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Purification and Kinetic Characterization of Statistically Optimized Cellulase Produced from *Aspergillus niger*

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Abstract

Bio-conversion of cellulosic based biomass materials for cellulase production is one among the major increasing demands for various biotechnological applications. Aspergillus niger was cultured in corn cobs based fermentation medium under some pre-optimized growth conditions. After four days of still culture incubation a large magnitude of cellulase (28.3±0.24U/mL) was achieved when the SSF medium containing 10 g corn cobs inoculated with 5 mL of inoculum at 30°C. The effects of different minerals were optimized through Response Surface Methodology (RSM) by adopting a Central Composite Design (CCD). The crude cellulase was purified 5.71 fold with specific activity of 232.5U/mg using ammonium sulfate precipitation, and Sephadex-G-100 gel filtration column chromatography. Enzyme was found to be a monomeric protein as evident by single band corresponding to 43 kDa on SDS-PAGE. Characterization revealed that the purified cellulase was optimally active and thermally more stable at pH 7 and 70°C, respectively. Using carboxymethyl cellulose as substrate, the enzyme showed maximum activity (V_{max}) of 45.5U/mL with its corresponding K_m value of 25µM. Among activators/inhibitors, different metal ions (EDTA, Hg^{2+} and Zn^{+2}) showed inhibitory effect up to different extents as different concentrations, whereas, the enzyme was activated by Co^{2+} and Mg^{2+} at the concentration of 5mM.

Keywords: *A. niger*, RSM, cellulase, CCD, purification, characterization, PAGE.

1. Introduction

In nature, cellulose, hemicellulose and lignin are the major components of plant cell walls. Among all of them, cellulose is about 35 to 50% which is the most common and most abundant component of all plant matter. They annually produce about 4×10^9 tons of cellulose which is a highly stable and linear biopolymer of anhydroglucose units consisting of β-1, 4-linked glycosyl residues, along with other polysaccharides (1, 2). A variety of micro-organisms have ability to produce enzymes like cellulases, hemicellulases, pectinases, esterases, oxidoreductases and proteases under suitable growth conditions to hydrolyze insoluble polysaccharides to soluble oligomers, and subsequently to monomers (3-5). *Aspergillus* and *Trichoderma* are one among the most efficient cellulases producer (2, 6), which are being studied for the production of cellulose degrading enzymes from various agro-

industrial waste materials and their by-products such as wood, wheat bran, rice straw, corncobs, corn stover, wheat straw, rice husk, and bagasse (2, 6-8).

Cellulose degrading enzymes system is a complex of three enzymes that can be divided into three types: (i) carboxymethyl cellulase also called as endoglucanase (endo-1,4-β-D-glucanase), (ii) Exoglucanases, including 1, 4-D-glucan glucanohydrolases also known as cellodextrinases and (iii) glucosidases or glucoside glucohydrolases (2, 6). CMCases converts the polymeric form of cellulose into oligosaccharide form, and then exoglucanase separates cellobiose into glucose units (9). Currently, cellulase is being used in many industrial applications, especially in the field of cotton processing, paper recycling, juice extraction, detergent, brewery, textile industries, animal feed, wine-making, agriculture as well as in the field of research and development (5-10). One of the potential applications of cellulase is the production of fuel ethanol from lignocellulosic biomass which is a good substitute for gasoline in internal combustion engines. The most promising technology for the conversion of the lignocellulosic biomass to fuel ethanol is based on the enzymatic breakdown of cellulose using cellulase enzymes (11).

With respect to the factors affecting culture conditions, productivity and properties of cellulase, it was considered of significance to purify and characterize this enzyme through kinetic studies to explore that factors. Therefore by following the current industrial and biotechnological scenario the present study was especially focused at producing the industrial important enzyme cellulase by growing *A. niger* in SSF of lignocellulosic substrate corn cobs by optimizing some parameters through RSM by adopting a central composite design.

2. Materials and Methods

2.1. Chemicals and substrate All the chemicals used in this study were of analytical laboratory grade. The agro-industrial waste, i.e. corn cobs was collected locally and crushed into pieces, oven dried at 70^{o}C and grinded mechanically to make it in powdered form, sieve to 40 meshes.

2.2. Fungal strain and inoculum development *A. niger* was selected for the production of cellulase. Figure 1 representing a characteristic morphology of the *A. niger* observed under stereoscope after staining with Lacto-phenol and Cotton blue. To develop homogeneous inoculums suspension, spores of the culture was cultivated using 250 mL capacity Erlenmeyer flask containing 50 mL of Potato Dextrose broth followed by incubation at 37^{o}C and 150 rpm for 72 h.

