World Applied Sciences Journal 9 (10): 1171-1179, 2010
ISSN 1818-4952

Some Critical Factors Affecting Cellulase(S) Production by *Aspergillus terreus* Mam-F23 and *Aspergillus flavus* Mam-F35 under Solid-State Fermentation of Wheat Straw

[1]M.A.M. Abo-State, [2]M. Swelim, [1]A.I. Hammad and [1]R.B. Gannam

[1]Department of Microbiology,
National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt
[2]Faculty of Science, Benha University, Egypt

Abstract: Two isolates of cellulase producer were isolated from agriculture waste and identified as *Aspergillus terreus* MAM-F23 and *Aspergillus flavus* MAM-F35. The results under solid state fermentation of wheat straw revealed that, the optimum pH values for the three cellulases (CMCase, FPase and Avicelase) and soluble protein production by *T. viride* as standard strain, *A. terreus* and *A. flavus* were ranging from 3.5 to 5.0 and the best inoculum size (level) was 0.5 ml spore suspension ($\simeq$ 2 x 10^7 spores/ml). Tap water was the best moisting agent in producing CMCase, FPase and Avicelase by *A. terreus* MAM-F23, but phosphate buffer was the best moisting agent for CMCase and Avicelase by *T. viride* and CMCase and FPase by *A. flavus* MAM-F35. The trend for cellulases production as the incubation period increased was almost the same in the three tested strains (*T. viride, A. terreus* and *A. flavus*). The maximum CMCase had been reached after 6 days incubation period and soluble protein increased as incubation period increased. On the other hand, Avicelase recorded the highest productivity after 12 hours incubation and decreased gradually as incubation period increased. However, FPase increased as incubation period increased to reach the maximum productivity after 48-60 hours, then decreased.

Key words: *Aspergillus* · Identification · CMCase · FPase · Avicelase

INTRODUCTION

Cellulose enzymes, which can hydrolyze cellulose forming glucose can be divided into three types: endoglucanase (endo, 1,4-D-glucanase, E.G. EC 3.2.1.4); cellobiohydrolase (exo-1,4,-D-glucanase, CBH, EC 3.2.1.91) and β-glucosidase (1,4-â-D-glucosidase BG, EC 3.2.1.21) [1,2]. Increasing interests in cellulases growing day by day because of their applications in industries as follows: in the textile industry for biobleaching of cotton fabries and treatment of denime garments to achieve a stone-wash effect, in the paper industry for de-inking and recycling of paper, in separation and isolation of starch and gluten from wheat flour, in animal food production, in grain alcohol fermentation, in extraction of fruit and vegetable juices and in bioethanol production [3-6]. The major bottle neck of comprehensive application of cellulases in industry is the highest cost of the enzymes production. It has been reported that solid state fermentation (SSF) is an attractive process to produce cellulase economically due to its lower capital investment, lower operating expenses, simpler equipment and higher productivity per reactor volume [7-9]. Production of cellulases in SSF using various substrate and microorganisms has been reported [10-12]. The growing approaches to reduce the cost of cellulases production can be achieved via using of cheap, renewable agriculture wastes which consider an excellent carbon source for microbial enzymes production and also looking for microorganism having the ability to hyper-production of cellulases.

In this study, two species of isolated *Aspergillus* proved to be good producer of cellulases on solid state fermentation of agriculture wastes, were used for studying some of the critical factors affecting cellulose(s) production to get enhanced cellulase production for industrial application.

MATERIALS AND METHODS

Isolation and Identification: Two strains of *Aspergillus* MAM-F23 and MAM-F35 were isolated previously from wheat straw agricultural waste on Sabroud's dextrose agar medium [13]. Potato dextrose agar medium (PDA, [13]) was

Corresponding Author: M.A.M. Abo-State, Department of Microbiology,
National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

used for strain maintenance. Identification of the two strains was performed at National Research Center, Dokki, Giza, Egypt. Identification on Sabroud medium according to Pitt and Hocking [14, 15] on the bases of cultural characters (colour, shape of colony, surface and reverse pigmentation, texture of the colony) as well as microscopical structure (septate or nonseptate hyphae, structure of hyphae and conidia) was performed.

Standard Strain: *Trichoderma viride* kindly provided by MERCIN, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

Substrate: Wheat straw as agricultural waste obtained from Upper Egypt Governorates was used. Wheat straw was firstly dried and then milled (3-5 mm) into small pieces.

Spore Suspension Preparation: Two *Aspergillus* species spore suspension had been prepared according to Abo-State [16] and the standard strain were inoculated on PDA plates and incubated for 7 days at 28°C. One disc of 9 mm diameter, from 7 days old culture was used to inoculate each flask (250 ml) containing 100 ml Sabroud's dextrose agar medium. Five flasks were used for each strain. The inoculated flasks were incubated for 7 days at 28°C. The spores were collected by adding 30 ml saline solution supplemented with 0.1% Tween 80 and the flasks were shacked at 180 rpm for 15 min. The spores were collected by centrifugation for 15 min at 8000 rpm, the supernatant was decanded and the spore pellets were washed with saline three times. The spore pellets were resuspended in saline and pooled in clean strile scrow capped flask as spore suspension. Spore suspension was kept at 4°C for further use. Spore suspension count had been determined.

