

Optimization of Culture Conditions Affecting Carboxy Methyl Cellulase Production by *Aspergillus* Species

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Received January 07, 2013; Revised July 25, 2013; Accepted July 27, 2013

Abstract The aim of this study was to determine the potential of new *Aspergillus* strain isolated from electroplating industry to produce Carboxymethyl Cellulase from agriculture waste. Agricultural wastes have great potential for the production of value added products with special reference to enzymes. Corn husk was used as substrate for Carboxymethyl Cellulase (CMCase) production by *Aspergillus species* through submerged fermentation. Optimization of parameters such as pH, temperature, substrate concentration was performed for the optimal production of CMCase. The fungal strain produced highest CMCase activity (3.3 ± 0.01 IU/ml) at 5% (w/v) level of corn husk as substrate at 28°C and pH 8 over 72 hrs of incubation.

Keywords: corn husk, Carboxymethyl Cellulase, agriculture waste, *Aspergillus species*

Cite This Article: Pavani, K. V., Gayathamma K., and N.Sunil kumar, "Optimization of Culture Conditions Affecting Carboxy Methyl Cellulase Production by *Aspergillus* Species." *World Journal of Agricultural Research* 1, no. 4 (2013): 65-59. doi: 10.12691/wjar-1-4-4.

1. Introduction

Agro-industrial wastes and by products is renewable form of resources generated round the year all over the world. Wheat and rice bran, sugar cane bagasse, corn cobs, citrus and mango peel etc. are one of important wastes of food industries. These by products or farm waste, if properly utilized, can widen the scope of economic growth. The role of micro-organisms in bioconversion of bio-products and bio-waste into value added products has been highlighted in the recent decades [1,2]. Escalating market trends in enzyme fermentation technology has made tremendous progress during the late 20th century. The enzymes produced are useful in the food processing and it is not only advantageous quantitatively but qualitatively as well.

Cellulases are among such enzymes that are gaining popularity in this regard. Cellulase is a complex multi-enzyme system comprised of at least the following major components; Carboxymethyl cellulase (CMCase) or Endo- β -glucanase (EC 3.2.1.4), Exo- β -glucanase (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21) [3]. Carboxymethyl cellulase, one of the members of the cellulase complex, cleaves the internal glycosidic bonds of cellulosic chains and acts synergistically with exoglucanases and β -D-glucosidases during the breakdown of cellulosic material. It has found wide applications in various fields such as - usage in textile industry, in laundry detergents, in pulp and paper industry and as a facilitator of biomass fermentation in bio fuel production [4,5,6,7].

Ligno-cellulosic wastes including sawdust [8], Corn cob [9], Bagasse [10] and Wheat straw [11] are employed

in the production of industrial enzymes such as Cellulase, Xylanase, Carboxy methyl Cellulase etc. Several fungi and bacteria are capable of naturally producing multiple groups of enzymes, collectively known as cellulases, acting synergistically to hydrolyze β -1, 4-glycosidic bonds within the cellulose molecules. Filamentous fungi, particularly the *Aspergillus* species have been reported to be efficient producers of cellulases. The members of *Aspergillus* species are major agents of decomposition and thus possess the ability to produce enzymes like cellulases [12]. This may be the first report on the production of CMCase using corn husk as substrate.

2. Materials and Methods

2.1. Substrate

Agro waste as Corn husk served as a substrate on which the fungus grew for the production of Carboxymethyl Cellulase (CMCase).

2.1.1. Isolation of Fungi

Soil samples were collected from the area in and around a metal plating industry, located in I.D.A-Balanagar, Hyderabad, India and a pure culture of the fungus *Aspergillus* species was isolated and established from it. Identification of strain was done by amplification of 18s rRNA gene using blast. The organism showed 90% similarity to *Aspergillus sp.*AS7 [13].

2.1.2. Optimization of Conditions

A series of variable levels of different parameters were utilised in this study, in order to arrive at the optimum

production conditions required for maximum output of CMCCase.

