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Submerged fermentation of wheat bran by *Aspergillus flavus* for production and characterization of carboxy methyl cellulase

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ABSTRACT

Objective: To elucidate the capabilities of carboxy methyl cellulase (CMCase) production with the selective species of *Aspergillus flavus (A. flavus)*. **Methods:** The carboxy methyl cellulase (CMCase) expression in *A. flavus* was evaluated under different environmental conditions using submerged fermentation (SmF) on different agricultural byproducts. **Results:** *A. flavus* produced high levels of CMCase under optimized culture conditions on 3rd day of incubation at an optimum pH 6.0, temperature 30 ℃, inoculum size 4% in Czapek Dox using wheat bran as a substrate by SmF. Enhanced production occurred on addition of 4% wheat bran and 1% peptone as nutritional factors. The enzyme hydrolyzed wheat bran exhibiting V_{max} value of 40 U/mg and K m value of 0.5 mg/mL at pH of 7. The enzyme was optimally activated 50 ℃. Except Mg^{2+}, all other metal ions such as Zn^{2+}, Cu^{2+}, Na^{+} and Ca^{2+} were found to be activators for the enzyme activity. **Conclusion:** The kinetic parameters showed the thermo stability, pH stability of the enzyme which reflects the potential and commercial significance of the enzyme.

1. Introduction

Fellulases are a group of hydrolytic enzymes capable of hydrolyzing the most abundant organic polymer i.e. cellulose to smaller sugar components including glucose subunits. Cellulases consist of three major components: endo-β-glucanase (EC 3.2.1.4), exo-β-glucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). These enzymes act together synergistically to converts crystalline cellulose to oligosaccharides and glucose. Endo-β-glucanase (CMCase) causes random scission of cellulose chains yielding glucose and cello-oligosaccharides. Exo-β-glucanase (avicelase) attacks on the non-reducing end of cellulase yielding cellobiose. β-glucanase (cellobiose) hydrolyses aryl- and alkyl-glycosides as well as cellobiose and cellodextrin[1, 2].

Cellulase production by different organisms in submerged state fermentation has received more attention and is found to be cost-prohibitive because of high cost of process engineering(3). Most commonly studied cellulolytic organisms include: Fungal species- *Trichoderma, Humicols, Penicillium, Aspergillus;* Bacteria- *Bacilli, Pseudomonads, Cellulomonas*; and Actinomycetes- *Streptomyces, Actinomucor* and *Streptomyces*[4].

Cellulases have enormous potential in industries and are used in food, beverages, textile, laundry, paper, waste management, medical/pharmaceutical industry, protoplast production, genetic engineering and pollution treatment and in other areas[5] and pulp industries etc. This study was aimed to screen the cellulytic ability of fungi from native environmental source. Furthermore, optimal condition for enzyme activity and induction of enzyme synthesis were also determined.

Commercial production and efficient utilization of increase process efficiency, one can search for more active cellulase, or develop cheap methods for pretreatment of the substrates, making them more accessible for enzymic degradation. So, for mitigation of this problem the present investigation describes the production and characterization of extracellular carboxy methyl cellulase from a newly isolated fungi *A. flavus* is reported.

2. Materials and methods

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2.1. Cellulosic material

In our preliminary studies, various agro wastes were used as carbon source and hence it could reduce the cost of enzyme production which is collected in dried form from cattle shop, Coimbatore. Substrates like wheat bran, cotton seed, rice bran, rice straw and pomegranate were screened for enzyme production, in which wheat bran showed higher CMCase production, so it was used for the further studies.

2.2. Organism and inoculum preparation

Fungal strains were isolated from soil of sugarcane field Coimbatore, India by serial dilution plate method. Fungus were isolated from 10^{-3} – 10^{-4} dilutions by plating into Potato Dextrose Agar (PDA) medium. Isolated fungal cultures were screened for protease enzyme production. The organisms were identified using lacto phenol cotton blue mounting method. The isolated culture (*A. flavus*) was purified by routine sub-culturing and stored at 4 ℃ for further use.