Culture Conditions: Ten grams of wheat straw was mixed with 25 ml distilled water (pH 7.0) as moisting agent into 250 ml Erlenmeyer flasks. The flasks were sterilized for 30 min at 121°C. Four milliliters of spores ($2x10^7$ spores/ml) were inoculated and incubated for 7 days at 30°C under static condition.

Experimental Design: The first experiment, the initial pH value of the moisting agent (distilled water) was adjusted to be 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 6.5, 7.0 or 8.0 and all, the other culture conditions as previously mentioned. The second experiment, the inoculum size was supplemented at 4 levels (0.5 ml, 1.0 ml, 3.0 ml or 4.0 ml spore suspension) one ml suspension contains $2x10^7$ spore/ml. All the culture conditions except that factors were applied as previously mentioned. In the third experiment, the only exception was the moisting agents, which were distilled water, tap water, acetate buffer (pH 5.0), citrate buffer (pH 5.0) or phosphate buffer (pH 7.0).The fourth experiment, all conditions as previously mentioned except, samples were taken after 12, 24, 36, 48, 60, 72, 96, 120 and 144 hours incubation.

Enzyme Extraction: The solid state cultures were prepared by adding 10-fold (v/w) distilled water and shaking (180 rpm) at 30°C for 60 min. The solid materials and fungal biomass were separated by centrifugation (8000 rpm, 15 min). The clear supernatant used for enzyme assays and soluble protein determination.

CMCase Assay: Endoglucanase (CMCase) activity was determined according to Wang *et al.* [17]. One ml of the crude enzyme supernatant was incubated with 1 ml of 1% (w/v) Carboxymethyl cellulose (Chemicals Co., St. Louis, MO, Sigma USA) in 0.1 M Sodium acetate buffer solution (pH 5.0) for 30 min at 63°C. The resulted reducing sugars were determined according to Miller [18] by dinitrosalisylic acid (DNS).

Fpase Assay: Total cellulase (FPase) activity was determined as described by Gadgil *et al.* [19]. One ml of the supernatant was incubated with 2 ml of 0.1 M citrate buffer (pH 4.8) containing 50 mg Whatman No. 1 filter paper. After incubation for 1 hour at 50°C, the resulted reducing sugars were determined.

Avicelase Assay: Exoglucanase (Avicelase) activity was determined according to Li and Gao [20]. One ml of supernatant was incubated with 1 ml of 2% (w/v) Avicel (Sigma) in 0.1 M phosphate-citrate buffer (pH 6.6) at 40°C for 2 hours. The resulted reducing sugars were determined.

Protein Determination: Protein was determined according to Lowry *et al.* [21]. One ml of the supernatant was used in a clean dry test tube and 5.0 ml of reaction mixture was added. The tubes were kept at room temperature for 10 min. Then 0.5 ml of Folin reagent (Fluka) was added. The tubes were leaved for 20 min. at room temperature and the absorbance was measured at 720 nm.

RESULTS AND DISCUSSION

Identification of the Isolates: Colonies of the two isolated *Aspergillus* strains were spreaded around with velvety, white hyphae after incubation for 2 days on Sabroud's dextrose agar medium. After 5 days, the first strain *Aspergillus* MAM-F23 became brownish yellow, while the second strain *Aspergillus* MAM-F35 became greenish cumin. The microscopic morphology showed that hyphae were septate and hyaline, conidial heads were columnar, condiophores were smooth-walled and hyaline and conidia were small, globose and smooth in case of *Aspergillus* MAM-F23, which identified as *Aspergillus terreus* MAM-F23. The second strain was identified as *Aspergillus flavus* MAM-F35, the hyphae were septated, with smooth unceptated conidiphore and rounded vesicle with small, smooth globose conidia.

Effect of Initial pH Values: The pH value of the substrate for cellulase production can be considered as the corner stone of the process of enzyme production. Cellulase(s) production influenced greatly by differing in pH values. The best CMCase produced by *T. viride* was recorded at pH 5.0 (159 U/ml), FPase at pH 4.0 (51 U/ml) and Avicelase at pH 3.5 (24 U/ml) and protein (338 µg/ml) as indicated in Fig. 1. The results of isolated *Aspergillus terreus* MAM-F23 revealed that, the best production for CMCase was at pH 4.5 (385 U/ml), FPase at pH 4.0 (113 U/ml), Avicelase and protein at pH 4.5 (31 U/ml) and (376 µg/ml) respectively as indicated in Fig. 2. In case of the isolated *Aspergillus flavus* MAM-F35, the best CMCase, FPase and Avicelase were at pH 4.0 (430 U/ml, 138 U/ml and 45 U/ml), respectively, while the best protein production was at pH 4.5 (476 µg/ml) as indicated in Fig. 3.

