

Optimization of Culture Conditions for Cellulase Production by Wild Strain of *Aspergillus Niger* Using Sugar Cane Bagasse as Substrate

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ABSTRACT

Studies were conducted to produce cellulase by cellulolytic fungi using sugar cane bagasse as substrate. *Aspergillus niger* was isolated from domestic wastes and assessed for cellulase production using sugar cane bagasse as substrate. The effect of factors such as incubation period, temperature, initial pH and inoculum size were analysed and optimized. Cellulase production by the *A. niger* was found to be optimum/highest with an incubation temperature of 40°C, pH 6.0, inoculum size of 1.0×10^6 spores/ml and incubation period of 5 days. The cellulases produced were partially purified using ammonium sulphate. Higher cellulose yield was found with the partially purified alkali treated sugarcane bagasse enzyme solution (0.74225U/mL) compared to the alkali treated sugarcane bagasse crude enzyme solution (0.3599U/mL).

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INTRODUCTION

Cellulose is the most common abundant, renewable biopolymer on earth and domestic waste materials from agriculture representing about 1.5×10^{12} tons of the total annual biomass production through photosynthesis in the tropics (Bhat, *et al.*, 2000; Klemm *et al.*, 2002). Cellulose is composed of D-glucose units linked together to form linear chain via β -1, 4-glycosidic linkages (Siddiqui *et al.*; 2000).

Cellulose is commonly degraded by an enzyme called cellulase. Cellulases are consortia of complex hydrolytic enzymes capable of hydrolyzing cellulose to smaller sugar components like glucose units. This enzyme is produced by several microorganisms, mainly by bacteria and fungi (Immanuel *et al.*, 2006, Kamble and Jadhav, 2012). Fungi are group of microorganism that can degrade mixtures of heterogeneous substrates such as municipal solid waste, cattle manure, and agricultural and industrial wastes (Singh, 2006) rapidly and for this, they possess a complex set of extracellular enzymes.

Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi. Fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al.*, 2002). Numerous microorganisms are capable of decomposing cellulose, although only a few of these microorganisms produce significant quantities of extracellular enzymes to decompose cellulose (Sukumaran *et al.*, 2005).

Cellulosic residues are renewable resources of which over a billion tons are

generated annually in world (Klemm *et al.*, 2009). The annual production of cellulose is estimated to be 4.0×10^7 tons (O'Sullivan, 1997). The non-use of these materials constitutes a loss of potentially valuable resources (Mussatto and Teixeira, 2010). In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol, organic acids, detergents and other chemicals. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen *et al.*, 2005).

Cellulase is expensive and the demand for more thermostable, highly active and specific cellulases is on the increase; and contributes only 50% to the overall cost of hydrolysis due to the low specific activity. This enzyme has enormous potential in industries and is used in food, beverages, textile, laundry, paper and pulp industries etc. (Lekh *et al.*, 2014).

Treatment of MSW with either cellulolytic enzymes or microorganism could lead to more efficient degradation of this waste material, promote cycling of nutrients in the environment and reduce the impact of waste accumulation on terrestrial and aquatic ecosystem as well as generation of end products of economic benefits like compost and biofuels (Klemm *et al.*, 2009).

In this investigation, a cellulase producing strain of *A. niger* isolated from municipal solid waste, were subjected to optimization of media and cultivation parameters for cellulase production.

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MATERIAL AND METHODS

Isolation, identification and screening of fungal isolates

Fungi were isolated from the refuse dump soil (Paul and Daniel, 2007), and identified by the use microscope (Domsch *et al.*, 1980). The isolates obtained were screened for cellulolytic activities (Albin *et al.*, 2014). Cellulolytic fungi create a clearing zone around the colony and these were measured in millimeters.

The powdered sugar cane baggase was treated with alkali solution of sodium hydroxide solution (0.25N) and autoclave at 121°C for 15 minutes. The treated sugar cane bagasse was washed several times with distilled water until pH 7.0 is attained. The residue were drained through muslin cloth and dried in an oven at 60° C until constant weight is attained. (Vyas *et al.*, 2005).

Inoculum Preparation

The inoculum was developed using stock culture of isolates obtained from PDA slants. The spore suspension was diluted to concentrations of 1.0×10^4 , 1.0×10^6 , and 1.0×10^8 per ml by counting with a haemocytometer (Damisa, 2007).