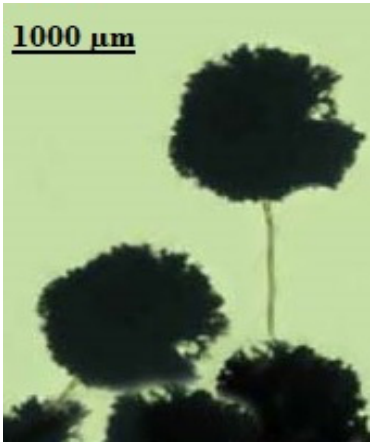

Figure 1. Stereoscopic morphology of *A. niger* after staining with lacto-phenol and cotton blue

2.3. Pre-treatment of corn cobs The fine-powdered size corn cobs (10 g) was pre-treated with 2% HCl in an Erlenmeyer flask (250 mL) at room temperature for 2 h of initial reaction time followed autoclaved at 121 ºC and 15 lb/in^2 pressure for 15 min. The slurry of corn cobs was filtered through Watman No 1 filter paper; both the filtrates and the residues were saved and used for the production of cellulase enzymes and further analysis.

2.4. Solid state fermentation protocol Solid State Fermentation was performed in duplicate Erlenmeyer flasks containing 10g of pre-treated corn cobs. The SSF medium was autoclaved at 121^{o}C for 15 min, inoculated with 5 mL of spore suspension of *A. niger* followed by incubation at 37°C in still culture incubator. After stipulated fermentation time, 100 mL of distilled water was added to all of the fermented cultures and the flasks were shaken at 140 rpm for 45 min. The contents were filtered through sterile Millipore filter and the filtrates were centrifuged at 4000 × g for 10 min. The collected supernatants were pooled that was used as crude enzyme extract for activity assay purposes.

2.5. Determination of Enzyme activity and protein contents Enzyme activity of supernatants collected was determined by UV-Vis spectrophotometeric method as described earlier by Iqbal et al. (7). The activity of each sample was measured at the wavelength of 540 nm using UV-Vis spectrophotometer. A unit activity was defined as the amount of enzyme required to produce a unit increase in absorbance at specific wavelength (nm) per mL of reaction mixture. Bovine serum albumin was used as a standard protocol to determine the protein contents of the crude and purified enzyme extracts.

2.6. Minerals optimization by Response Surface Methodology To achieve maximum yield of cellulase effect of different minerals were optimized through RSM under pre-optimized culture conditions. By adopting a Central Composite Design 30 treatments (T_1-T_{30}) were used to investigate the best one treatment for its significant effect on the cellulase activity. Each treatment was the combination of four factors (N, Ca^{+2}, Mg^{+2} and K^+) along their ranges and levels. For nitrogen, percentage level was from 0.1 to 0.7 (left to right) and similarly, the levels of Ca^{+2}, Mg^{+2} and K^+ are given (Table 1). These percentage levels were taken as a reference for 30 treatments (Table 2). Counter plots and Response surface 3D curves were plotted to understand the interactive effects of variables and also for identifying the optimal concentrations of each parameter for attaining maximal cellulase yield.

Table 1. Levels of experimental factors for minerals optimization

Sr. #	Factors	Ranges and levels (%)				
			Low	Medium	High	
		-2	**-1**	**0**	**1**	**2**
1	Nitrogen	0.1	0.2	0.3	0.5	0.7
2	Calcium	0.01	0.05	0.1	0.15	0.2
3	Magnesium	0.05	0.001	0.015	0.02	0.025
4	Potassium	0.1	0.2	0.3	0.4	0.5

2.7. Purification of cellulase The enzyme concentrate was placed in ice bath and crystals of ammonium sulfate were added to attain 80% saturation at 0^{o}C followed by centrifugation at 10,000 g. The resulting pellets were dissolved in 0.2M Tris-HCl buffer and dialyzed against distal water. Then it was lyophilized and used for further purification and molecular weight determination through gel filtration chromatography and PAGE, respectively. Gel filtration chromatography was carried out using Sephadex-G-100 column with the flow rate of 0.5mL/min.