From the previous results it is clear that the optimum pH for cellulases and protein production ranging from pH 3.5 to 5.0. These results were confirmed by other previous researches. Garg and Neelakantan [22] found that, the optimum pH for fungal cellulases and crude protein production varies from species to species. In most of the cases, the optimum pH ranges from 3.0 to 6.0. In case of *Aspergillus terreus* GN1, the maximum biomass protein content and protein recovery were obtained with initial pH 4.0. However, the optimum pH of the multi-form of *T. viride* was ranging between 4.0 and 5.5 [23]. *Phanerochaete chrysosporium* gave the maximum yield of cellulase on soya hull SSF at pH 4.0 [24].

Endoglucanase produced by the thermophilic fungus, *Scytalidium thermophilum* type culture *Torula thermophila* gave the optimum activity at pH 6.0 [25]. Meanwhile, the optimum pH for cellulases production by the newly isolated strain of the fungus *Mucor circinelloides* (NRRL 26519) was ranged from 4.0 to 6.0 [26]. The highest production of cellulase was noted at pH 4.0 [27]. On the other hand, Gao *et al.* [11] reported a lower range of pH value between 2.0 and 3.0 for endoglucanase and β-glucosidase respectively, produced under solid state cultivation on corn stover by *Aspergillus terreus* M 11.

Effect of Inoculum Size: Inoculum size (level) has also significant effect on the production of cellulases and protein. The best inoculum size on cellulases production by *T. viride, A. terreus* MAM-F23 and *A. flavus* MAM-35 have been recorded at 0.5 ml inoculum size and as the inoculum size increased the production decreased as indicated in Figures 4-6, except Avicelase for *T. viride* the best (46 U/ml) was by inoculum size 4.0ml and the best CMCase (283 U/ml) of *A. flavus* was at 3.0ml. These results are confirmed by previously studies of Sharma *et al.* [28]. They found that small inoculum size controls and shortens the initial lag phase whereas larger inoculum size increased the moisture content to significant extent. The free excess liquid presents an additional diffusion barrier together with that imposed by solid nature of the substrate and leads to a decreased in growth and enzyme production [23, 29-31]. However, Garg and Neelakantan [22] reported that the highest CMCase by *A. terreus* was found at 5.0% v/v inoculum size.

Effect of Moisting Agent: Moisting agent plays another important role in cellulases production by SSF. Phosphate buffer was the best moisting agent for CMCase (107 U/ml) and Avicelase (15 U/ml) by *T. viride*, while acetate buffer was the best for FPase (46 U/ml) and protein (166 µg/ml) as indicated in Figure 7. The results also, revealed that CMCase production in case of using tap water (101 U/ml) or distilled water (105 U/ml) was nearly comparable with phosphate (107 U/ml). Tap water was the best moisting agent in producing CMCase (319 U/ml), FPase (49 U/ml) and Avicelase (148 U/ml) by *A. terreus* as indicated in Fig. 8.

However, phosphate buffer supporting CMCase production (268 U/ml) and FPase (36 U/ml) by *A. flavus* as indicated in Fig. 9. The best Avicelase was recorded in

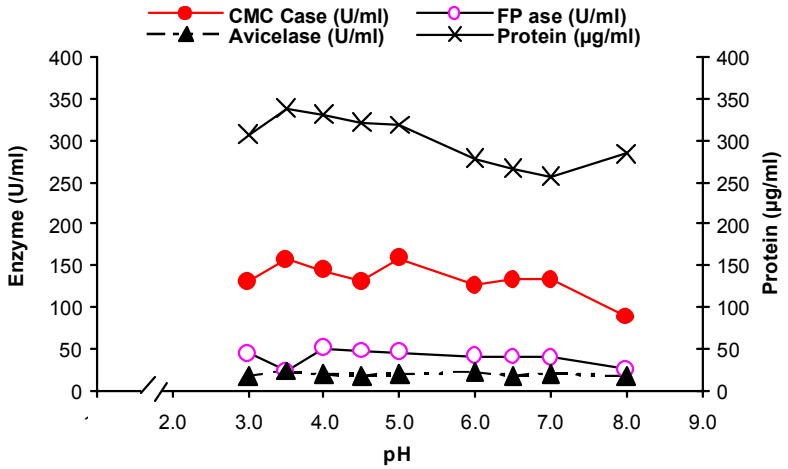

Fig. 1: Effect of different pH values on cellulases produced by *T. viride* on SSF.