Cultivation medium and enzyme production

The fungus were cultivated at ambient temperature of 28°C in Mandel's medium (Mandels *et al.*, 1999) The cultivation was carried out in 250 ml capacity Erlenmeyer flasks in a rotary shaker at ambient temperature of 28°C and 350 rpm. The samples were withdrawn for analysis after every 24 hours, centrifuged at $13,000 \times g$ for 30 minutes in a table top high speed refrigerated centrifuge at 4°C and the supernatant was taken as the crude enzyme suspension (Damisa, *et al.*, 2008).

Optimization of Culture Conditions for Cellulase Production

The effect of temperature (20, 30 and 40°C), pH (4.0, 5.0 and 6.0), inoculums size (1.0×10^4 , 1.0×10^6 and 1.0×10^8 spores/ml) (Damisa, 2007). And incubation period of ten days (Tanzila *et al.*, 2013) were determined.

Partial Purification of Cellulase

Crude extract of enzyme was centrifuge at 10,000 rpm for 15 min at 4°C to increase clarity. Solid crystals of ammonium sulfate were added until it is 50% saturated and kept for 4-6 hrs at 4°C. The resulting precipitate were collected and centrifuge at 10,000 rpm for 15 min at 4°C. The pellet of precipitated protein was discarded and more crystals of ammonium sulfate were added to attain 85% saturation at 0°C and again kept for 4-6hr at 4°C and centrifuged at 10,000 rpm for 15

min at 4°C. After centrifugation, the supernatant were kept separate and the sediments were dissolved in small amount of acetate buffer (pH 5.5). The solution were 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 was used for assay (Lokhande and pethe, 2016).

CELLULASE ASSAY

Endoglucanase activity-carboxyl methyl cellulose:

Carboxy methyl cellulase activity was determined following a method described by the International Union of Pure and Applied Chemistry (Ghose, 1987). The reaction mixture comprise of 0.5 ml carboxyl methyl cellulose (0.2w/v) in 0.05 M citrate buffer, pH 4.8 and 0.5ml crude enzyme in test tubes. The mixture was incubated at 50°C for 1 hour. After incubation 1 ml of dinitro-salicylic acid (DNSA) reagent were added to stop the reaction. The mixture was boiled for 5 minutes in a boiling water bath and 10ml water was added and absorbance was read at 540 nm using a spectrophotometer. The spectrophotometer was blanked with a mixture treated in the same way as the reaction mixture but with 0.05 M citrate buffer replacing the crude enzyme. Glucose standards were prepared with varying concentrations of glucose ranging from 0.1 to 2.0 mg ml⁻¹ and the absorbance were read at 540 nm. Absorbance of the reaction mixture was plotted against concentrations of glucose to obtain a calibration curve. The quantity of reducing sugar produced by the action of crude enzyme was read off from the curve. One unit of enzyme activity will defined as the amount of enzyme that released 1μM of reducing sugar per milliliter of substrate per minute (Saliu, 2012).

RESULTS

Determination of optimum conditions for cellulase production by *Aspergillus niger*

Effect of Incubation Period on Cellulase Production

The effect of incubation period on cellulase activity by *Aspergillus niger* using sugar cane bagasse as substrate is shown in figure 1. The highest cellulase enzyme activity was observed after the 5th day of incubation (0.243U/mL) there was a decline in enzyme activity beyond day 5.

Effect of Incubation Temperature on Cellulase Production

The effect of temperature on cellulose activity is shown in figure 2. The highest enzyme activity was obtained at temperature of 40°C

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(0.268U/mL) while the lowest was observed at 20°C (0.055U/mL).

Effect of Initial pH of Fermentation Medium on Cellulase Production

The effect of initial pH of the fermentation medium on cellulase activity is shown in figure 3. The highest activity was

obtained at pH 6.0 (0.273U/mL) while the lowest (0.079U/mL) was obtained at pH 4.0.

Effect of Inoculum Size on Cellulase Production

Figure 4 shows the effect of inoculums size on cellulase activity. The highest enzyme activity was recorded at an inoculums concentration of 1.0×10^6 spores/ml (0.407U/mL) while the lowest activity was recorded at a concentration of 1.0×10^4 spores/ml (0.199U/mL).