2.3. Fermentation condition

The liquid media (modified Czapek-Dox media) contained (g/L): 3.0 $NaNO_3$, 0.5 KCl, 1.0 KH_2PO_4, 0.5 $MnSO_4$. $7H_2O_4$, 0.1 $FeSO_4.7H_2O$ and 1.0% carbon source (wheat bran). One liter (1 L) of the media was supplemented with 1.0 mL of trace metal solution containing (g/L) 1.0 $ZnSO_4$ and 0.5 $CuSO_4.5H_2O$. The pH of each media was adjusted to 5.6 with 0.1M HCl before sterilizing in an autoclave at 121 ℃ for 15 min. After inoculation (10^6 spores/mL), the flasks were incubated at 30 ℃ for seven days in a incubator shaker at 125 rpm. At the end of fermentation, the supernatant was harvested by centrifugation at 10 000 rpm for 10 min (4 ℃) and was used as crude enzyme extract.

2.4. Optimization of CMCase

Various process parameters affecting CMCase production in SmF were optimized. The strategy was to optimize each parameter independently and subsequently optimum conditions were employed in each experimental run. The best substrate was selected for optimum production of CMCase. The tested process parameters in this study were incubation time (1-6 days), pH (3-8), temperature (20-60 ℃), inoculum level (1%-5%), nitrogen source (beef extract, KNO_3, peptone, yeast extract and urea) and its concentration and concentration (1%-5%) of substituted carbon source.

2.5. Characterization of enzyme

2.5.1. Effect of pH on enzyme activity

The activity of the purified protease was measured at different pH values. The pH was adjusted using, the following buffers: 50 mM sodium citrate (pH 3.0-6.0) and 50 mM sodium phosphate (pH 7.0 & 8.0). The reactions were incubated at room temperature for 3 days, and the activity of the enzyme was measured by using DNS method(6) and the protein content also estimated by using Lowry *et al*.[7].

2.5.2. Effect of temperature on enzyme activity

The optimum temperature of the CMCase activity of the purified enzyme was assayed at pH 8.0 and different temperature of incubation 20 to 70 ℃ for 72 hours at pH 5.6 with the addition of substrate (wheat bran). The residual activity was then measured by using DNS method[6] and the protein content also estimated by using Lowry *et al*.[7].

2.5.3. Determination of kinetic parameters

Michaelis constant (Km) and maximal velocity (V_{max}) of the purified enzyme (for CMCase) was determined as follows: varying concentrations of wheat bran ranging from 1.0 to 5.0 mg/mL as substrate, add 0.1 mL diluted enzyme solution and incubated at room temperature for 72 hours. The reducing sugar produced was measured calorimetrically with DNS reagent[6]. The Vmax and Km were calculated from double-reciprocal plots according to the method of Lineweaver and Burk[8].

2.5.4. Effect of cations on enzyme activity

The effect of different metal ions on endoglucanase activity was determined by the addition of the corresponding ion at a concentration of 0.5 mM to the reaction mixture, and the assay was performed under standard conditions. The tested ions included the following corresponding salts: NaCl, $CaCl_2$, $MgCl_2$, $CuSO_4$, and $ZnSO_4$. All experiments were conducted with triplicates and their mean values represented.

2.5.5. CMCase activity assay

CMCase activity was measured following the method of Miller[6]. Briefly, a reaction mixture composed of 0.2 ml of crude enzyme solution plus 1.8 ml of 0.5% carboxymethyl cellulose (CMC) in 50 mM sodium phosphate buffer (pH 7.0) was incubated at 37 ℃ in a shaking water bath for 30 min. The reaction was terminated by adding 3.0 mL of DNS reagent. The color was then developed by boiling the mixture for 5 min. Optical densities of samples were measured at 575 nm against a blank containing all the reagents minus the crude enzyme[9].