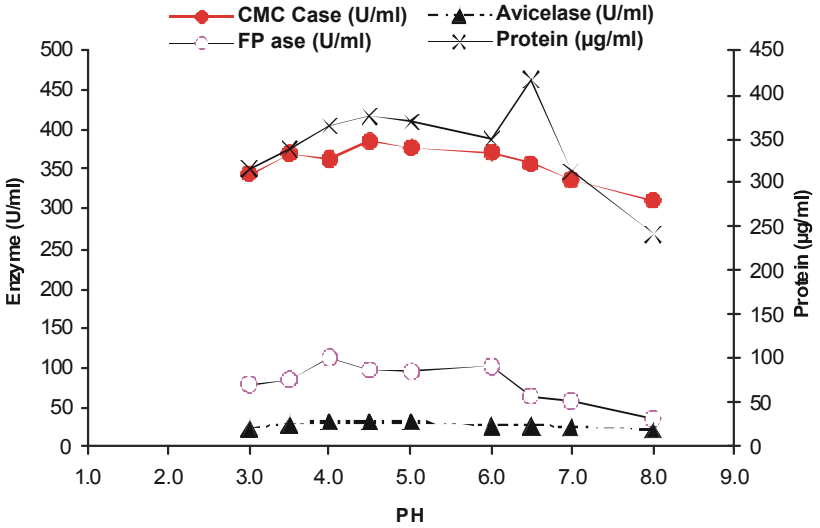

Fig. 2: Effect of different pH values on cellulases produced by *Aspergillus terreus* MAM -F23 on SSF.

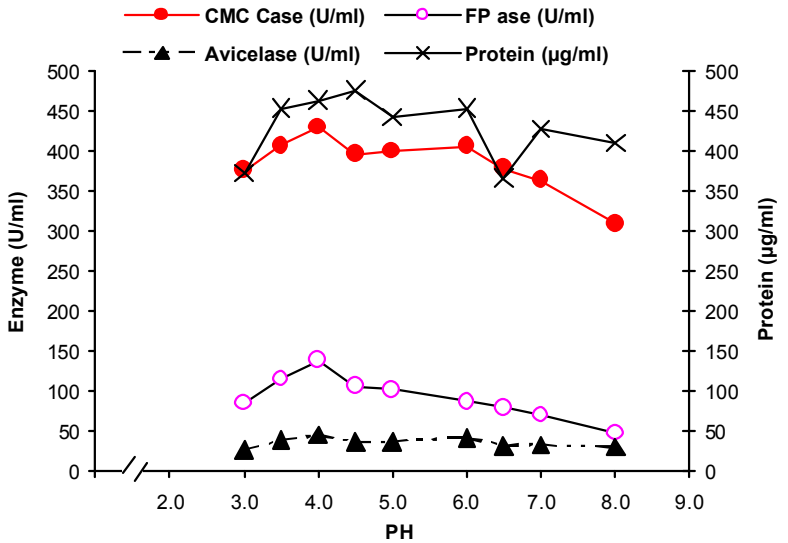

Fig. 3: Effect of different pH values on cellulases produced by *Aspergillus flavus* AM -F35 on SSF.

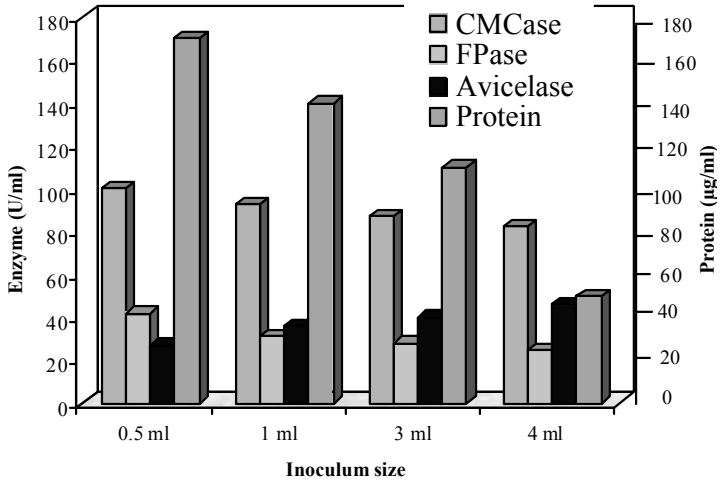

Fig. 4: Effect of inoculum size on cellulases produced by *T. viride* on SSF

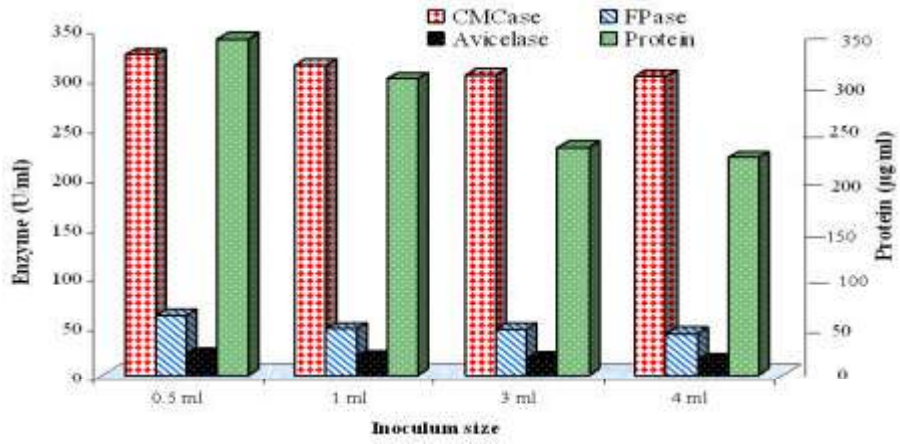

Fig. 5: Effect of inoculum size on cellulases produced by *Aspergillus terreus* MAM-F23 on SSF

