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ISOLATION, SCREENING AND PARTIAL PURIFICATION OF CELLULASE FROM CELLULASE PRODUCING FUNGI

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ABSTRACT

The present investigation was undertaken to isolate and Screen the Cellulase Producing fungi. Microbial cultures were isolated from the soil sample collected from different villages of saline belt of Akola and Buldhana District, Maharashtra India. A Total of 146 isolates were isolated and identified based on Morphology and Biochemical characterization. Among all isolated organisms 72 fungal species were isolated, the one cellulolytic bacterial isolate shows maximum enzyme activity, and identified as *Aspergillus niger*. The best conditions like pH, carbon source, temperature and incubation period were also observed for cellulase producing organisms. Carboxy methyl cellulose [1.0% (w/v)] were found to be the best carbon source for the production of cellulase from *Aspergillus niger* and Optimum temperature and pH of the medium for the cellulase production were 40°C and 6, whereas it shows maximum enzyme activity after fifth day of incubation period. Further partial purification of cellulase was carried out by dialysis and ammonium sulphate precipitation and also to determine molecular weight by SDS-PAGE. The partial purification of the cellulase enzyme produced by *Aspergillus niger* had protein bands with the molecular weight of 45 kDa and FTIR was performed.

KEYWORDS: *Aspergillus niger*, Cellulase, Partial purification.

INTRODUCTION

Cellulase is a generic name for the group of enzymes which catalyze the hydrolysis of cellulose and related cellu-oligosaccharide derivatives. Cellulose consists mainly of long polymers of β 1-4, linked glucose units and forms a crystalline structure.[1] The cellulase complex is comprised of three major components: Carboxymethyl cellualase (CMCases) or Endo-ß-glucanase (EC 3.2.1.4), Exo-ß-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21).[2and3] This enzyme is produced by several microorganisms, commonly by bacteria and fungi.

Cellulases have attracted much interest because of the diversity of their application. The major industrial applications of cellulases are in textile industry for 'bio-polishing' of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness.[4] Cellulase isused in the fermentation of biomass into biofuels.[5] Fibre modification and they are even used for pharmaceutical applications. Application of enzymes in textile, food, detergent, leather and paper industries demands identification of highly stable enzymes active at extreme pH and temperature.[6]

The aim of this study was to isolate and screening of cellulase producing fungi from Soil. optimization of conditions, production of cellulase, partial purification determination of Molecular weight.

MATERIALS AND METHODS

Isolation and screening of Cellulase producing fungi

Fungi were isolated from the soil sample collected from, different villages of saline belt of Akola and Buldhana District, Maharashtra India. Serial dilution method was used for the isolation of cellulolytic fungi. The medium used contains 1.0 % peptone, 1.0 % carboxymethyl cellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and pH 7. The Plates were incubated for 48 hours at 30°C. Samples were spread on the surface of potato dextrose agar and incubated for 7 days at 28ºC. Colonies were picked and sub-cultured to obtain pure culture. PDA Plates were spot inoculated with spore suspension of pure culture and incubated at 30ºC.After 3 days, plates were flooded with 1% Congo red solution for 15 minutes then de-stained with 1M NaCl solution for 15 minutes. The diameter of zone of de-colorization around each colony was measured. The fungal colony showing largest zone of de-colorization was selected for cellulase production and the isolated strains were carefully identified by morphological characteristics include color of the colony and growth pattern studies, as well as their vegetative and reproductive structures observed under the microscope.[7]

Inoculum development
Pure cultures of selected bacterial isolates were inoculated in broth medium containing basal salt medium containing 1% Carboxy methylcellulose (CMC) as a sole carbon source and incubated at 28 ± 2°C for 4-8 days for the fungi .After 24h of fermentation period these vegetative cells were used as inoculum source.