3. Result

Cellulases are inducible enzymes and their induction and activity depends on the nature of substrate. Enzyme production by micro organisms is greatly influenced

by media components, especially carbon and nitrogen sources, and physical factors such as temperature, pH, incubation time and inoculum density. It is important to produce the enzyme in inexpensive and optimized media on large scale for the process to be commercially viable. So the studies on the influence of various physico-chemical parameters such as incubation periods, inoculam size, temperature, pH, carbon, and nitrogen sources. Agricultural byproducts rich in cellulosic biomass can be exploited as cheap raw material for the industrially important enzymes and chemicals[10].

Time course of enzyme production plays a very critical role in enzyme synthesis. The *A. flavus* was incubated for 1-6 days (Figure 1). The production of CMCase was increased with increase in the incubation period and found maximum on 3rd day after inoculation.

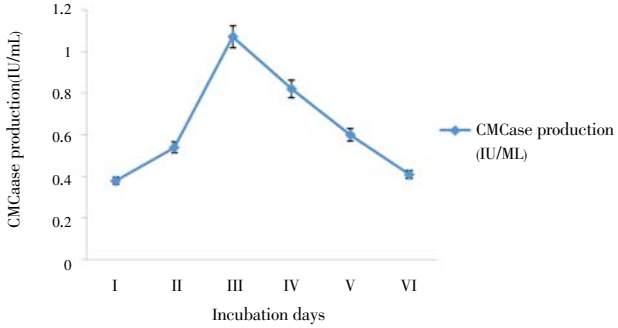

Figure 1. Effect of incubation days on CMCase production
Results are mean of three independent determinations. Bars correspond to standard deviation.

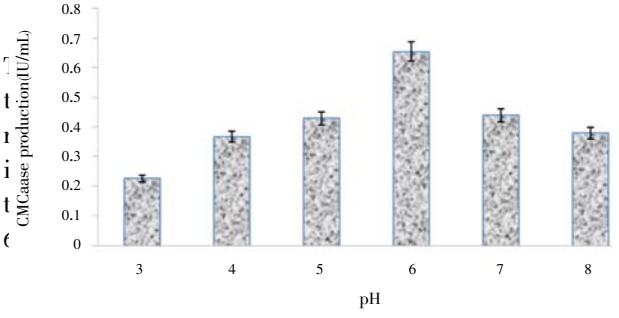

Figure 2. Effect of pH on CMCase activity.
Results are mean of three independent determinations. Bars correspond to standard deviation.

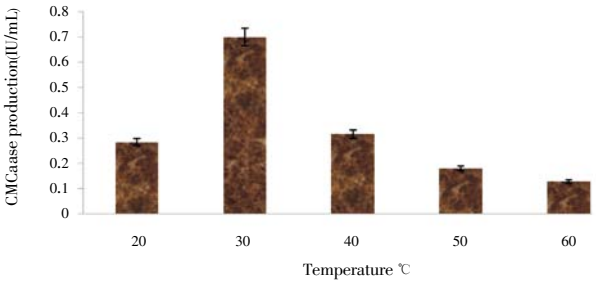

Figure 1. Effect of temperature on CMCase production
Results are mean of three independent determinations. Bars correspond to standard deviation.

Incubation temperature of the fermentation medium is a critical factor has insightful influence on metabolic activities of microorganisms. The effect of different incubation temperature (20-70 ℃) on the CMCase production was investigated. The production of enzyme was maximal in flasks incubated at 30 ℃ (Figure 3). As the temperature increased, there was a gradual decrease in the enzyme production.

Inoculums size certainly has an effect on the rate of production[12]. In the present study, effect of different sizes of inoculums was also explored (Figure 4). CMCase production found to be optimal (3.3 IU/mL) when flasks were inoculated with 4% of inoculums size by *A. flavus*.