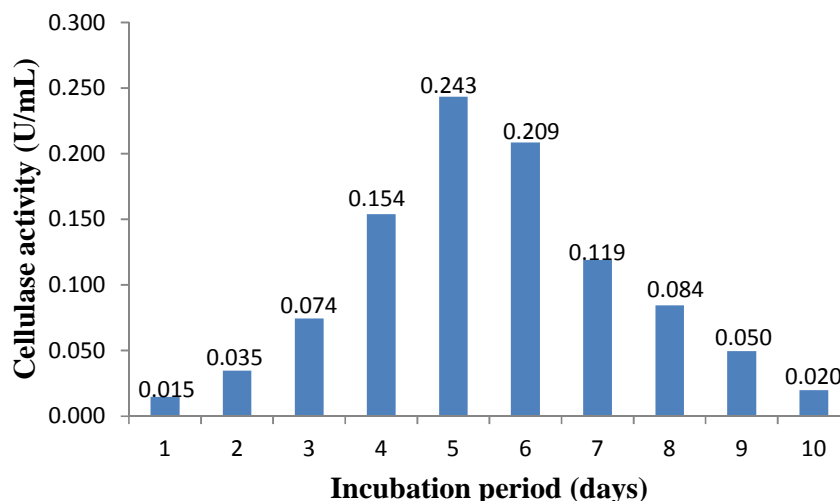


Figure 1: Effect of Incubation Period on cellulase activity by *Aspergillus niger* using sugar cane bagasse as substrate.

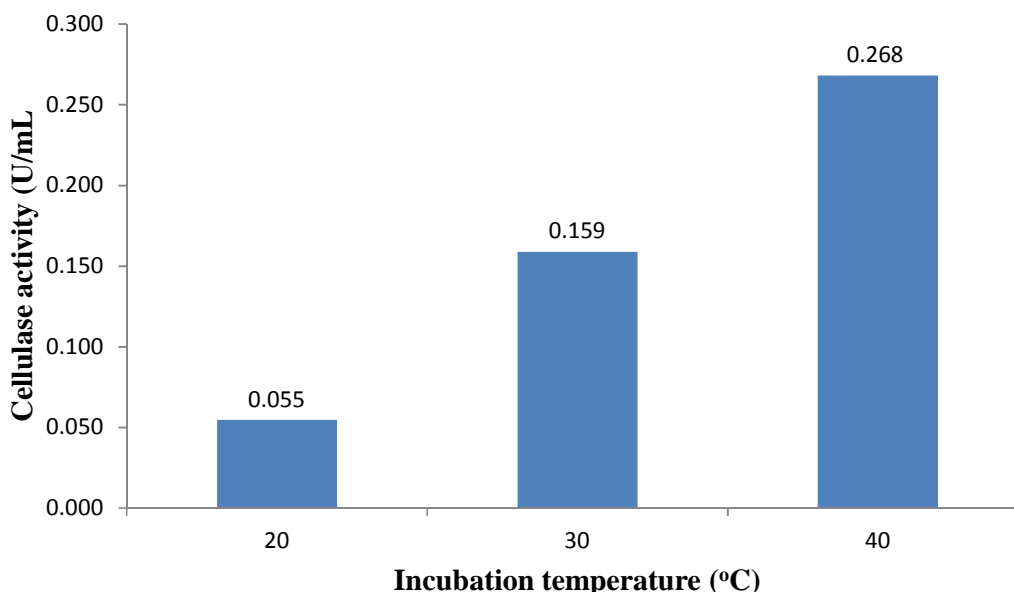


Figure 2: Effect of Incubation Temperature on cellulase activity by *Aspergillus niger* using sugar cane bagasse as substrate.