Table 2. Central composite experimental design from response surface methodology to optimize different minerals for induced production of cellulase

Treatments	Levels				Combination of four minerals			
	N	Ca	Mg	K	N %	Ca %	Mg %	K %
T_1	1	-1	1	-1	0.5	0.05	0.02	0.2
T_2	-1	-1	-1	-1	0.2	0.05	0.001	0.2
T_3	0	0	0	0	0.3	0.1	0.015	0.3
T_4	1	-1	-1	1	0.5	0.05	0.001	0.4
T_5	-1	-1	1	1	0.2	0.05	0.02	0.4
T_6	-1	1	-1	1	0.2	0.15	0.001	0.4
T_7	1	1	-1	-1	0.5	0.15	0.001	0.2
T_8	0	0	0	0	0.3	0.1	0.015	0.3
T_9	1	1	1	1	0.5	0.15	0.02	0.4
T_{10}	-1	1	1	-1	0.2	0.15	0.02	0.2
T_{11}	1	1	1	-1	0.5	0.15	0.02	0.2
T_{12}	-1	1	1	1	0.2	0.15	0.02	0.4
T_{13}	-1	1	-1	-1	0.2	0.15	0.001	0.2
T_{14}	0	0	0	0	0.3	0.1	0.015	0.3
T_{15}	-1	-1	1	-1	0.2	0.05	0.02	0.2
T_{16}	1	-1	1	1	0.5	0.05	0.02	0.4
T_{17}	0	0	0	0	0.3	0.1	0.015	0.3
T_{18}	-1	-1	-1	1	0.2	0.05	0.001	0.4
T_{19}	1	-1	-1	-1	0.5	0.05	0.001	0.2
T_{20}	1	1	-1	1	0.5	0.15	0.001	0.4
T_{21}	0	0	-2	0	0.3	0.1	0.005	0.3
T_{22}	0	0	2	0	0.3	0.1	0.025	0.3
T_{23}	0	0	0	0	0.3	0.1	0.015	0.3
T_{24}	-2	0	0	0	0.1	0.1	0.015	0.3
T_{25}	0	0	0	2	0.3	0.1	0.015	0.5
T_{26}	0	-2	0	0	0.3	0.01	0.015	0.3
T_{27}	0	0	0	0	0.3	0.1	0.015	0.3
T_{28}	0	2	0	0	0.3	0.2	0.015	0.3
T_{29}	2	0	0	0	0.7	0.1	0.015	0.3
T_{30}	0	0	0	-2	0.3	0.1	0.015	0.1

2.8. SDS-PAGE To determine the molecular weight of purified cellulase Sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% resolving gel according to the method as described earlier (2). The molecular weight of the purified cellulase was determined in comparison to marker protein (standard protein marker, 21-116kDa; Sigma, USA) after documentation of the gel.

2.9. Characterization of purified cellulase The purified cellulase was subjected to characterization through kinetic studies by studying the effect of different pH values (3-10), incubation temperatures (30-80ºC), substrate concentrations (100-1000µM) and various compounds metal ions (EDTA, Hg^{2+}, Zn^{+2}, Co^{2+} and Mn^{2+}). The enzyme activities for each case were determined under standard assay conditions using carboxymethyl cellulose as substrate as described earlier.

2.10. Statistical analysis All experiments and enzyme assays were performed in triplicates; statistically evaluated and results have been presented as mean ± S.E. (standard error). The S.E values have been displayed as Y-error bars in figures.

3. Results and Discussion

3.1. Production of cellulase *A. niger* was cultured in the fermentation medium containing 10g of 2% HCl pre-treated corn cobs as growth supported substrate under some pre-optimized growth conditions. After four days of still culture incubation a large magnitude of cellulase (28.3±0.24U/mL) was achieved when the SSF medium containing 10 g corn cobs substrate (50% w/w moisture) inoculated with 5 mL of culture inoculum at pH 8 and 30°C. In t has been reported in literature that a low cost substrates like wheat flour, wheat bran, corn and rice straws, corn cobs, and molasses are suitably effective for fungal growth and enzyme production (2, 7). Ojumu et al. (12) reported highest cellulase activity from saw dust, bagasse and corn cob as growth substrates, respectively. In an earlier study, Quiroz-Castaneda et al. (13) has achieved maximum activity of the cellulases after 8 days of inoculation using wheat straw as a growth substrate. In this context, in present study, *A. niger* produced higher titters of cellulase without any additional supplements in comparison to previously studied different fungi which produced maximum enzymes after 6-8 days of fermentation (13).

3.2. Minerals optimization by RSM Minerals were optimized by RSM using *A. niger* under pre-optimized culture conditions (96 h, 30ºC and pH, 9.0). Out of total 30 treatments the best treatment, which significantly enhanced the cellulase activity, was T_{13} (33.6U/mL). The regression coefficients of four variables (C_1=N, C_2=Ca^{+2}, C_3=Mg^{+2} and C_4=K^+), probability value (p-value) for each variable and p-value for their interactions on the response were used to evaluate the significance levels ($p<0.01$ or $p<0.05$). The p-value suggest that the coefficient for linear effect of calcium, C_2 and potassium, C_4 were found to be significant model terms with p values 0 and 0.001, respectively and N*Ca, Ca*K, K*K, N*Mg, Ca*Mg and N*N were significant insight mineral interactions with p values 0, 0, 0.011, 0.001, 0.008 and 0.027, respectively. Whereas, the coefficient of determination (R^2) and R^2-adj were 90.7% and 82%, respectively this ensured the satisfactory adjustment and significance of RSM model to experimental data. The interaction between all four tested mineral variables i.e. N, Ca^{+2}, Mg^{+2} and K^+ and their effcet on cellulase production has been displayed in contour plots (figure 2A) and response surface 3D curves (figure 2B).