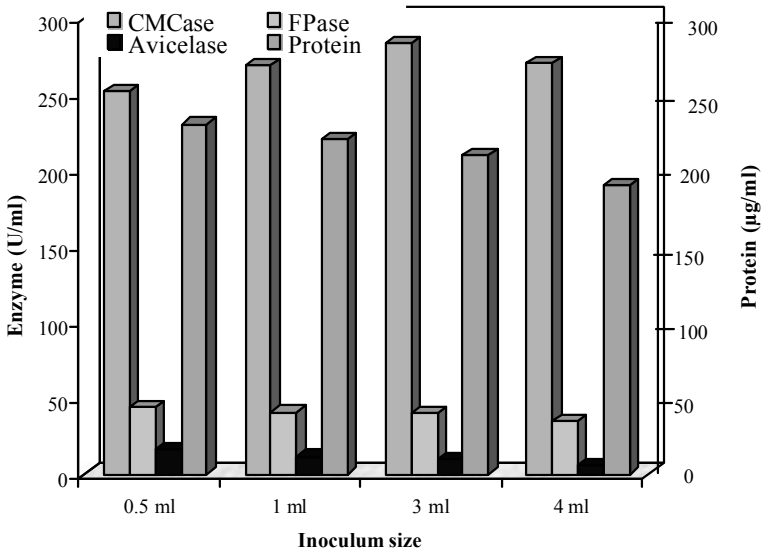

Fig. 6: Effect of inoculum size on cellulases produced by *Aspergillus flavus* MAM-F35 on SSF.

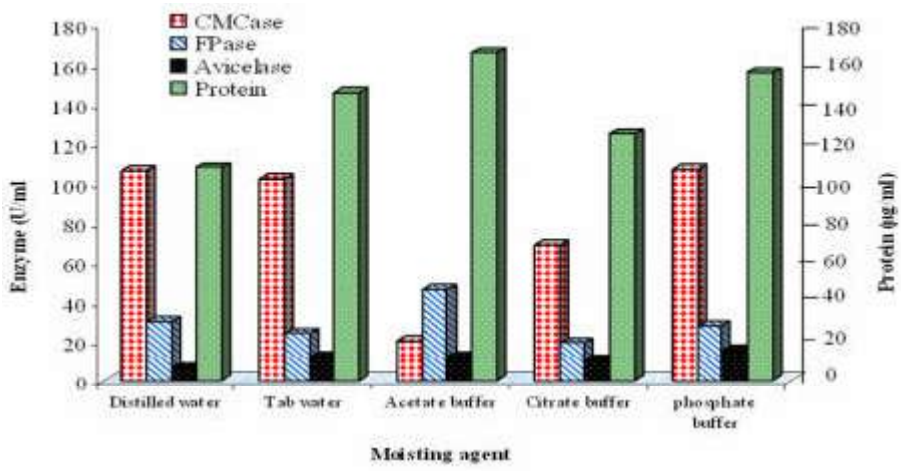

Fig. 7: Effect of moisting agent on cellulases produced by *T. viride* on SSF

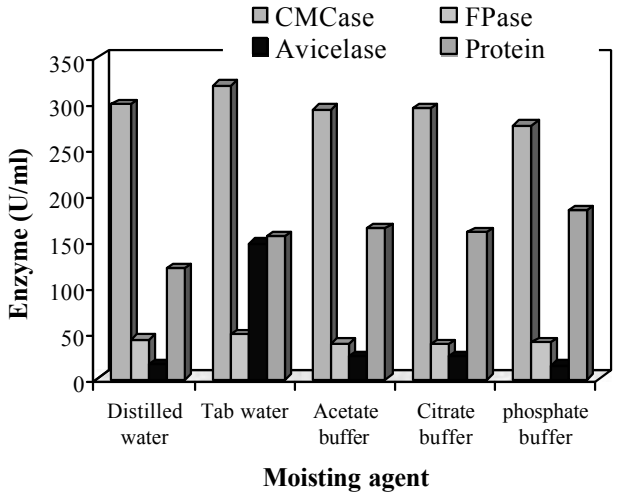

Fig. 8: Effect of moisting agent on cellulases produced by *Aspergillus terreus* MAM-F23 on SSF