Submerged fermentation process
The Identified cellulolytic species were screened for cellulase enzyme production in submerged fermentation process**,** having composition of basal salt medium. The flasks were incubated at 28°C at 200 RPM for 4-5days.[7]

Preparation of Crude enzyme
After termination of the fermentation period the fermented broth was centrifuged at 6000 RPM for 15 min at 4˚C and supernatant was used as crude cellulase source.[7]

Enzyme Assay
Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method.[8] Aliquots of appropriately diluted cultured filtrate as enzyme source were added to Whatman's no. 1 filter paper strip (1 × 6 cm; 50mg) immersed in one milliliter of 0.05M Sodium citrate buffer of pH 5.0. After incubation at 50°C for 1 hr, the reducing sugar released was estimated by dinitrosalicylic acid (DNS) method.[9] One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 μmole of reducing sugar from filter paper per ml per min. Endoglucanase activity (CMCase) was measured using a reaction mixture containing 1mL of 1% carboxymethyl cellulose (CMC) in 0.5M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50°C for 1 h, and the reducing sugar produced was determined by DNS method. β-glucosidase activity was assayed by the method of.[10] One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µ mole of reducing sugar per min.

Optimization of Culture Conditions for Enzyme Production

Effect of Incubation Period on Enzyme Production
Fermentation period was an important parameter for enzyme production by *Aspergillus niger*. In this study, fermentation experiment was carried out up to 7 days and production rate was measured at 24 h intervals.

Effect of Temperature on Enzyme Production
In order to determine the effective temperature for cellulase production by *Aspergillus niger* fermentation was carried out at 10°C intervals in the range of 20 to 80°C.

Effect of pH on Enzyme Production
To determine optimal pH, *Aspergillus niger* was cultivated in a 150mL flask containing 50 mL optimized medium with different pH ranges from 3.0 to 8.0. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The flasks were kept in stationary stage at 28°C for 4-7 days of cultivation.

Effect of Temperature on Enzyme Production
In order to determine the effective temperature for cellulase production by *Aspergillus niger* fermentation was carried out at 10°C intervals in the range of 20 to 80°C.

Effect of substrate concentration.
To evaluate the effect of substrate concentration on cellulase production the production medium was supplemented with different concentration of CMC including, 0.2%, 0.5%, 1% and 1.5%.[11]

Partial purification of cellulase
Crude extract of enzyme were centrifuge at 10,000 RPM for 15 min at 4°C to increase clearity .Solid crystals of ammonium sulfate were added until it was 50% saturated and kept it for 4-6 hrs at 4°C.The resulting precipitate were collected by centrifugation at 10,000 RPM for 15 min at 4°C.The pellet of precipitated protein were discarded and more crystals of ammonium sulfate were added to attain 85% saturation at 0°C and it is again kept for 4-6hr at 4°C and centrifuged at 10,000RPM for 15 min at 4°C.After centrifugation the supernatant was kept seperate and the sediments were dissolved in small amount of Tris–HCL buffer (pH 8). The solution was kept in a dialysis bag and after sealing securely, dialyzed against distilled water with 4 regular changes of the water after every 6 h. The partially purified cellulase and used for further studies SDS –PAGE.[12]

Sodium Dodecyl Sulfate –Polyacrylamide Gel Electrophoresis (SDS –PAGE)
Sample was purified by salt precipitation and Dialysis. The final product was saved in 1mL of 10mM TrisHCl buffer. Molecular weight determination by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a 3-mm slab gel with

- Polyacrylamide separating gel (12%):- 12% polyacrylamide gel consists of 30% acrylamide mixture, 1.5 M Tris-HCl buffer, pH 8.8, 10% SDS (w/v), 10% (w/v) ammonium per sulfate and TEMED. It was prepared by mixing 1.68 ml of water, 2 ml of 30 % acrylamide mixture, 1.25 ml of 1.5 M Tris-HCl (pH 8.8), 50 µl of 10 % SDS, 25 µl of 10 % ammonium per sulfate and lastly 2.5 µl of TEMED to final volume of 5ml.
- Stackinggel (4%) :- It was prepared by mixing 3.05 ml of water, 665µ of 30% acrylamide mixture, 1.25 µl of 0.5 M Tris-HCl (pH 6.8), 50 µl of 10 % SDS, 25 µl of 10 % ammonium per sulfate and lastly 5 µl of TEMED to final volume of 5ml.