3.3. Purification and PAGE analysis of cellulase The supernatant with cellulase activity of 6720U/200mL and specific activity of 40.7U/mg was used as crude enzyme solution and subjected to purification by ammonium sulfate precipitation followed by passing through the Sephadex-G-100 gel filtration column. By gel filtration the enzyme was purified to 5.71 fold with a specific activity of 232.5U/mg (Table 3). In literature, Sephadex G-100 column gel filtration technique to purify various fungal enzymes mainly cellulases from *Trichoderma harzianum* & *Trichoderma viridi*, protease from *A. niger*, laccase and MnP, and LiP from *Trametes versicolor* IBL-04 have been reported by many authors (2, 14-17). Recently, Asgher and Iqbal (18) have also been used the Sephadex-G-100 gel filtration technique to purify MnP enzyme. The purified cellulase resolved on SDS-PAGE was found to be a homogenous

monomeric protein as evident by a single band corresponding to 43kDa relative to the standard molecular weight markers (Figure 3).

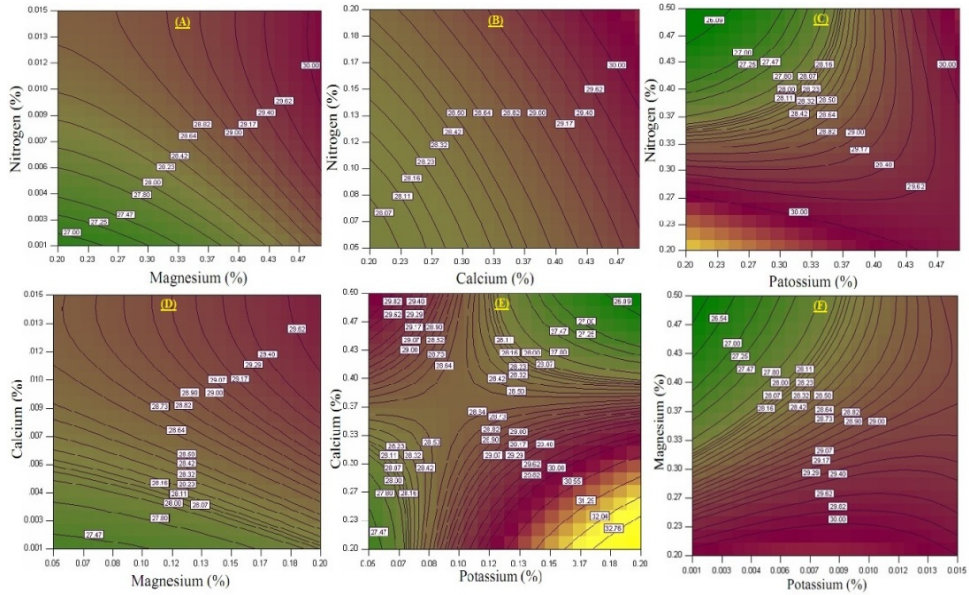

Figure 2A. Contour plots showing the interactive effect of the minerals (A) nitrogen vs. magnesium, (B) nitrogen vs. calcium, (C) nitrogen vs. potassium, (D) calcium vs. magnesium, (E) calcium vs. potassium, and (F) magnesium vs. potassium on cellulase production by *A. niger* under optimum conditions