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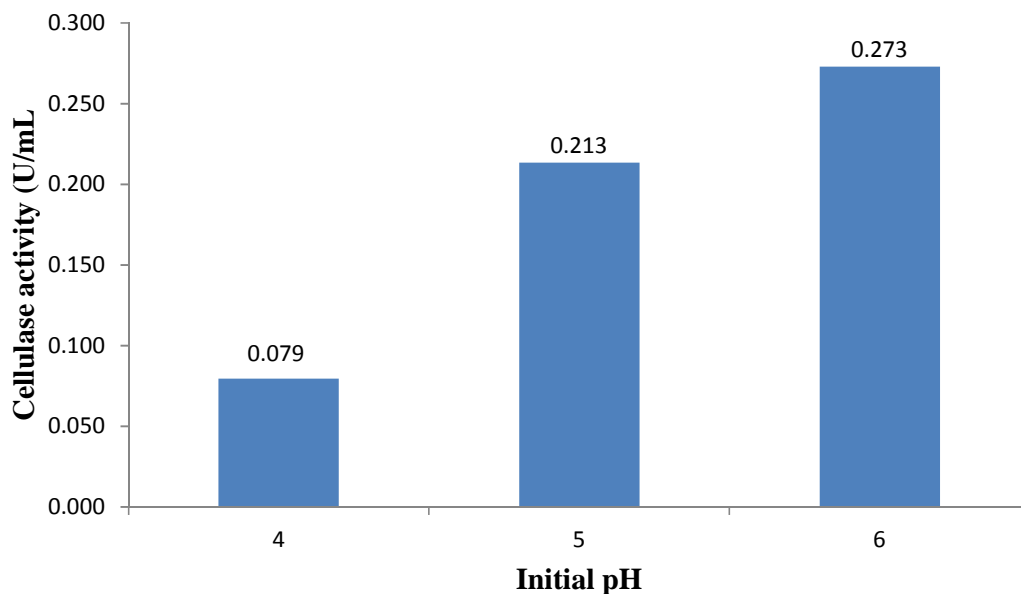


Figure 3: Effect of Initial pH on cellulase activity by *Aspergillus niger* using sugar cane bagasse as substrate.

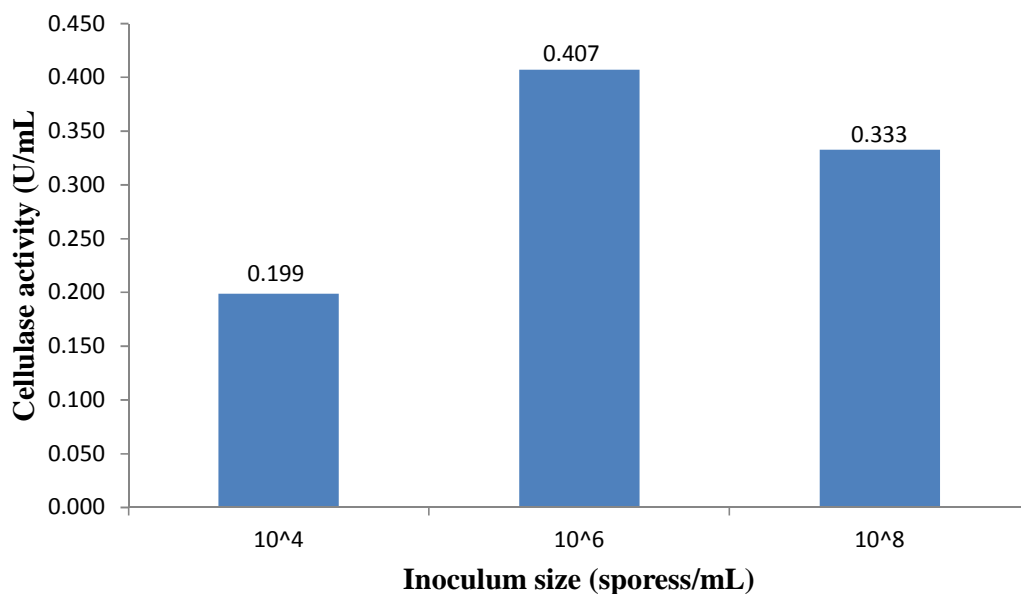


Figure 4: Effect of Inoculum size on cellulase activity by *Aspergillus niger* using sugar cane bagasse as substrate.

Partial Purification of Enzymes Using the Optimum Parameters Obtained

Table 1 shows the comparison of the cellulase yield of crude enzyme solution of the alkali treated and untreated sugar cane bagasse and partially purified enzyme solution of the alkali

treated and untreated sugar cane bagasse. The partially purified alkali treated sugar cane bagasse had higher cellulase yield (0.7422U/mL) compared to the crude enzyme (0.3624U/mL).