The gels were stained with Coomassie brilliant blue R-250 and destained. The standard proteins with 66, 45, 35, 25, 18 and 14 KDa weight were used as the marker for the

molecular weight determination.

FTIR (Fourier Transform Infrared Spectroscopy)

1) Mixture of sample and KBr (5 % sample, 95 % KBr) were passed into a disk for Fourier Transform Infrared Spectroscopy.
2) Record the spectra with 32 scans in the frequency range of 4000 – 400 cm-1with a resolution of 4 cm-1. Disks were prepared in triplicates to obtain a constant spectrum.

RESULTS AND DISCUSSION

Isolation and screening of Cellulase producing fungi

The fungi were isolated and screened from the samples collected from soil for cellulase production by Congo red assay. The isolated strains were carefully identified by morphological Characteristics include color of the colony and growth pattern studies. Some of the microscopic characteristics examined under the microscope include spore formation and color. The diameter of hydrolytic zone and colony diameter were measured (Fig:1)**.** Isolate ABS 228 showed highest zone of hydrolysis as 49 mm.

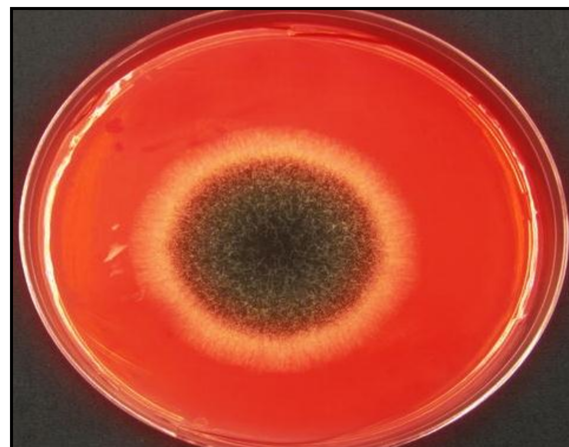


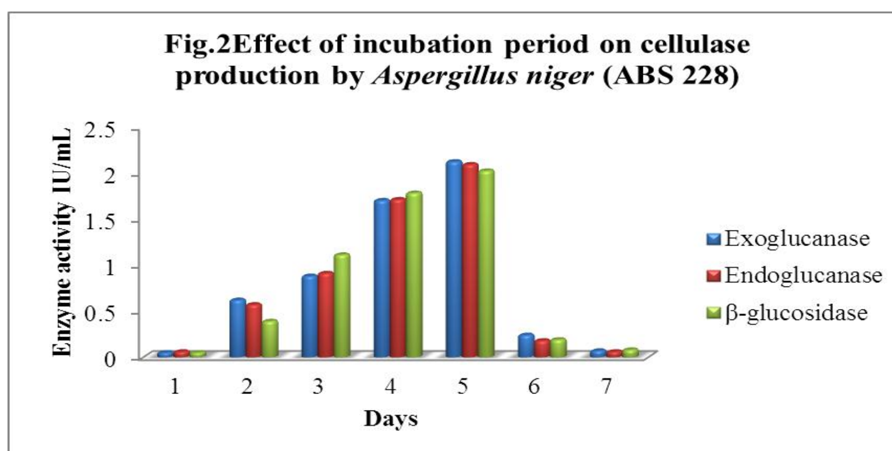
Fig: 1 Congo red test for cellulase production.

