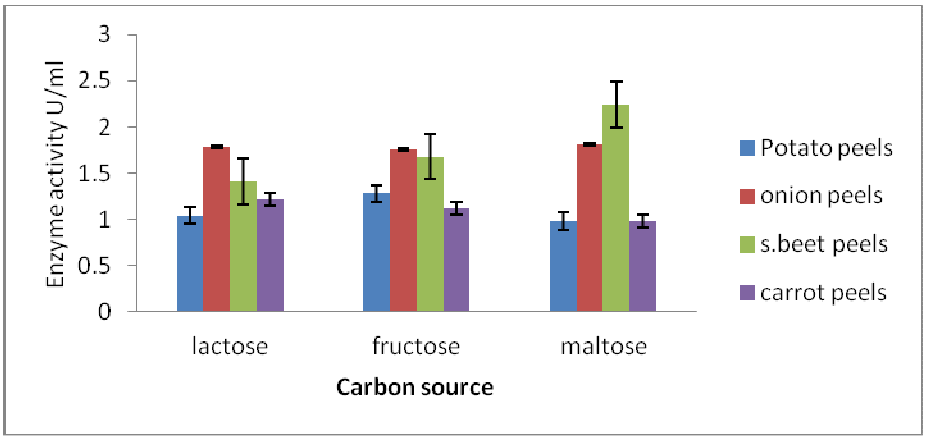


Figure 6
Effect of carbon sources on enzyme activity by using different substrates

CONCLUSION

In the present study we have isolated and identified efficient fungi producing cellulase from environment rich in cellulose. The fungus isolated was identified as *Cladosporium cladosporioides.* It showed a potential to produce cellulase using agro wastes as a substrate and its enzyme production efficiency was increased by optimization of culture conditions and media components. The potato peel, onion peel, carrot peel and, sugar beet peel, may be a good alternative for cellulase production from agro wastes which are very cheap and available in abundant.

ACKNOWLEDGEMENT

We would wish to acknowledge ofJain University, management Bangalore, providing laboratory facilities to accomplish this work.

REFERENCES

1. Jamil, A., Naim, S. Ahmed S. and Ashraf, M. Production of Industrially important enzymes using molecular approaches; cellulases and xylanases. In: Genetic resources and Biotechnology II, Volume Two, eds by D. Thangadurai, T. Pullaiah, Pedro A. Balatti. Regency publications, New Delhi. (2005).
2. Ahmed, S., Aslam, N. Latif, F. Rajoka M. I. and Jamil. A. Molecular cloning of cellulose genes from Trichoderma harzianum. (Eds.): Atta-ur-Rehman/ Choudhary/ Khan, Frontiers in Natural Product Chemistry. Bentham Science Publishers. The Netherlands. 1: 73-75 (2005).
3. Li, Y. H., Ding, M. Wang, J. Xu, G. J. and Zhao, A novel thermoacidophilic endoglucanase, Ba-EGA, from a new cellulose degrading bacterium, Bacillus sp. AC-1. Appl. Microbiol. Biotechnol. 70: 430–436 (2006)
4. Gao, J., Weng, H. Zhu, D. Yuan, M. Guan F.and Xi. Y. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal Aspergillus terreus M11 under solid-state cultivation of corn stover. Bioresour. Technol. 99: 7623–7629 (2008)