Table 1: Comparison of the Cellulase Yields of Crude Enzyme and Partially Purified Enzyme Solutions of the Alkali Treated and Untreated Sugar Cane Bagasse

Enzyme Product	Mean of Enzyme Activity (U/ml)
Crude enzyme	
Treated SB	0.3599±0.088
Untreated SB	0.1415±0.011
Partially purified enzyme	
Treated SB	0.7422±0.067
Untreated SB	0.3624±0.042

Key: SB- Sugar Cane Bagasse

* Values are means of duplicate samples.

DISCUSSION

Cellulase is an inducible enzyme and it is affected by the nature of the substrates used for its production. Optimization of various physical and nutritional growth parameters can cause an increase in the enzyme activity. Such parameters include: incubation period of 5 days, incubation temperature of 40°C, initial pH of fermentation media 6.0 and inoculums size of 1.0×10^6 spores/ml. Therefore, media optimization is an important aspect to be considered in the development of fermentation technology.

Maximal enzyme activity (0.243U/ml) was observed on the 5th day of incubation with fresh spores of *Aspergillus niger* using sugarcane bagasse as substrates (figure 1) which is suitable from a commercial point of view (Kang *et al.*, 2004). The decline in cellulase activity observed from 6th to 10th day of incubation might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretory machinery of the enzyme (Nochure *et al.*; 1993).

Lokhande and Pethe; (2016) recorded peak cellulase activities at day 5 of incubation with *Aspergillus niger* while Gautam *et al.*; (2010) reported the maximum time of cellulase production of 4 days of fermentation period for *Aspergillus niger* by using municipal solid waste residue. Ogel *et al.*; (2001) reported that time course required to reach maximum levels of activity may be affected by several factors like the presence of different ratios of amorphous to crystalline cellulose. In process economics, longer periods of fermentation are quite uneconomical and such processes are prone to contamination (Gautam *et al.*; 2010).

Temperature of incubation is an important factor that affects enzyme production through fermentation. The enzyme activity obtained in this study increased as the incubation

temperature increased until a peak of (0.268U/ml) was obtained at 40°C and then there was a decline in enzyme activity beyond that temperature (figure 2). This may be because enzyme are easily denatured at high temperatures and though the growth temperature of *A. niger* is at ambient, it does not always follow that it is best for enzyme activity (Kotchoni *et al.*; 2003). This agrees with the reports of Gautam *et al.* (2010) and Lokhande and Pethe (2016) who recorded an optimum incubation temperature of 40°C for cellulase production by *Aspergillus niger*. This is in contrast with the findings of Vinod *et al.* (2014) who reported 35°C as the optimum temperature for cellulase activity.

Maximum enzyme activity was obtained at pH 6.0 (0.273U/ml) in this study (figure 3). This is not surprising as most fungal cultures require slightly acidic pH for their growth and enzyme biosynthesis (Muhammad *et al.*, 2010). This is in agreement with the findings of Gautam *et al.* (2010) and Lokande and Pethe (2016) who reported optimum pH of 6.0 of the medium for the production of cellulase by *Aspergillus niger*.

Number and density of spores is an important factor in the fermentation experiments. Maximum cellulase activity (0.407U/ml) was obtained in this study at inoculums size of 1.0×10^6 cells/mL (figure 4). This is because in order to achieve good enzyme activity, the spore concentration in fermentation medium must be high enough to colonize the substrate particles (Damisa, 2007). In this regard therefore, the inoculums size of 1.0×10^6 spore/ml gave maximum cellulase production level in comparison to other inoculums sizes used. This is an agreement with the work conducted by Damisa (2007) where he recorded highest cellulase activity at the inoculums concentration of 1.0×10^6 spore/ml.

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Partial purification of the crude enzyme by ammonium sulphate precipitation technique significantly enhanced the cellulase activity. Higher cellulose yield was observed with the partially purified sugarcane bagasse enzyme solution (0.7422U/ml) as compared to the crude enzyme solution (0.3599U/ml). This is an agreement with the work of Saliu; (2012) also Charita and Sunil (2012) where they observed higher cellulase yield in the partially purified enzyme solution.