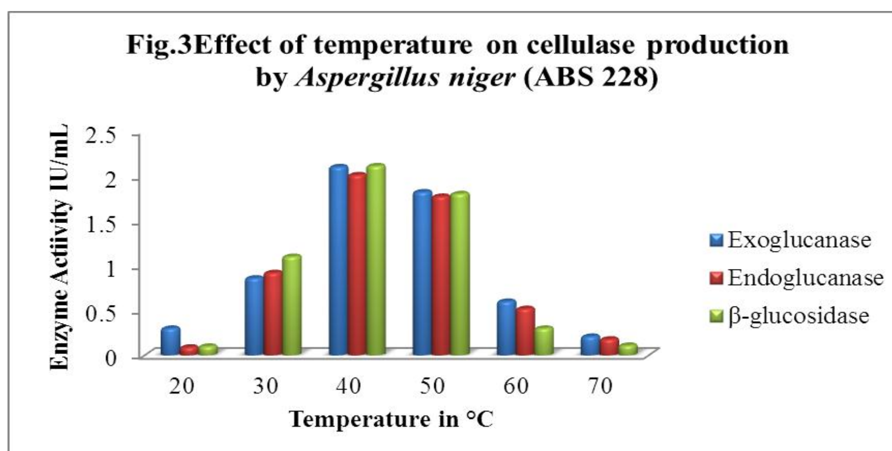
Enzyme Assay

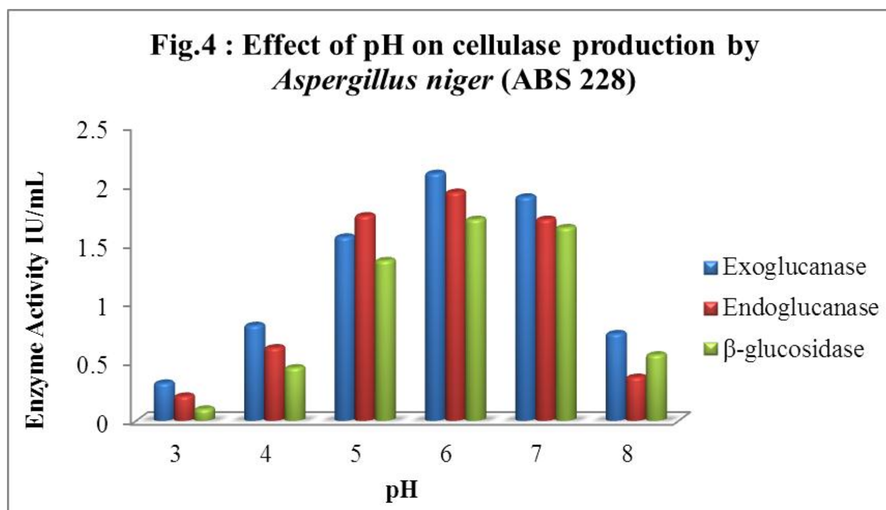
Enzyme assay of selected isolates were performed *.Aspergillus niger* ABS 228 showed highest exoglucanase (2.22 IU/ml), Endoglucanse (2.02 IU/ml) and β-glucasidase (1.97 IU/ml) activity.

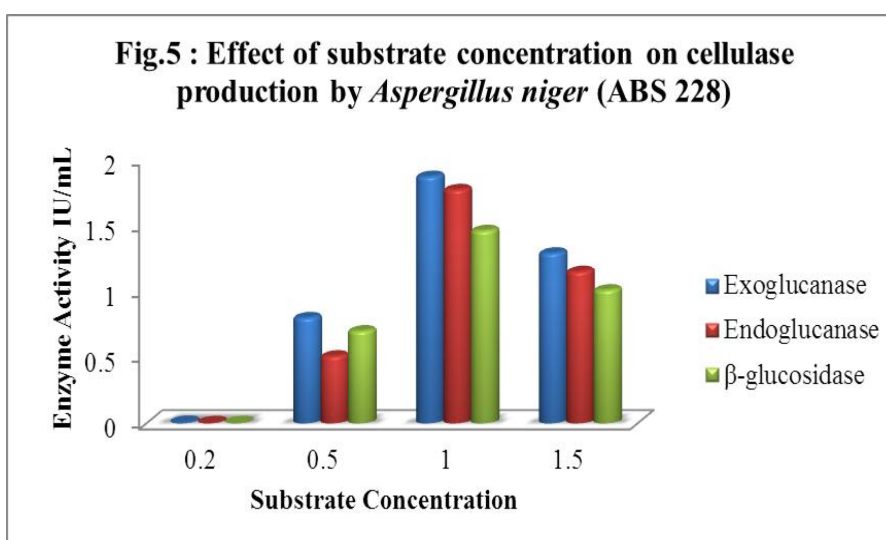
Optimization of production of cellulase enzyme

Maximum activity was achieved at the cultural optimized conditions recorded by *Aspergillus niger* **ABS228** with temperature of 40°C, pH of 6 at the growth of 5th day. And 1% carboxy methyl cellulose as a substrate concencentration.