Substrate levels

The experiment setup and performed for the selection of optimum concentration of the substrate (corn husk) for maximum production of CMCCase. The substrate was employed in various concentrations of 2, 3, and 5 % of the growth medium.

pH

Four different pH levels (range 4, 5, 6 and 8) in the medium were utilized and evaluated for the optimal pH required for maximum enzyme production.

Incubation Temperature

Five different incubation temperatures (28, 30, 32, 35 and 37°C) were evaluated for the maximum enzyme production.

Fermentation period

Five fermentation periods of 24, 48, 72, 96 and 120 hours were given for each treatment to analyze for the best time period for fermentation under pre-optimized conditions and maximum enzyme production.

Ammonium sulphate precipitation

The method of De-Moraes, et.al., [14] was followed for purification of Carboxymethyl Cellulase (CMCase). Different concentrations of ammonium sulphate (30, 60 and 80 % w/v in Citrate buffer at pH 4.8) were used for the protein (enzyme) precipitation.

Dialysis

Subsequent to the ammonium sulphate precipitation, the enzyme salt solution was dialyzed against the Citrate buffer with changes for 24 hours at 4°C to remove ammonium salt [15].

Gel filtration

The dialysed solution (5.0 ml) was subjected to gel filtration on Sephadex G-75 column (1.6 cm x 60 cm), pre-equilibrated with 0.05 mol/L Citrate buffer (pH 4.8) at a flow rate of 1.0 ml/min. Fractions of 5.0 ml each were collected and analysed using a Spectrophotometer at 280 nm and assayed for CMCCase activity. The active fractions of the elute containing CMCCase activities from the column were pooled and dialyzed against the Citrate buffer for further analysis [15].

Protein determination

The protein concentrations in the enzyme preparations were determined using the Lowry et.al, method [16] and comparing the results with a standard curve for Bovine serum albumin.

2.1.3. Statistical Analysis

All data are given as the mean \pm SD of triplicates (n = 3). Analysis of variance (ANOVA) was performed.

2.1.4. Results and Discussion

In a study reported by Ojumu et. al., on *Aspergillus flavus* reported that saw dust, corn cobs and bagasse were found to be the best substrates for the production of Cellulase [17]. In this study we were able to produce CMCCase using corn husk as a substrate for the *Aspergillus* species during submerged fermentation. Growth conditions regarding the incubation time, temperature, pH of the medium, different concentrations of substrate were optimized for maximizing enzyme production.

2.1.5. CMCase Production at 2% Corn Husk Concentration

It was observed that incubation time, pH and temperature played significant role at all the concentrations of corn waste. As shown in (Figure 1), the CMCase was produced by *Aspergillus* species up to an incubation period of 120 hrs, at 28°C and at a substrate concentration of 2% corn waste in growth medium. In the beginning, less enzyme activity was noticed; however it increased after 24 hrs of incubation and reached maximum enzyme activity (2.7 ± 0.02 IU/ml) at pH 5 up to 48 hrs of incubation. The results are supported by Fadel [18] who reported that the maximum CMCase was produced by *Aspergillus niger* at pH 4.5. A progressive decline in the enzyme production at all pH levels is observed after 48 hrs of incubation. The decrease in the production may be attributed to the depletion of the nutrients in the medium.

In (Figure 2), maximum enzyme activity (1.1 ± 0.06 IU/ml) was observed after 48 hrs of incubation, at pH 8 and at 30°C; (Figure 3) depicts maximum enzyme activity (1.5 ± 0.03 IU/ml) up to 48 hrs of incubation at pH 6 and temperature of 32°C. *Aspergillus oryzae* also produced CMCase at an optimum temperature of 55°C and pH range between 3.8 to 8.0 [19]. Maximum enzyme activity (0.41 ± 0.07 IU/ml) was noticed after 48 hrs of incubation at pH 8.0 and at a temperature 35°C (Figure 4). The organism showed maximum enzyme activity (1.18 ± 0.01 IU/ml) after 48 hrs of incubation, pH 8 and the temperature 37°C (Figure 5). It is apparent that when the incubation temperature of 28°C, the enzyme activity increased and subsequently started decreasing with rise in temperature.