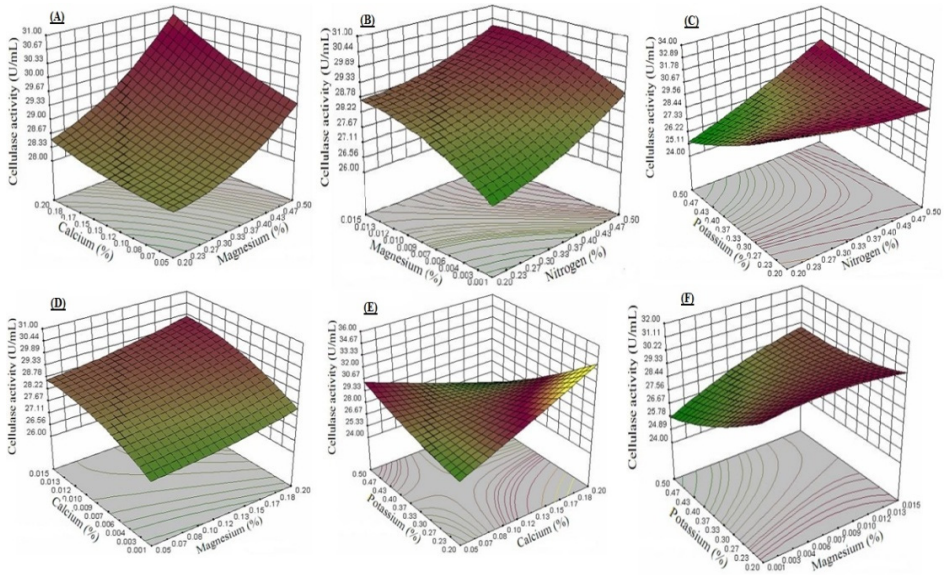

Figure 2B. 3-D response surface plots showing the interactive effect of the minerals (A) calcium & magnesium, (B) magnesium & nitrogen, (C) potassium & nitrogen, (D) magnesium & calcium, (E) potassium & calcium, and (F) potassium & magnesium on cellulase production by *A. niger* under optimum conditions

Table 3. Purification summary of cellulase produced from *A. niger*

Sr. No.	Purification Steps	Volume (mL)	Enzyme Activity (U)	Protein Content (mg)	Specific Activity (U/mg)	Purification fold	% Yield
1	Crude Enzyme	200	6720	165	40.7	1	100
2	$(NH_4)_2SO_4$ Precipitation	30	1275	30	42.5	1.04	18.97
3	Dialysis	25	1400	18	77.7	1.91	20.83
4	Sephadex-G-100	12	1860	8	232.5	5.71	27.67

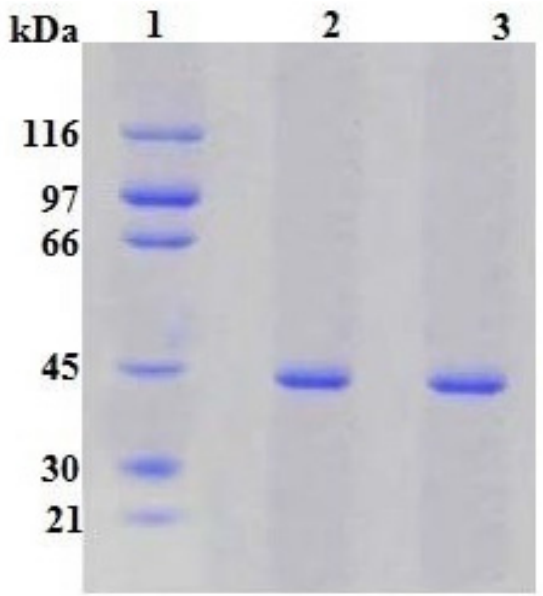

Figure 3. SDS-PAGE for cellulase produced by *A. niger*

3.4. Effect of pH and temperature on cellulase activity and stability Results of enzyme assay showed that the cellulase was completely stable in a large pH range (5-9) and retained 88.8% of its original activity at a pH value of 7 (Figure 4) which was higher than previously reported cellulases from *Mucor circinelloides*, 4.0-7.0 (19) and *Bacillus circulans*, 4.5-7.0 (20). Our results are in line with Odeniyi et al. (21) who reported that the cellulase activity possessed a relatively broad pH, and progressively tends to decrease in the direction of high alkalinity up to pH 10. Temperature optimum for purified cellulase was observed at 70°C with 95% of original activity. Results of figure 5 showed cellulase activity and stability profile with ascending trend from 30-70°C and revealed that at temperatures higher than 70°C enzyme starts to losses its activity rapidly. For a variety of industrial applications relatively high thermo-stability is an attractive and desirable characteristic of an enzyme (22). Most of the earlier reported CMCase were found to lose their activities at temperatures around 60°C. Cellulase from *Bacillus coagulans* was optimally active and stable when incubated at an optimum temperature of 50°C (21).