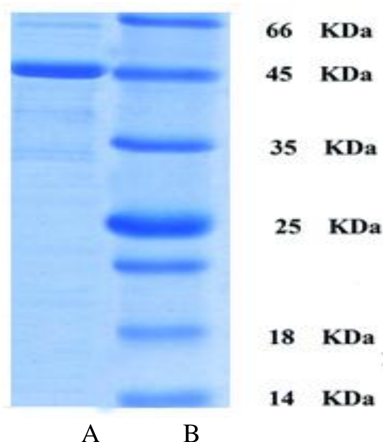






Sodium Dodecyl Sulfate –Polyacrylamide Gel Electrophoresis (SDS –PAGE) of bacteria

Molecular weight determination by Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out. Molecular weight of enzyme was determined and found out to be 45 KDa. (Fig.6).



A- Fungal Dialyzed Cellulase

B- Marker

FTIR analysis of the fungal enzyme

The purified cellulase enzyme was characterized by FTIR spectroscopy. Fig.4.8(a). The IR spectrum of purified enzyme indicated that there are some peaks in 765.23cm^{-1} regions which showed presence of C-2…O2,absorption at 850.66 cm^{-1} revealed the presence of CH/COC,absorption at 1010.34 cm^{-1} showed presence of C3…O-3 , absorption at 1259.99 cm^{-1} showed presence of CH+COH,absorption at 1560.13 cm^{-1} showed presence of C=C.The absorption at 2170.13 cm^{-1} revealed the presence of C=O, The absorption at 3028.43 cm^{-1} revealed the presence of CH2, The absorption at 3256.67 cm^{-1} revealed the presence of 3-OH….O-5 (Fig.7).