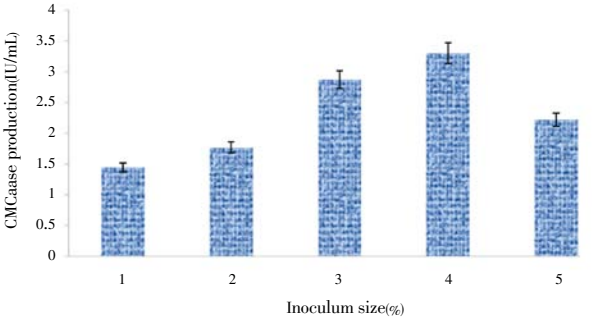

Figure 4. Effect of inoculum size on CMCase production.
Results are mean of three independent determinations. Bars correspond to standard deviation.

Different agricultural byproducts such as wheat bran, rice bran, rice straw, cotton seed and pomegranate were tested for the production of enzyme (Table 1). Of all the substrates tested, wheat bran was found to be the best substrates for the production of CMCases. The other substrates

Table 1
Effect of various substrate on carboxymethyl cellulase (CMCase) production.

Various substrates	CMCase production (IU/mL) Incubation days					
	I	II	III	IV	V	VI
Cotton seed	0.240±0.010	0.315±0.000	0.77±0.01	0.070±0.000	0.067±0.000	0.051±0.020
Pomegranate	0.384±0.000	0.96±0.010	0.54±0.01	0.383±0.000	0.180±0.020	0.108±0.000
Rice bran	0.098±0.000	0.32±0.020	0.24±0.01	0.080±0.010	0.067±0.000	0.044±0.000
Rice straw	0.16±0.010	0.52±0.010	0.63±0.02	1.100±0.000	0.458±0.000	0.308±0.000
Wheat bran	0.367±0.000	0.458±0.000	1.23±0.02	0.540±0.020	0.334±0.020	0.298±0.000

gave comparatively less production of cellulases. CMCases production in fermentation medium was found to be maximal when 4.0% of wheat bran was used (Figure 5). Further increase in amount of wheat bran resulted decrease in the production of enzyme.

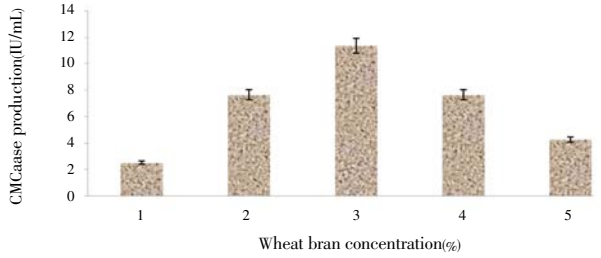

Figure 5. Effect of wheat bran concentration CMCase production. Results are mean of three independent determinations. Bars correspond to standard deviation.

The higher C/N ratio of the medium composition results in pH decrease and lower C/N ratio of the medium composition results in pH increase. Consequently, increasing nitrogen concentration is another way to prevent pH decreasing during cell cultivation. The nitrogen source used in the production medium is one of the major factors affecting enzyme production and level. All the nitrogen sources (beef extract, KNO_3, peptone, yeast extract and urea) used in this study (Table 2) favored for the synthesis of CMCases complex. In that, peptones where found to be the most suitable nitrogen sources for CMCase production. The influence of various peptone concentrations was investigated (Figure 6). In that, 1% peptone was found to be best in CMCase production.

Table 2
Effect of various nitrogen source on carboxymethyl cellulase (CMCase) production.

Nitrogen sources	CMCase production (IU/mL) Incubation days					
	I	II	III	IV	V	VI
Beef extract	0.134±0.080	0.491±0.100	1.191±0.040	0.705±0.280	0.408±0.020	0.223±0.090
KNO_3	0.325±0.080	0.534±0.160	1.200±0.030	0.834±0.050	0.208±0.030	0.116±0.080
Peptone	0.416±0.010	1.312±0.060	1.634±0.030	0.91±0.020	0.624±0.080	0.198±0.080
Urea	0.134±0.050	0.168±0.020	0.238±0.020	0.175±0.080	0.108±0.020	0.093±0.070
Yeast extract	0.208±0.020	0.853±0.080	1.015±0.010	0.534±0.050	0.300±0.030	0.108±0.050