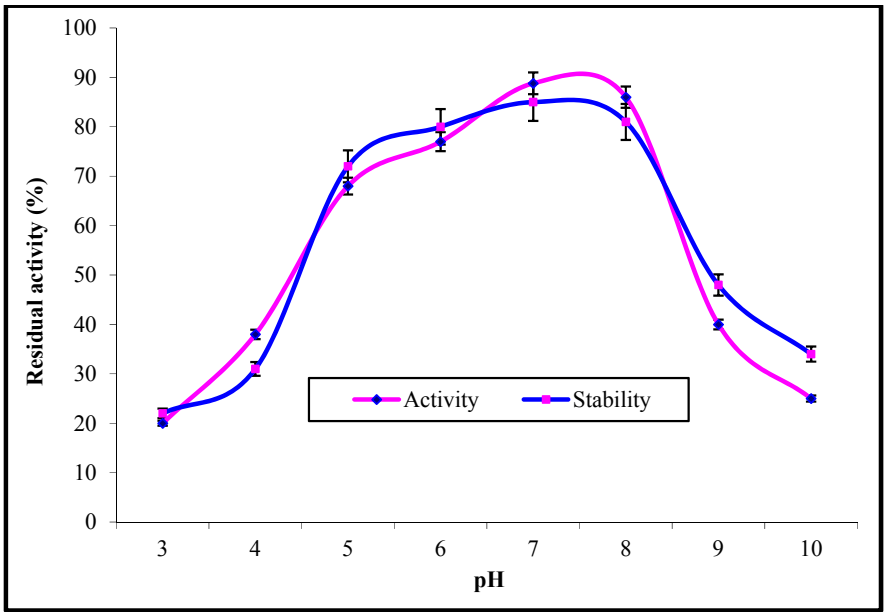

Figure 4. Effect of pH on activity and stability of cellulase produced by *A. niger*

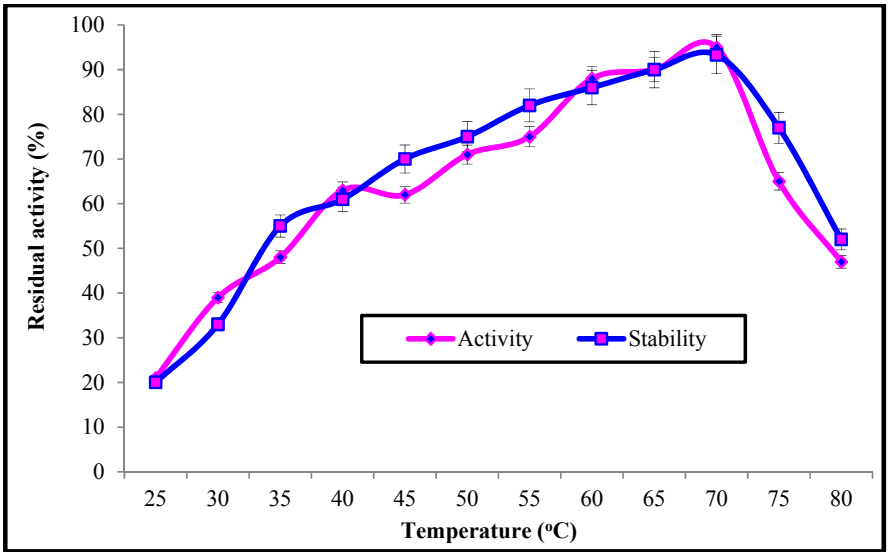

Figure 5. Effect of temperature on activity and stability of cellulase produced by *A. niger*

3.5. Effect of substrate concentration: determination of K_m and V_{max} From the catalytic properties, K_m and V_{max} values of purified cellulase from *A. niger* were 25µM and 45.5U/mL respectively. An enzyme with low K_m has a greater affinity for its substrate. In literature, different ranges of K_m and V_{max} for different fungal species have been reported. Ekperigin, (23) reported K_m values of 0.32 and 2.54 mM using cellobiose as substrate for two different spp. i.e., *A. anitratus* and *Branhamella* spp. respectively. In some other studies a bit higher K_m

values i.e., 3.6mg/mL for *Pseudomonas fluorescens* and 1.1 mM for *Trichoderma reesei* have been reported in literature by different authors (24, 25). The K_m value reported in this present study for cellulase obtained from *Aspergillus niger* is lower than the *Branhamella* sp. and showing greater affinity for its substrate. The difference in K_m value of the presently purified cellulase from *A. niger* and other reported fungal species may be due to the genetic variability among different species.