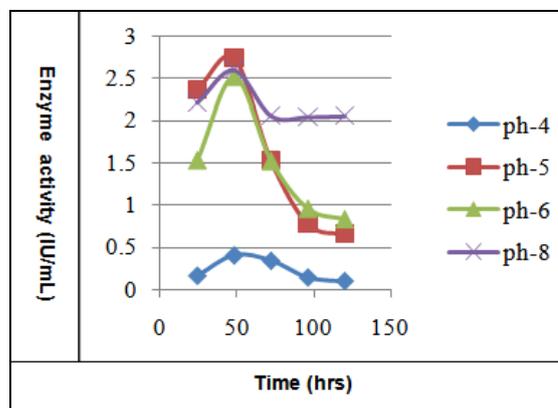


Figure 1. CMCase production during 120hrs incubation at 28°C at different pH levels

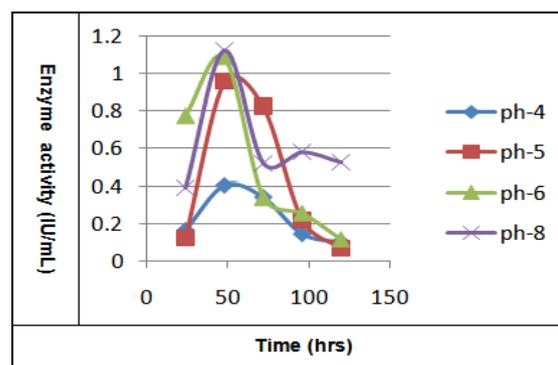


Figure 2. CMCase production during 120hrs incubation at 30°C at different pH levels

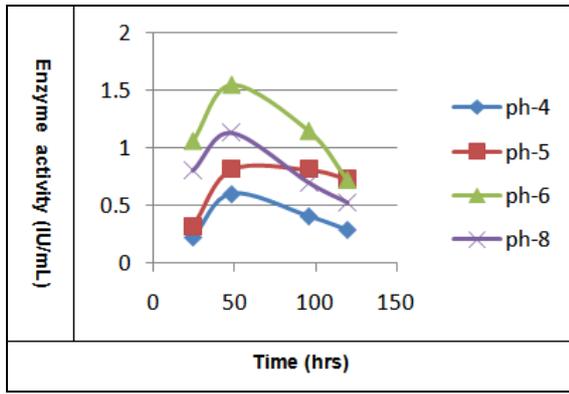


Figure 3. CMCase production during 120 hrs incubation 32°C at different pH levels

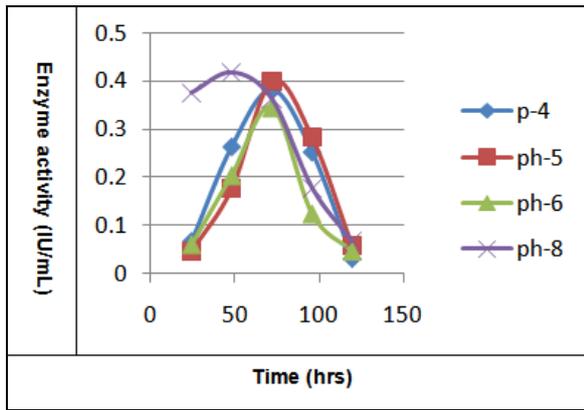


Figure 4. CMCase production during 120hrs at incubation at 35°C at different pH levels