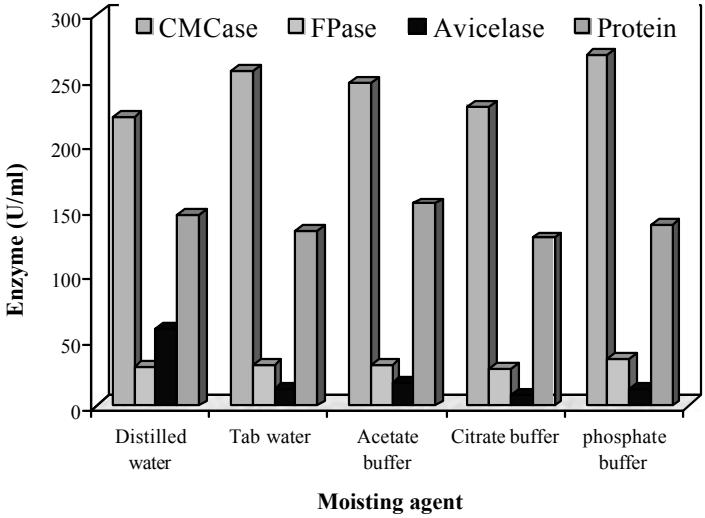

Fig. 9: Effect of moisting agent on cellulases produced by *Aspergillus flavus* MAM-F35 on SSF.

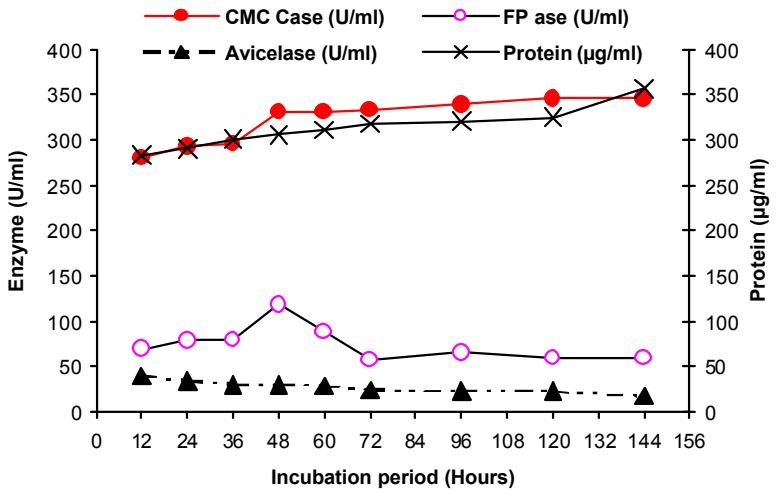

Fig. 10: Effect of incubation period on cellulases produced by *T. viride* on SSF.

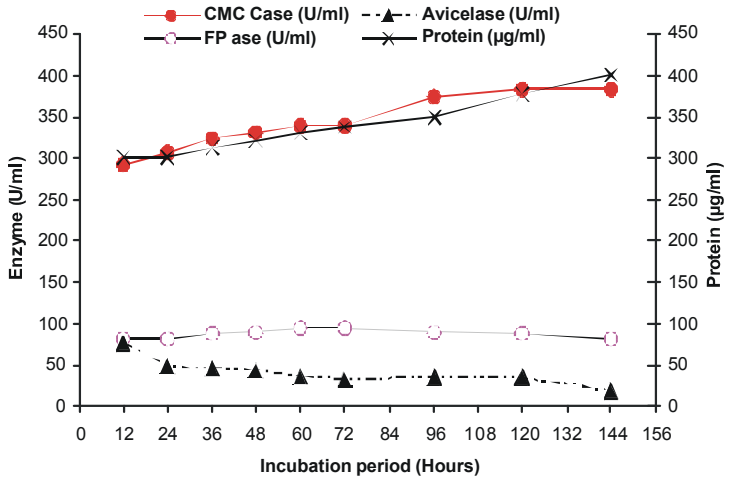

Fig. 11: Effect of incubation period on cellulases produced by *Aspergillus terreus* MAM-F23 on SSF.

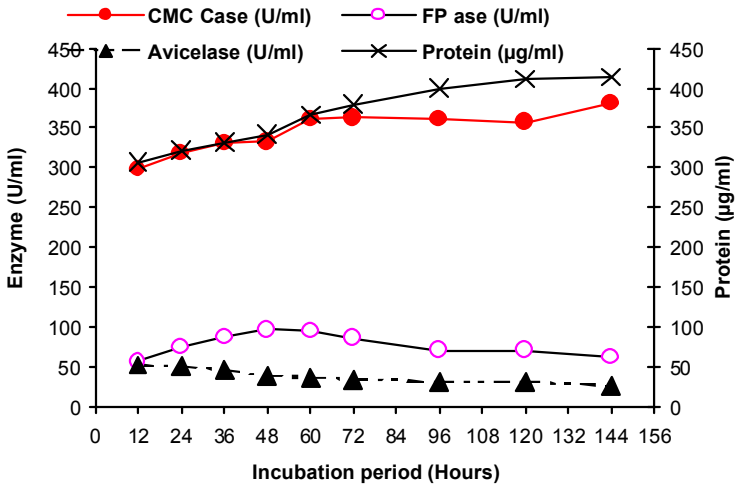

Fig. 12: Effect of incubation period on cellulases produced by *Aspergillus flavus* MAM-F35 on SSF.