CONCLUSION

The conclusions that could be drawn from the present findings include:

1. Different fungi were isolated from different locations of municipal solid waste soils within Zaria metropolis with *Aspergillus* being the most prevalent (86.4%).
2. *Aspergillus niger*-MR2 was observed to be the best cellulase-producing isolate after screening with 73mm zone of diameter of hydrolysis.
3. Incubation period of 5 days, temperature of 40°C, pH 6.0 and inoculum size of 1.0×10^6 spores/mL were found to be the optimum for cellulase production.
4. Partial purification of the cellulase with solid ammonium sulphate revealed an increase in cellulose yield (0.7422±0.067).

RECOMMENDATIONS

At the end of the research work the following recommendations are made:

1. Alkali treated sugar cane bagasse should be used as substrate for cellulase enzyme production as it is cheap and readily available.
2. *Aspergillus niger* should be used for cellulase enzyme production because it is easily isolated from the environment especially refuse dump soil.
3. Further studies should be carried out by modification of the best producing strain to improve enzyme production.
4. Other agro-industrial wastes and residues should be tested for their potentials to serve as substrates for cellulase production by *Aspergillus niger*.

REFERENCES

Albin, G. J., Shyamji, S., Harshita, S., and Sardul, S. (2014). Isolation and Screening of Potential Cellulolytic Fungi for the management of municipal solid waste. *International Journal of Recent Scientific Research*, 5(12):2298-2300

- Bhat, M. K. (2000). Cellulases and related Enzymes in Biotechnology. *Biotechnology Advances*, 18:355-383.
- Charitha, M. D., and Sunil M. K; (2012). Production, Optimization and Partial Purification of Cellulase by *A. niger* fermented with paper and timber saw mill industrial wastes. *Journal of Microbiology Research*. 2 (1): 120-128.
- Damisa, Duro. (2007). The effects of mutagenic treatment on the rate of cellulose production by *Aspergillus niger* isolated from the soil.
- Damisa, D., Ameh, J. B., and Umoh, V. J. (2008). Effects of chemical pretreatment of some lignocellulosic wastes on the recovery of cellulose from *Aspergillus niger* AH3 mutant. *African journal of Biotechnology*, 7 (14): 2444-2450.
- Domsch, K. H; Games, W. and Anderson, T. H. (1980). *Compendium of Soil Fungi*. Academic Press, London, UK, 1:1-858.
- Gautam, S.P., Bundela, P.S., Pandey, A.K., Jamaluddin, Awasthi, M.K., Sarsaiya, S. (2010). *Fusarium* sp. from Municipal Solid Waste Compost: Ability to Produce Extracellular Cellulases Enzyme. *International Journal of Biotechnology and Bioengineering Research*, 2(2): 239–245.
- Immanuel, G., Dhanusa, R., Prema, P. and Palavesam, A. (2006). Effect of Different Growth Parameters on Endoglucanase Enzyme Activity by Bacteria isolated from coir retting effluents of estuarine environment. *International Journal of Environmental Science Technology*, 3 (1): 25-34.
- Kang, S.W., Ko, E.H., Lee, J.S. and Kim, S.W. (2004). Over production of β -glucosidase by *Aspergillus niger* mutant from lignocellulosic biomass. *Biotechnology Letters* 21: 647-650.
- Kamble, D. R. and Jadhav, A. R. (2012). Isolation, Identification and Screening of Potential Cellulase-free Xylanase Producing Fungi and its Production. *African Journal of Biotechnology*, 11(77): 14175-14181.
- Klemm, D; Schmauder, H. P. and Heinze, T. (2002). *Biopolymers*, Volume 4, Edited by E. Vandamme, S De Beats and A Steinb chel (Wiley-VCH, Weinheim), 290 – 292.
- Klemn, D. H.P. and Schmauder, T. H. (2009). In biopolymer edited by E. Vandamme, S. De Beats and A. steind_Chel (Wiley-VCH, Weinheim), 6:290-292.