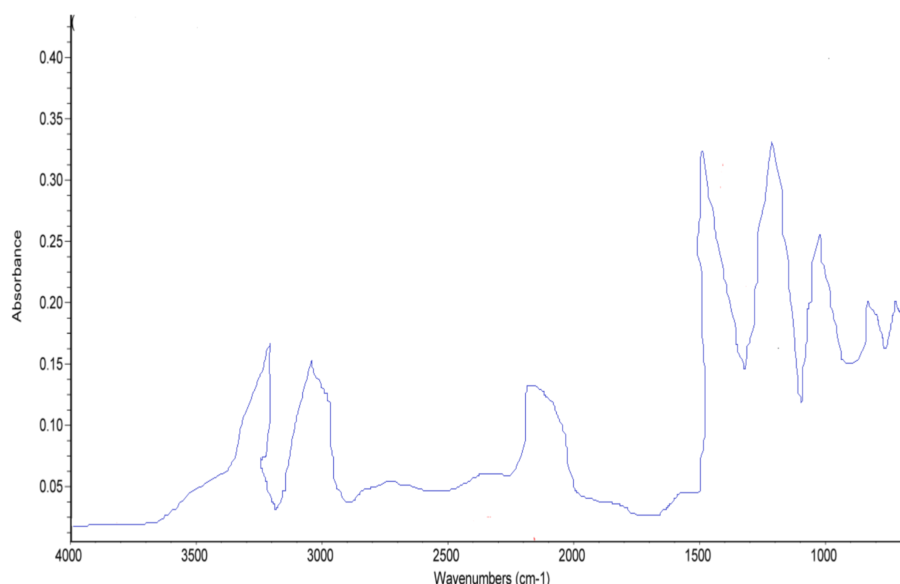


Fig.7 Fungal Cellulase FTIR

DISCUSSION

In the present study, total 146 organisms were obtained from soil samples, 72 fungal isolated were isolated .All the isolated strains of organism were evaluated for their efficiency of cellulose degradation. Efficiency was checked in the form of zone of clearance around colony by congo red test. Among the tested organism *Aspergillus niger* (ABS 228) showed highest zone of cellulose degradation i.e.49 mm. Gautam *et al.,* 2010[13] reported that *Trichoderma spp.*I, *Aspergillus fumigatus* showed excellent cellulose producing results in the screening. Morederate cellulose producers were also recorded among other fungi. Acharya *et al*., 2012[14] also selected cellulolytic organisms on the basis of zone of hydrolysis in which *Bacillus subtilis* shows highest zone i.e.26mm. Gautam *et al*., 2010[13] isolated 20 fungal culture isolates from environmental sources including 8 different zones, out of these 16 fungi were found to passes cellulose degrading ability. Cellulolytic fungi belonging to *Aspergillus funmigatus*, *Trichoderma spp*. I and *Chaetomium spp.* It is evident from the result that maximum cellulase activity was observed in *Aspergillus niger*, Gautam *et al*., in 2012[15] studied the enzyme activity 49 isolates and reported that *Trichoderma viride* shows maximum exoglucanase activity (2.22 IU/mL). Maximum endoglucanase activity was also observed in *Trichoderma viride* (2.03 IU/mL) whereas, highest β glucosidase activity also reported in *Trichoderma viride* (1.98 IU/mL).

Optimization of production of cellulase enzyme

Maximum activity was achieved at the cultural optimized conditions recorded by *Aspergillus niger* **ABS228** with temperature of 40°C, pH of 6 at the growth of 5th day.

And 1% carboxy methyl cellulose as a substrate concencentration. Gautam *et al*.,[16] found that in case *Aspergillus niger* the maximum yield of exoglucanase (1.64U/mL) and endoglucanase (1.84U/mL) activity was obtained after 4 days. However, maximum *β*-glucosidase (1.61U/mL) activity was shown after 3–5 days incubation. Immanuel *et al*., (2007)[17] also found high level of cellulase production at 40°C (0.292 and 0.258 U/ml) by *Aspergillus fumigatus* and *A. niger* using coir waste. Also, Bansal *et al*. (2012)[18] found the highest cellulases production by A. nigerNS-2 was at pH 7.0. Verma *et al*., 2012[19] found the Maximal cellulase production was obtained after 48 hr of incubation at 45°C in medium containing 1.5% carboxymethyl cellulose (CMC) as substrate.

This study reveal that the crude filtrate prepared using above treatments could be partially purified using ammonium sulphate precipitation shows protein band 45 kDa and FTIR analysis. CMCase had a strong absorption peak at 1655.46 cm^{-1},which is the characteristic peak of the alpha helix caused by symmetric stretching vibration of C=O stretching vibration and NH bond.[20 and 21]

CONCLUSION

The present work was carried out to optimize the nutritional and environmental parameters for improving cellulase production by the cellulolytic bacteria. The cellulase producing *Aspergillus niger* was isolated soil and characterized by, biochemical analysis and partial purification. From this study, the result showed that cellulase producing fungi can grow at optimized condition. Thereby, partial purification of cellulase enzyme was done and determined molecular weight of the enzyme. The *Aspergillus niger* species showed a potential to convert cellulose into reducing sugars which could be readily used in many applications such as animal foods and a feed stock for production of valuable organic compounds. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production and as a possible food supplement or in pharmaceutical industry.