case of using distilled water (59 U/ml). The previous results may be attributed to enhanced growth of *T. viride* and *A. flavus* when phosphorous was added to SSF as phosphate buffer. Mean- while, tap water which containing trace elements, needed for enhanced growth and consequently enhanced cellulase production by *A. terreus*.

Effect of Incubation Period: Incubation period is considered one of the most important factors affecting cellulases production. The trend for cellulase production as the incubation period increased was almost the same in the three tested species (*T. viride, Aspergillus* sp. MAM-F35 and *Aspergillus* sp. MAM-F23), as indicated in Figures 10-12. The maximum CMCase had been reached after 6 days incubation period. Also protein increased as the incubation period increased. FPase increased gradually to reach their maximum productivity after 48-60 hours, then decreased. On the other hand, Avicelase surprisingly recorded the highest productivity after 12 hours incubation and decreased gradually as the incubation period increased. This may be explained on the bases that, exoglucanases began the degradation of the cellulosic mater, so it was secreated at the beginning of the incubation period followed by FPase to reach maximum productivity after 48 hours. But CMCase increased gradually from the beginning to reach the maximum after 5-6 days incubation. The highest CMCase activity had been recorded after 7 days for *A. terreus* [22]. Velkovska *et al.* [32] reported that, *T. reesei* produces primary and secondary mycelium and suggested that secondary mycelium has a higher capacity of cellulolytic protein synthesis. Fang *et al.* [33] proposed that batch culture of *Acremonium cellulolyticus* has three distinguishable phases according to time course of FPase activity. Phase I (0-24 hrs.) is the primary growth period. This phase is characterized by undetectable FPase activity. Phase II (24-48 hrs.) is the period of secondary mycilial growth. Most of the cellulolytic protein is synthesized and a part of protein is released during phase II. Phase III (48hrs.) is characterized by high release of cellulolytic enzymes due to autolysis of hyphae. So the maximum cellulase activity obtained at phase III.

In the present study, it is clear that phase I was not reported. FPase and Avicelase had been detected from the beginning. This observation may be attributed to the difference between species used by Fang *et al.* [33] and the tested strains in this study. Also may be attributed to the difference between batch culture and SSF. Leite *et al.* [12] found that *Thermoascus aurantiacus* exhibited maximum β-glucosidase production after 48-72 incubation and pH 4.5.

REFERENCES

1. Hong, J., H. Tamaki, S. Akiba, K. Yamamoto and H. Kumagai, 2001. Cloning of a gene encoding a highly stable endo â-1,4-glucanase from *Aspergillus niger* and its expression in yeast. J. Biosci. Bioeng., 92: 434-441.
2. Li, Y.H., M. Ding, J. Wang, G.J. Xu and F. Zhao, 2006. A novel thermoacidophilic endoglucanase, Ba-EGA, from a new cellulose degrading bacterium, *Bacillus* sp. AC-1. Appl. Microbiol. Biotechnol., 70: 430-436.
3. Bhat, M.K., 2000. Cellulases and related enzymes in biotechnology. Biotechnol. Adv., 18: 355-383.
4. Adsul, M.G., K.B. Bastawde, A.J. Varma and D.V. Gochale, 2007. Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production. Bioresour. Technol., 98: 1467-1473.
5. Kaur, J., B.S. Chadha, B.A. Kumar and H.S. Saini, 2007. Purification and characterization of two endo- glucanases from *Melanocarpus* sp. MTCC 3922. Bioresour. Technol., 98: 74-81.
6. Dogaris, I., G. Vakontios, E. Kalogeris, D. Mamma and D. Kekos, 2009. Induction of cellulases from *Neurospora crassa* under solid state cultivation for bioconversion of sorghum bagasse into ethanol. Ind. Crops Product, 29: 404-411.
7. Xia, L. and P.L. Cen, 1999. Cellulase production by solid state fermentation on lignocellulosic waste from the xylose industry. Process Biochem., 34: 909-912.
8. Viniegra-Gonzáles, G., E. Favela-Torres, C.N. Aguilar, S.J. Rómero-Gomez, G. Diaz-Godinez and C. Augur, 2003. Advantages of fungal enzyme production in solid- state over liquid fermentation system. Biochem. Eng. J., 13: 157-167.
9. Pérez-Guerra, N., A. Torrado-Agrassar, C. Lopez-Macias and L. Pastrana, 2003. Main characteristics and applications of solid-state fermentation. Electron. J. Environ. Agric. Food Chem., 2: 343-350.
10. Latifian, M., Z. Hamidi-Esfahani and M. Barzegar, 2007. Evaluation of culture conditions for cellulase production by two *Trichoderma reesei* mutants under solid-state fermentation conditions. Bioresour. Technol., 98: 3634-3637.