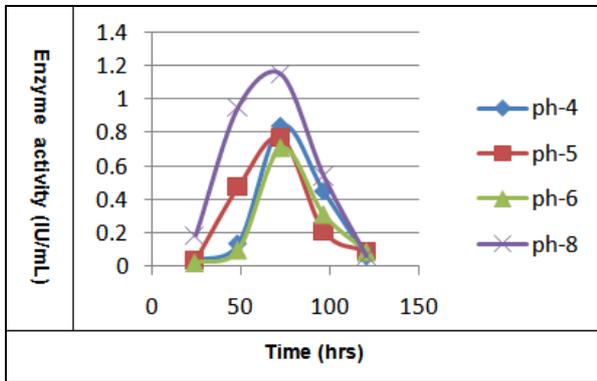


Figure 5. CMCase production during 120 hrs incubation at 37°C at different pH levels

2.1.6. CMCase Production at 3% Corn Husk Concentration

Enzyme biosynthesis by *Aspergillus* species at four different pH levels, 3% corn husk concentration, different temperatures and various incubation periods is depicted in (Figure 6). The mean values indicate that when the medium was kept initially at pH 4, the organism produced maximum enzyme activity (3.2±0.05 IU/ml) at 48 hours of incubation which subsequently decreased after 72 hours. The CMCase biosynthesis by *Aspergillus* species as illustrated in (Figure 7) indicates that at 30°C and with 3% corn husk concentration maximum enzyme activity (3.2±0.08 IU/ml) was obtained at 48 hours of incubation. However less amount of CMCase production was observed at pH 4.

In another study, the enzyme production was carried out by *Aspergillus* species at 32°C as depicted in (Figure 8) that explicated gradual increase in enzyme activity (1.7±0.01 IU/ml) which started decreasing after 48 hours of incubation.

The enzyme synthesis by *Aspergillus* species that was carried out at 35°C showed maximum enzyme activity (1.18±0.04 IU/ml) at pH 8 (Figure 9). It was reported that the optimal pH for CMCase production in *A. niger* was found to lie between 6.0 and 7.0 range [20]. In another study the optimal pH for CMCase production by *A.niger* was found to be between 4.0 and 4.8 [21]. Such varied observations may be due to the metabolic differences within the same genus. Enzyme production at 37°C is depicted (Figure 10), where the incubation was carried out for 120 hours. The organism showed more enzyme activity at pH 8 (1.7±0.04 IU/ml).

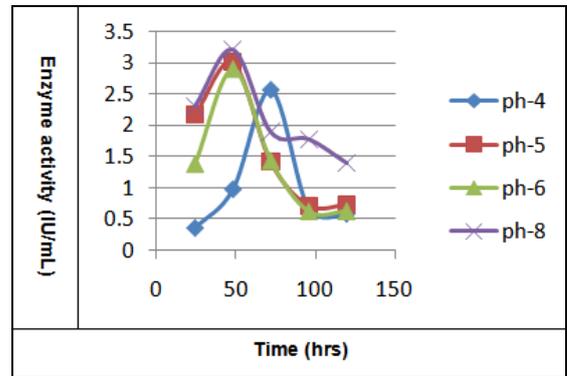


Figure 6. CMCase production during 120 hrs incubation 28°C at different pH levels

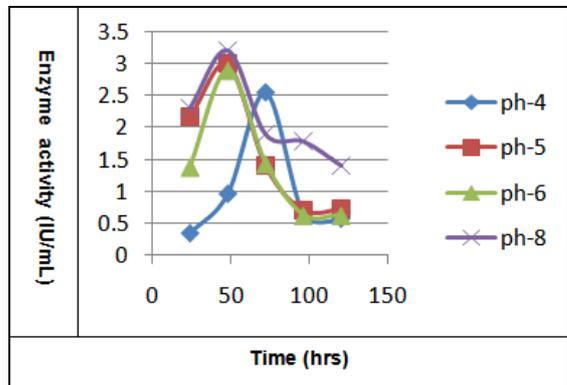


Figure 7. CMCase production during 120 hrs at incubation at 30°C at different pH levels