5. Chahal DS. Solid state fermentation with Trichoderma reeseifor cellulose production. Appl Environ Microbiol. 49: 205-210 (1985).
6. Pothiraj, C. and. Eyini, M. Enzyme activities and substrate degradation by fungal isolates on cassava waste during solid state fermentation. Mycobiology, 35: 196-204 (2007)
7. Schlesinger WH.. Biogeochemistry: an Analysis of Global Change. San Diego: Academic. 443 pp. (1991)
8. Conclin A.R., Jr., Soil Microorganisms. AEHS Magazine, 1Ð2 (internet) (2002)
9. K. Chandel and O. V. Singh, Appl. Microbiol. Biotechnol. 89, 1289 (2011).
10. R. R. Singhania, R. K. Sukumaran, A. K. Patel, C. Larroche, and A. Pandey, Enzyme Microb. Technol. 46, 541 (2010).
11. El-Morsy EM. Fungi isolated from the endorhizosphere of halophytic plants from the Red Sea Coast of Egypt. Fungal Diversity 5: 43–54 (2000).
12. Ellis, M.B. Dematiaceous Hyphomycetes. Commonwealth Mycological Institute: Kew Surrey, UK. (1971)
13. Kumaresan V, Suryanarayanan TS. Endophyte assemblage in young, mature and senescent leaves of Rhizophora apiculata: evidence for the role of endophytes in mangrove litter degeneration. Fungal Diversity 9: 81–91 (2002).
14. Riesen T, Sieber T. Endophytic fungi in winter wheat (Triticum aestivum L.). Swiss Federal Institute of Technology, Zürich (1985).
15. Deschamps F, Huet MC. Xylanase production in solid-state fermentation: a study of its properties. Appl.Microbial. Biotechnol.22:177-180 (1985)
16. Kim KC, Yoo SS, Oh YA, Kim SJ. Isolation and characteristics of T. harziamum FJ1 producing cellulases and xylanase, J. Microbial. Biotechnol, 13: 1-8 (2003).
17. Lee, B.H, B.K Kim, Y.J Lee, C.H Chung and J.W. L Industrial scale of optimization for the production of carboxymethylcellulase from rice bran by marine bacterium, Bacillus subtilis subsp. Subtilis, A-53. Enz. Microb. Technol., 46:38-42 (2010).
18. Singh, A., N. Singh and R. Bishnoi. Production of cellulase by Aspergillus Heteromorphus from wheat straw under submerged fermentation. Inter. J. Env.Sci.Eng., 1:23-26 (2009).
19. Baig, M.M.V. Cellulolytic enzyme of trichoderma lignorum production on banana agro waste: Optimization of culture medium and condition. J. Sc and Ind Research., 64:57-60 (2005).
20. Alam, M.Z.,N. Muhammad and M.E. Mahmat. Production of cellulase from oil palm Biomass as substrate by solid state bioconversion. American J. appl. Sc., 2: 569-572 (2005).
21. Hang, Y.D and E.E. Woodams. Apple Pomace: A potential substrate for production of β-glucosidase by Asprgillus foetidus, Lebnsm. Wiss.u. technol., 27: 587-587 (1994).
22. Ali, S., A. Sayed, R.T. Sarker and R. Alam. Factors affecting cellulose production by Aspergillus terreus. World J. Microbiol and Biotechnol., 7: 62-66 (1991).
23. Oxoid. Manual of Culture Media, Ingredients and other Laboratory Services. Published by Oxoid Limited, Wade Road, Basingstoke, Hampshire RG24 OPW. UK (1982).
24. Miller, G.L. Use of dinitrosalicyclic acid reagent for determination of reducing sugar, Anal. Chem., 31: 426-428, 1959.
25. Teodor Vintila, Veronica Croitoriu, Monica Dragomirescu, Dragos Nica, Animal Science and Biotechnologies. 43 (1), 337 (2010).
26. S. W. Kang, Y. S. Park, J. S. Lee, S. I. Hong, and S. W. Kim, “Production of cellulases and hemicellulases by Aspergillus niger KK2 from lignocellulosic biomass,” Bioresource Technology, vol. 91, no. 2, pp. 153–156, (2004).
27. Gautam, S. P., Bundela, P. S., Pandey, A. K., Jamaluddin, Awasthi, M. K. and Sarsaiya, S. Cellulase production by

pseudomonas spp. isolated from municipal solid waste compost. Int. J. Academic Research, 2:330-333 (2010).

28. Xing-hua Lia, Hua-junYanga, BhaskarRoya, EnochY.Parkb, Li-junJianga, Dan Wanga, Yun- gen Miaoa. Enhanced cellulase production of the Trichoderma viride mutated by microwave and ultraviolet.Micribiological research, 160:190-168 (2010)
29. Haltrich, D., Nidetzky, B., Kulbe, K.D., Steiner, W. and Zupaneie, S. Production of fungal xylanases. Bioresource Techn., 58:137-161 (1996).
30. Tolan, J. S. and Foody, B.Cellulase from Submerged Fermentation. Advances in Biochemical Eng. Biotech., 65: 42-66 (1999).
31. Shafique, S. and Bajwa, R. Cellulase biosynthesis by selected Trichoderma species. Pak. J. Bothech., 41: 907-916 (2009).
32. S. Murao, R. Sakamoto, and M. Arai, “Cellulase of Aspergillus aculeatus,” in Methods in Enzymology, W. A. Wood and S. T. Kellog, Eds.Academic Press, London, UK, vol. 160, pp. 275–284 (1988).
33. W. Lu, D. Li, and Y. Wu, “Influence of water activity and temperature on xylanase biosynthesis in pilot-scale solid-state fermentation by Aspergillus sulphureus,” Enzyme andMicrobial Technology, vol. 32, no. 2, pp. 305–311(2003).
34. Desai, J.D., A.J. Deasi and N.P.Patel. Production of cellulose and glucosidases by shake culture of Scytaidum lignicoia. Journal of Fermentation Technology, 60: 117-124 (1982).
35. Szakacs, G., Tengerdy, R. T. and Nagy, V. (2006). Cellulases. In: Enzyme Technology ed., pp (Pandey, A., Colin, W., Soccol, C. R. and Lorroche, C., eds). Spring Science +Besiness Media, Inc. and Asiatech) (2006).