11. Gao, J., H. Weng, D. Zhu, M. Yuan, F. Guan and Y. Xi, 2008. 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.
12. Leite, R.S.R., H.F. Alves-Prado, H. Cabral, F.C. Pagnocca, E. Gomes and R. Da-Silva, 2008. Production and characteristics comparison of crude â-glucosidases produced by microorganisms *Thermoascus aurantiacus* and Aureobasidium *pullulans* in agriculture wastes. Enzyme Microb. Technol., 43: 391-395.
13. Oxoid, 1982. The Oxoid Manual of culture media ingredients and other laboratory services. Trunsr Graphic, England.
14. Pitt, J.I. and A.D. Hocking, 1985. Fungi and Food Spoilage, Academic Press, Australia.
15. Pitt, J.I. and A.D. Hocking, 1997. Fungi and Food Spoilage, 2nd Edition, Blacki Academic Press, London, UK.
16. Abo-State, M.A.M., 2003. Production of carboxymethyle cellulase by *Fusarium oxysporium* and *Fusarium neoceras* from gamma-pretreated lignocellulosic wastes. Egypt. J. Biotechnol., 15: 151-168.
17. Wang, C., T. Hseu and C. Huang, 1988. Induction of cellulase by cello-oligosaccharides in *Trichoderma konigii* G-39. J. Biotechnol., 9: 47-60.
18. Miller, G.L., 1959. Use of dinitrosalysilic acid reagent for the determination of reducing sugars. Anal. Chem., 31: 426-428.
19. Gadgil, N.J., H.F. Daginawala, T. Chakakrabarti and P. Khanna, 1995. Enhanced cellulose production by mutant of *Trichoderma reesei*. Enzyme Microb. Technol., 17: 942-946.
20. Li, X. and P. Gao, 1997. Isolation and partial properties of cellulose decomposing strain of *Cytophaga* sp. LX-7 from soil. J. Appl. Microb., 82: 73-80.
21. Lowry, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with folin phenol reagent. J. Biol. Chem., 193: 265-267.
22. Garg, S.K. and S. Neelakantan, 1981. Effect of cultural factors on cellulase activity and protein production by *Aspergillus terreus*. Biotechnol. Bioeng., 23: 1653-1659.
23. Voragen, A.G., J.G. Beldman and F.N. Rombouts, 1988. Cellulases of mutant strain of *Trichoderma viride* QM9414. Methods Enzymol., 160: 243-251.
24. Jha, K., S.K. Khare and A.P. Ghandi, 1995. Solid-state fermentation of soy hull for the production of cellulase. Bioresour. Technol., 54: 321-322.
25. Arifoğlu, N. and Z.B. Ögel, 2000. Avicel-adsorbable endoglucanase production by the thermophilic fungus *Scytalidium thermophilum* type culture *Torula thermophila*. Enzyme Microb. Technol., 27: 560-569.
26. Saha, B.C., 2004. Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Process Biochem., 39: 1871-1876.
27. Sohail, M., R. Siddiqi, A. Ahmad and S. Ahmed Khan, 2009. Cellulase production from *Aspergillus niger* MS82: Effect of temperature and pH. New Biotechnol., 66: 1-5.
28. Sharma, D.K., M. Tiwari and B.K. Behere, 1996. Solid state fermentation of new substrates for production of cellulase and other bio-polymer hydrolyzing enzymes. Appl. Biochem. Biotechnol., 15: 495-500.
29. Muniswaran, P.K.A. and N.C.L. Charyulu, 1994. Solid state fermentation of coconut Cairo pith for cellulase production. Enzyme and Microbial. Technol., 16: 438-446.
30. Jatinder, K., B.S. Chadha and H.S. Saini, 2006a. Optimization of medium components for production of cellulases by *Melanocarpus* sp. MTCC 3922 under solid-state fermentation. World J. Microbiol. Biotechnol., 22: 15-22.
31. Jatinder, K., B.S. Chadha and H.S. Saini, 2006b. Optimization of culture conditions for production of cellulases and xylanases by *Scytalidium thermophilum* using Response Surface Methodology. World J. Microbiol. Biotechnol., 22: 169-176.
32. Velkovska, S., R.M. Mark and F.O. Davis, 1997. Kinetic model for batch cellulase production by *Trichoderma reesei* RUTC30. J. Biotechnol., 54: 83-94.
33. Fang, X., S. Yano, H. Inoue and S. Sawayama, 2009. Strain improvement of *Acremonium cellulolyticus* for cellulase production by mutation. J. Biosci. Bioeng., 107: 256-261.