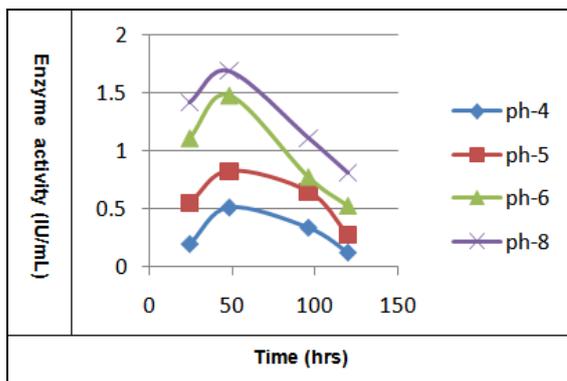


Figure 8. CMCase production during 120 hrs incubation at 32°C at different pH levels

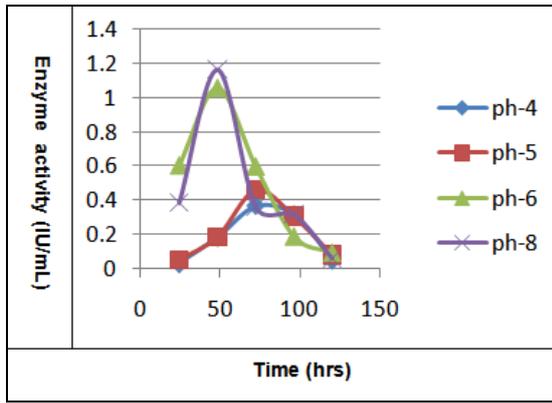


Figure 9. CMCCase production during 120 hrs incubation at 35°C at different pH levels

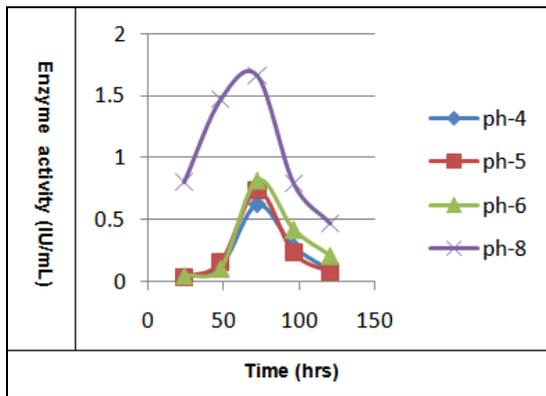


Figure 10. CMCCase production during 120 hrs incubation at 37°C at different pH levels

2.1.7. CMCCase Production at 5% Corn Waste Concentration

CMCase activity showed by *A. species* at 5% corn waste concentration and 28°C temperature at pH 8 and 120hrs incubation period was (3.3±0.01 IU/ml) as in Figure 11. Ahmed et al also reported *Trichoderma harziaumum* producing higher levels of cellulases on incubation at 28°C [22].

The graphical illustration indicates enhanced enzyme activity (1.6±0.05 IU/ml) at pH 5 and 30°C and then decrease in enzyme activity was noticed at pH 6 and 8 (Figure 12). The CMCase synthesis by *A. species* is represented in Figure 13, wherein incubation with 5 % corn waste as substrate at 32°C, the mean values exhibit an increasing trend in CMCase activity (1.7±0.04 IU/ml) up to a period of 72 hrs, then a decline was observed.

The Figure 14 elucidates the CMCase activity by *Aspergillus* species on fermentation at 35°C and 5% corn waste concentration up to a period of 120 hrs. The fungus exhibited maximum CMCase activity (0.9±0.01 IU/ml) up to 72 hrs of fermentation at pH 8 of the culture medium, which then decreased at 120 hrs of incubation.