3.6. Effect of various activators and inhibitors As shown in figure 6 that among various compounds and metal ions; EDTA, Hg^{2+} and Zn^{+2} showed inhibitory effect on purified cellulase whereas, the enzyme was activated by Co^{2+} and Mg^{2+} at a concentration of 5mM as compare to control (0mM). EDTA is a metal chelating agent and found inhibitory to the carboxymethyl cellulase activity due to its inactive complex formation with inorganic groups of enzyme. Bakare et al. (24) also reported an inhibitory effect of EDTA to the activities of cellulase from wild type of *Pseudomonas fluorescens*. According to Saha, (19) Co^{2+} and Mn^{2+} activate the enzyme that from *Mucor circinelloides* and *Chalara paradoxa* respectively. In case of Zn^{+2} at 0 mM the cellulase activity was the highest 26.7U/mL and the lowest at 20 mM (8.7U/mL), showing decline in activity up to 67.38%. Our data suggest that the presently purified cellulase was 50% more stable against higher concentration of inhibitors like Zn^{+2} in comparison to the earlier reported cellulase with maximum activity up to 32% at a concentration of 10 mM (26).

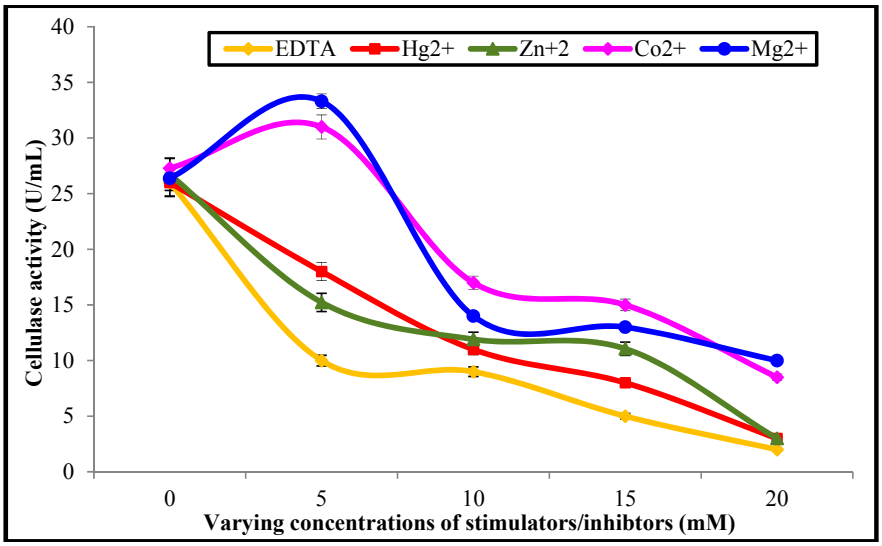

Figure 6. Effect of activators/inhibitors on purified cellulase produced by *A. niger*

4. Conclusions

The results of this study indicate a remarkable enzyme production potential of *A. niger* from agro-industrial corn cobs. CCD from RSM was successfully applied for hyper-production of cellulase under varying concentrations of different minerals. Hence, this cellulase was purified to electrophoretical homogeneity by ammonium sulfate precipitation, and Sephadex-G-100 gel filtration column chromatography. The further optimization on the commercial scale production for cellulase using this strain is on-going currently.