REFERENCES

1. Shallom, D. and Shoham, Y. Microbial hemicellulases. *Current Opinion in Microbiology*, 2003; 6: 219-228.
2. Kaur, J., *et al.* Purification and characterization of two endoglucanases from *Melanocarpus* sp. MTCC 3922. *Bioresource Technology*, 2007; 98: 74-81.
3. Thongekkaew, J., *et al.* An acidic and thermosta-ble carboxymethyl cellulase from the yeast Cryptococcus sp. S-2: Purification, characterization and improvement of its recombinant enzyme production by high cell-den- sity fermentation of *Pichia pastoris*. *Protein Expression and Purification*, 2008; 60: 140-146.
4. Cavaco-Paulo A. Mechanism of cellulase action in textile processes. *Carbohydr. Polym,* 1998; 37: 273-277.
5. Cherry JR, Fidantsef AL. Directed evolution of industrial enzymes: an update. *CurrOpin Biotechnol,* 2003; 14: 438-443.
6. Abdelnasser SSI, Ahmed IE. Isolation and Identification of New Cellulases Producing Thermophilic Bacteria from an Egyptian Hot Spring and Some Properties of the Crude Enzyme. *Australian Journal of Basic and Applied Sciences,* 2007; 1(4): 473-478.
7. Charitha M. Devi and Sunil Kumar M. Production, optimization and partial purification of callulase by *Aspergillus niger* fermented with paper and timber sawmill industrial wastes. *J. Microbiol. Biotech. Res.,* 2012; 2(1): 120 – 128.
8. Hankin L. and S. L. Anagnostakis, Solid media containing carboxymethylcellulose to detect C*x* cellulase activity of micro organisms. *Journal of General Microbiology*, 1977; 98(1): 109–115.
9. Miller G. L. Use of dinitrosalicylic acid reagent for determination of reducing sugar, *Analytical Chemistry*, 1959; 31(3): 426–428.
10. Pointing S. B., "Qualitative methods for the determination of lignocellulolytic enzyme production by tropical fungi," *Fungal Diversity,* 1999; 2: 17–33.
11. Shaikh N. M.., Patel A. A., Mehta S. A., Patel N. D. Isolation and Screening of Cellulolytic Bacteria Inhabiting Different Environment and Optimization of Cellulase Production. *Universal Journal of Environmental Research and Technology*, 2013; 3(1): 39–49.
12. Triyat Scientific, Nagpur.
13. Gautam S. P., Bundela P. S., Pandey A. K., Awasthi M. K. and Sarsaiya S. Screening of Cellulolytic Fungi for Management of Municipal Solid Waste. *Journal of Applied Sciences in Environmental Sanitation,* 2010; 5(4): 391-395.
14. Acharya, D. R. Joshi, K. Shrestha and D.R. Bhatta Isolation and screening of thermophilic cellulolytic bacteria from compost piles. *Scientific World,* 2012; 10(10): 43-46.
15. Gautam S. P., Bundela P. S., Pandey A. K., Jamaluddin, Awasthi M. K.,and Sarsaiya S. Diversity of Cellulolytic Microbes and the Biodegradation of Municipal Solid Waste by a Potential Strain *International Journal of Microbiology,* 2012; 1–12.
16. Gautam, S. P., Bundela, P. S., Pandey, A. K., Khan, J.,Awasthi, M. K., and Sarsaiya, S. Optimization for the production of cellulase enzyme from municipal solid waste residue by two novel cellulolytic fungi. *Biotechnology Research International*, 2011; 1: 1–8.
17. Immanuel G., Dhanusha R., Prema P., and Palavesam A. Effect of different growth parameters on endoglucanase enzyme activity by bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science and Technology*, 2006; 3(1): 25–34.
18. Bansal, N., Tewari, R., Soni, R., and Soni, S. K.. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. *Waste Management*, 2012; 32: 1341-1346.
19. Verma V., Verma A. and Kushwaha A. "Isolation and production of cellulase enzyme from bacteria isolated from agricultural fields in district Hardoi, Uttar Pradesh, India" *Advances in Applied Science Research*, 2012; 3(1): 171-174
20. Dong, A. Caughey, B., Caughey, W. S., Bhat, K. S. and Coe, J. E. Secondary structure of the pentraxin female protein in water determined by infrared spectroscopy: Effect of calcium and phosphorylcholine. *Biochemistry,* 1992; 31(39): 9364–9370.
21. Byler, D. M., and H. Susi. Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers*, 1986; 25: 469–487.