Time scale of production of CMCase by *Aspergillus* species is represented in Figure 15. The mean values explicate the maximum enzyme activity (1.5±1.01 IU/ml) up to 72 hrs at pH 8. The optimum pH required for in vitro production of fungal cellulases varies from species to species, though in most cases the pH ranges from 3.0 to 6.0 [23]. The CMCase production increased with increase in substrate (Corn husk) concentration.

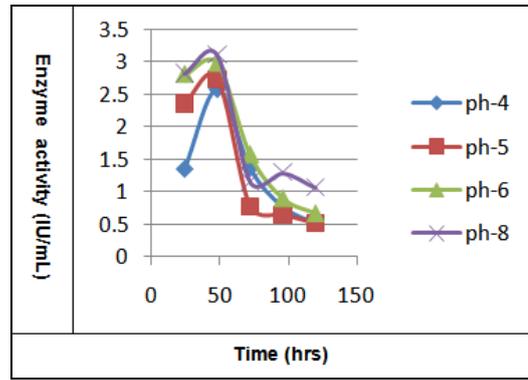


Figure 11. CMCCase production during 120 hrs incubation at 28°C at different pH levels

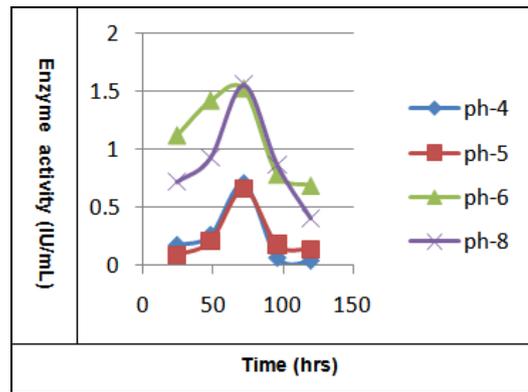


Figure 12. CMCCase production during 120 hrs incubation at 30°C at different pH levels

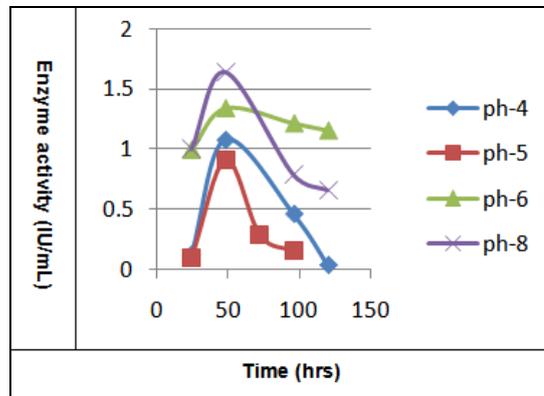


Figure 13. CMCCase production during 120 hrs incubation at 32°C at different pH levels

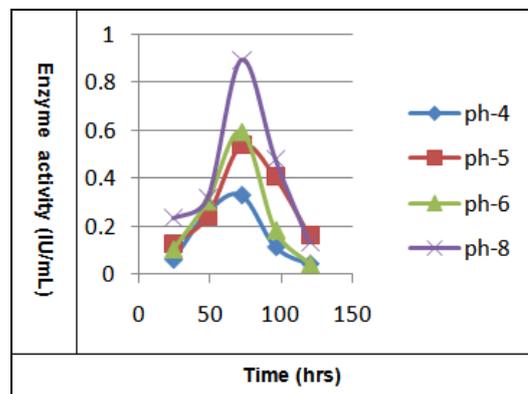


Figure 14. CMCCase production during 120 hrs incubation at 35°C at different pH levels

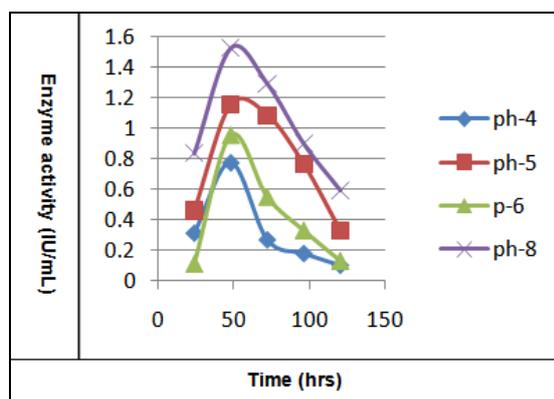


Figure 15. CM Case production during 120 hrs incubation at 37°C at different pH levels