- Kotchoni, O.S., Shonukan, O.O., and Gachomo, W.E. (2003). *Bacillus pumilus* BpCRI6, a promising candidate for cellulase production under conditions of catabolite repression. *African Journal of biotechnology* 2:140-146.
- Lokhande, S. and Pethe, A. S. (2016). Isolation, Screening and Partial Purification of Cellulase from Cellulase Producing Fungi. *European Journal of Pharmaceutical and Medical Research*. 3(9), 301-306.
- Lekh R., Kuldeep K. and Sandeep S. (2014). Screening Isolation and Characterisation of Cellulase Producing Microorganisms from Soil. *International Journal of Pharmaceutical Science Invention*. 3 (3): 12-18.
- Lynd, L.R., Weimer, P.J., Vanzyl, W.H. and Pretorius, I.S. (2002). Microbial Cellulose Utilization: Fundamentals and Biotechnology. *Microbiology Molecular Biology Review*, 66(3): 506-77.
- Mandels, M. and Reese, E.T. (1999). Fungal Cellulase and Microbial Decomposition of Cellulosic Fibers. *Development of Industrial Microbiology and Biotechnology*, 5(4): 225-240.
- Miller, G.L. (1972). Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugar. *Biotechnology and Bioengineering Symposium*, 5:193-219.
- Mussatto, S. I., and Teixeira, J. A. (2010). Lignocellulose as raw material in fermentation processes. *Biotechnology advances*, 28(6): 817-830.
- Muhammad, I., Quratualain, S., Muhammad, Y., Muhammad, N., Shahjhan, B. and Saghir, A. J. (2010). Studies on Pre-treatment of Wheat straw for improve Production of Carboxymethyl Cellulase by thermophilic *Trichoderma Viride* – FBL 1 in Solid State Fermentation. *Academia Arena*. 2 (7): 18-30.
- Naveenkumar, K. J. and Thippeswamy, B. (2013). Isolation and Screening of Potential Cellulolytic Fungi from Areca nut Husk Waste. *International Journal of Curriculum Science*, 8: 125-132.
- Nochure, J.V., Roberts, M.F. and Demain, A.I. (1993). True Cellulases Production by *Clostridium thermocellum* grown on different carbon sources, *Biotechnology letters*. 15: 641-646.
- O'Sullivan, A.C. (1997) Cellulose: The Structure Slowly Unravels. *Cellulose*, 4:173-207
- Ogel. Z.B., Yarangumeli, K., Du, H. and Ifrij, I. (2001). Submerged Cultivation of *Scytalidium thermophilum* on complex lignocellulosic biomass for endoglucanase production. *Emzyme Microbiology Tech*. 28: 689-695.
- Paul, J.J.A., Daniel, T. (2007). Lignolytic and Phosphate Solubilizing Efficiency of Fungal Species Isolated from Municipal Solid waste. *Asian Journal of Microbial Biotechnology*, 9(4):837-840.
- Saliu, B. K. (2012). Production of Ethanol from some Cellulosic Waste Biomass Hydrolyzed using Fungal Cellulases.
- Singh, H. (2006). Mycoremediation: Fungal Bioremediation. John Wiley & Sons, Hoboken, New Jersey, Pp: 360-362
- Sukumaran, R.K., Singhania, R.R. and Pandey, A. (2005). Microbial Cellulases-production, Applications and Challenges. *Journal of Sciences and Industrial Researches*, 64:832– 844.
- Siddiqui, K. S; Saqio, A. A. N; Rashid, M. H. and Rajoka, M. I. (2000). Carboxyl Group Modification Significantly Altered the Kinetic Properties of Purified Carboxymethyl Cellulase from *Aspergillus niger*, *Enzyme Microbial Technology*, 27: 467–474.
- Tanzila, Shahzadi, Nadia, Ikram., Umer, Rashid., Amber, Afroz., Hamma, Islam-But, Zahid, Anwar., Muhammad, Irshad and Sajid, Mahmood. (2013). Optimization of physical and nutritional factors for induced production of cellulose by co-culture solid- state bio-processing of corn stover.
- Vyas, A., Vyas, D. and Vyas, K. M. (2005). Production and optimization of cellulases on pretreated groundnut shell by *Aspergillus terreus* AV49. *Journal of Scientific and Industrial Research*, 64: 281-286.
- Wen, Z., Liao, W. and Chen, S. (2005). Production of Cellulase by *Trichoderma reesei* from Dairy Manure. *Bio resource Technology*, 2005, 96: 491- 499.