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References

1. C. POTHIRAJ, P. KANMANI, P. BALAJI, Bioconversion of lignocellulose materials. Mycobiol., **34**(4), 159-165, (2006).
2. H.M.N. IQBAL, I. AHMED, M.A. ZIA, M. IRFAN, Purification and characterization of the kinetic parameters of cellulase produced from wheat straw by *Trichoderma viride* under SSF and its detergent compatibility. Adv. Biosci. Biotechnol., **2**(3), 149-56, (2011).
3. N. BEUKES, B.I. PLETSCHKE, Effect of sulfur-containing compounds on *Bacillus* cellulosome-associated 'CMCase' and 'avicelase' activities. FEMS Microbiol. Lett., **264**, 226-231, (2006).
4. C.E. TODERO RITTER, M. CAMASSOLA, D. ZAMPIERI, M.M. SILVEIRA, A.J.P. DILLON, Cellulase and xylanase production by *Penicillium echinulatum* in submerged media Containing Cellulose Amended with Sorbitol. Enz. Res., **2013**, Article ID 240219, (2013).
5. H.M.N. IQBAL, S. KAMAL, I. AHMED, M.T. NAVEED, Enhanced bio-catalytic and tolerance properties of an indigenous cellulase through xerogel immobilization. Adv. Biosci. Biotechnol., **3**, 308-313, (2012).
6. K.H. BRIJWANI, S. OBEROI, P.V.VADLANI, Production of a cellulolytic enzyme system in mixed-culture solid-state fermentation of soybean hulls supplemented with wheat bran. Proc. Biochem., **45**, 120-128 (2010).
7. Z. ANWAR, M. GULFRAZ, M. IRSHAD, Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. J. Radiat. Res. Appl. Sci., In-press, (2014).
8. T. SHAHZADI, S. MEHMOOD, M. IRSHAD, Z. ANWAR, A. AFROZ, N. ZEESHAN, U. RASHID, K. SUGHRA, Advances in lignocellulosic biotechnology: A brief review on lignocellulosic biomass and cellulases. Adv. Biosci. Biotechnol., **5**(3), 246-251, (2014).
9. K. KURODA, M. UEDA, Arming technology in yeast novel strategy for whole-cell biocatalyst and protein engineering. Biomolecules., **3**(3), 632-650, (2013).
10. S. YANO, H. OZAKI, S. MATSUO, M. ITO, M. WAKAYAMA, K. TAKAGI, Production, purification and characterization of D-aspartate oxidase from the fungus *Trichoderma harzianum* SKW-36. Adv. Biosci. Biotechnol., **3**(1), 7-13, (2012).
11. A. AHAMED, P. VERMETTE, Culture based strategies to enhance cellulase enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture conditions. Biochem. Eng. J., **40**, 399-407, (2008).
12. T.V. OJUMU, B.O. SOLOMON, E. BETIKU, S.K. LAYOKUN, B. AMIGUN, Cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 fermented in sawdust, bagasse and corncob. Afr. J. Biotechnol., **2**, 150-152, (2003).
13. R.E. QUIROZ-CASTANEDA, E. BALCAZAR-LOPEZ, E. DANTAN-GONZALEZ, A. MARTINEZ, J. FOLCH-MALLOL, C.M. ANAYA, Characterization of cellulolytic activities of *Bjerkandera adusta* and *Pycnoporus sanguineus* on solid wheat straw medium. Elect. J. Biotechnol., **12,** Issue of October 15, (2009).
14. I. AHMED, M.A. ZIA, T. IFTIKHAR, H.M.H. IQBAL, Characterization and detergent compatibility of purified protease produced from *Aspergillus niger* by utilizing agro wastes. BioRes., **6**, 4505-4522, (2011).
15. M. ASGHER, H.M.H. IQBAL, Characterization of a novel manganese peroxidase purified from solid state culture of *Trametes versicolor* IBL-04. BioRes., **6**, 4302-4315, (2011).
16. M. ASGHER, H.M.H. IQBAL, M.J. ASAD, Kinetic characterization of purified laccase produced from *Trametes versicolor* IBL-04 in solid state bio-processing of corncobs. BioRes., **7**, 1171-1188, (2012).
17. M. ASGHER, H.M.H. IQBAL, M. IRSHAD, Characterization of purified and xerogel immobilized novel lignin peroxidase produced from *Trametes versicolor* IBL-04 using solid state medium of corncobs. BMC Biotechnol., **12**, 46, (2012).
18. M. ASGHER, H.M.N. IQBAL, Enhanced catalytic features of sol-gel immobilized MnP isolated from solid state culture of *Pleurotus ostreatus* IBL-02. Chin. Chem. Lett., **24**(4), 344-346 (2013).
19. B.C. SAHA, Production purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*. Proc. Biochem., **39**, 1871-1876, (2004).
20. C.H. KIM, Characterization and substrate specificity of an endo-beta-1,4-D-glucanase I (Avicelase I) from an extracellular multienzyme complex of *Bacillus circulans*. Appl. Environ. Microbiol., **61**, 959-965, (1995).
21. O.A. ODENIYI, A.A. ONILUDE, M.A. AYODELE, Production characteristics and properties of cellulase/polygalacturonase by a *Bacillus coagulans* strain from a fermenting palm-fruit industrial residue. Afr. J. Microbiol. Res., **3**, 407-417, (2009).

22. H.M.H. IQBAL, M. ASGHER, Characterization and decolorization applicability of xerogel matrix immobilized manganese peroxidase produced from *Trametes versicolor* IBL-04. Prot. Pept. Lett., **20**, 591-600, (2013).
23. M.M. EKPERIGIN, Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. Afr. J. Biotechnol., **6**, 028-033, (2007).
24. M.K. BAKARE, I.O. ADEWALE, A. AJAYI, O.O. SHONUKAN, Purification and characterization of cellulase from the wild-type and two improved mutants of *Pseudomonas fluorescens*. Afr. J. Biotechnol., **4**, 898-904, (2005).
25. J.F. CASCALHEIRA, J.A.QUEIROZ, Kinetic study of the cellobiase activity of *Trichoderma reesei* cellulose complex at high substrate concentrations. Biotechnol. Lett., **21**, 651-655, (1999).
26. J.A. JAMES, G. DIXON, O. LAMIKANRA, Characterization of partially purified cellulase from muscadine grapes (*Vitis rotundifolia* Michx.). Am. J. Enol. Vitic., **1**, 19-24, (1999).