2.1.8. Partial Purification of CMCCase

The crude enzyme present in the culture filtrate obtained from fermentative action of *A. species* on 3% corn husk at 28°C, pH 8 and 72 hours of incubation period was used for partial purification of enzyme. The specific activity of CMCCase was recovered at different ammonium sulfate saturation fractions and 60 % saturation fraction was selected for the enzyme precipitation. After centrifugation the precipitated enzyme was loaded on to a column packed with Saephadex G-75 and fractions of 5 mL volume were collected and analyzed for specific enzyme activity. The two step purification process yielded a partially purified CMCCase with a specific activity of (1.9815±0.01U/mg). The study revealed that the new strain isolated showed optimum activity at pH 8 and at 28°C of incubation and a substrate of 3% Corn Husk concentration.

2.1.9. Conclusion

The literature surveyed thus far and to the best of our knowledge reveals that currently little efforts are being made to prepare the hydrolytic enzymes like Carboxymethyl Cellulase by fermenting the agricultural wastes and agro-by products using microorganisms. The best possible and cost effective solution would then be the utilization of indigenous, cheaper and underutilized biomass as substrate for the production of this valuable enzyme. Utilization of agro wastes for the production of enzymes from microorganisms is yet become an attractive way to resolve the environmental pollution problems.

References

- [1] Pandey, A., Soccol, C.R., Nigam, P., Soccol, V.T., "Biotechnological potential of agro- industrial residues: Sugarcane bagasse", *Biores Technol*, 74 (1), 69-80, Aug, 2000.
- [2] Ahuja, S.K., Ferreira, G.M., Moreira, A.R., Utilization of enzymes for environmental applications, *Crit. Rev. Biotechnol*, 24 (2-3), 125-154, Apr, 2004.
- [3] Kaur, J., Chadha, B.S., Kumar, B.A., Saini, H.S., "Purification and characterization of two endoglucanases from *Melanocarpus* sp. MTCC 3922", *Bioresour. Technol*, Jan, 2007, 98 (1), 74-81.
- [4] Bhat, M.K., "Cellulases and related enzymes in biotechnology", *Biotech. Adv*, 18 (5), 355-383, Aug, 2000.
- [5] Adsul, M.G., Bastawde, K.B., Varma, A.J., Gochale, D.V., "Strain improvement of *Penicillium janthinellum* NCIM 1171 for increased cellulase production", *Bioresour. Technol*, 98 (7), 1467-1473, May, 2007.
- [6] Harada, O., Lysenko, E.D., Edwards, N.M., Preston, K.R., "Effects of commercial hydrolytic enzyme additives on Japanese- style sponge and dough bread properties and processing characteristics", *Cereal Chem*, 82 (3), 314-320, May/June, 2005.
- [7] Dogaris, I., Vakontios, G., Kalogeries, E., Mamma, D., Kekos, D., "Induction of cellulases and hemicellulases from *Neurospora crassa* under solid- state cultivation for bioconversion of sorghum bagasse into ethanol", *Ind. Crops Product*, 29 (2-3), 404-411, Mar, 2009.
- [8] Acharya, P.B., Acharya, D.K., Modi, H.A., "Optimization for cellulase production by *Aspergillus niger* using sawdust as substrate", *Afr J Biotechnol*, 7 (22), 4147 -4152, Nov, 2008.
- [9] Muhammad Irfan, Quratulain Syed, Muhammad Gulsher, Saijad Abbas, "Muhammad Nadeem, Shahjahan Baig. Pretreatment of corn cobs for the production of hydrolytic enzymes from *Aspergillus niger*-IR01", *IJAVMS*, 4 (3), 81-87, Jul, 2010.
- [10] Muhammad Irfan, Saijad Abbas, Shahjahan Baig, Muhammad Gulsher, Muhammad Nadeem and Quratulain Syed, "Pretreatment: A Potential Technique to enhance the enzymatic hydrolysis", *World journal of Agricultural sciences*, 6(4), 440- 445, Jul, 2010.
- [11] Sadia Aslam and Muhammad Asgher, "Partial purification and characterization of ligninolytic enzymes produced by *Pleurotus ostreatus* during solid state fermentation", *African Journal of Biotechnology*, 10 (77), 17875 - 17883, Dec, 2011.
- [12] Coral, G. B., Arian, M. N., Unaldi and Guvenmez, H., "Some Properties of Crude Carboxymethyl Cellulase of *Aspergillus niger* Z10 Wild-Type Strain", *Turk. J. Biol*, 26 (4), 209-213, Nov, 2002.
- [13] Pavani, K.V., Balakrishna, K., Nagarjuna reddy Cheemarala, "Synthesis of zinc nanoparticles by *Aspergillus species*", *Int. j. of nanotechnology and applications*, 51 (1), 27-36, Jun, 2011.
- [14] De-Moraes, L.M.P., Filho, SA., and Ulhaa, C.J., "Purification and some properties of an alpha amylase and glucoamylase fusion protein from *Saccharomyces cerevisiae*", *World Journal of Microbiology and Biotechnology*, *World J. Microbiol. Biotechnol*, 15 (5), 561-564, Jul, 1999.
- [15] Peshin, A and Mathur J.M.S., "Purification and characterization of β -glucosidase from *Aspergillus niger* strain 322", *Lett. Appl. Microb*, 28 (5), 401-404, May, 1999.
- [16] Lowry, O.H., Rosebrough, N.J, Farr, A.L, Randall, R.J, "Protein measurement with the Folin phenol reagent", *J. Biol. Chem*. 193 (1) 265-275, Nov, 1951.
- [17] Ojumu, T.V., Solomon, B.O., Betiku, E., Layokun, S.K., Amigun, B., "Cellulase Production by *Aspergillus flavus* Linn Isolate NSPR 101 fermented in sawdust, bagasse and corncob", *Afr. J. Biotechnol*, 2 (6), 150-152, Jun, 2003.
- [18] Fadel, M., "Production physiology of cellulases and β -glucosidase enzymes of *Aspergillus niger* grown under solid state fermentation conditions", *Online Biol. Sci*, 1 (5) 401-411, Jun, 2000.
- [19] Ghada A. Youssef, Physiological studies of cellulase complex enzymes of *Aspergillus oryzae* and characterization of carboxymethyl cellulase, *African Journal of Microbiology Research*, 5(11) 1311-1321, Jun, 2011.
- [20] Akiba S, Kimura Y, Yamamoto K, Kumaga PH., "Purification and characterization of a protease- resistant cellulase from *Aspergillus niger*", *J.Fermen. Bioengin*, 79 (2), 125-132, Jun, 1995.
- [21] McCleary, B.V., Glennie- Holmes, M., "Enzymic quantification of (1-3) (1-4)- β -D-glucan in barley and malt", *J. Inst. Brew*, 91 (3), 285-295, Jul, 1985.
- [22] Ahmed, S., Aslam, N., Latif, Farooq., Rajoka, M. I., Jamil, A., "Molecular cloning of cellulase genes from *Trichoderma harzianum*", *Frontiers in natural product chemistry*, 1 (1), 73-75, Jan, 2005.
- [23] Niranjane, A.P., Madhou, P and Stevenson, T.W, The effect of carbohydrate carbon Sources on the production of cellulase by *Phlebia gigantea*. *Enzyme and Microbial Technology* *Enzyme.Microbial.Technol*. 40 (6), 1464-1468, May, 2007.