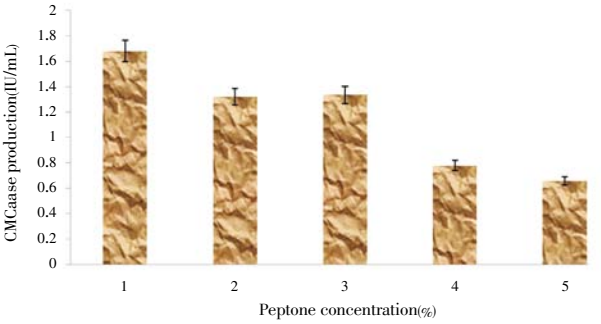

Figure 6. Effect of differnt concentration of peptone on CMCase production.
Results are mean of three independent determinations. Bars correspond to standard deviation.

Activity assay of CMCase was done in reaction mixture at varying pH by using appropriate buffers. It was found that enzyme has got activity over a broad range of pH (Figure 7). Maximum activity was expressed at pH 7 in case of wheat bran as substrate.

For estimation of optimum temperature of enzyme, the enzyme activity was determined by carrying out the assay at several temperatures between 30 and 70℃ (Coral *et al*., 2002). The optimum temperature was observed around 50℃ (Figure 8).

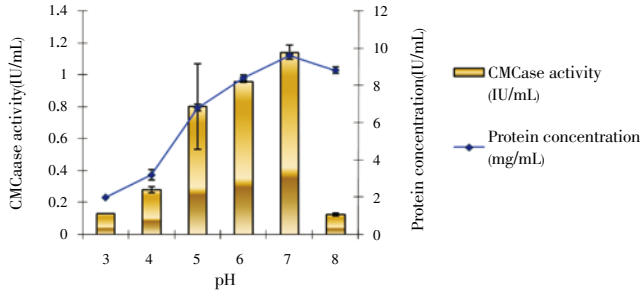

Figure 7. Effect of pH on CMCase activity.
Results are mean of three independent determinations. Bars correspond to standard deviation.

The Michaelis-Menten constants, Km and V_{max}, of the purified CMCase were estimated from the double reciprocal plot of the data obtained for *A. flavus* cellulase at varying

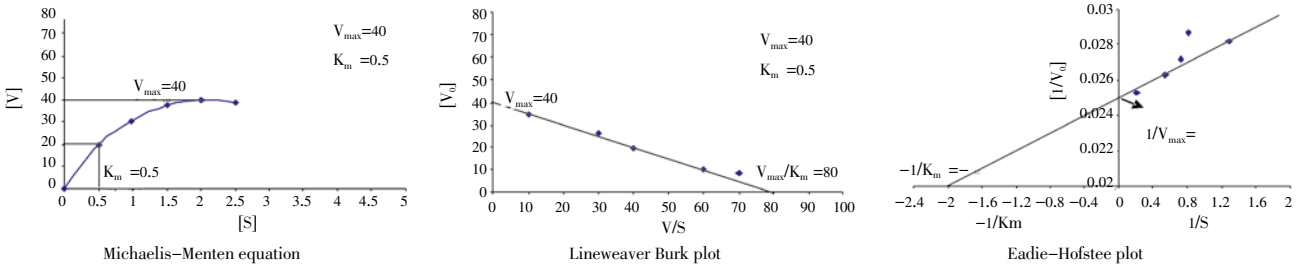

Figure 9. Enzyme kinetics of *A. flavus*

substrate concentrations. The K_m and V_{max} obtained for the purified CMCase was 0.5 mg/ml and 40.0 U/ml (Figure 9).

The effects of various ions on activity of the carboxy methyl cellulae of *A. flavus* is shown in Figure 10. Significant inactivation of the CMCase of A. flavus was observed with Mg^{2+} but Zn^{2+}, Cu^{2+}, Na^{+} and Ca^{2+} enhanced the enzyme activity.

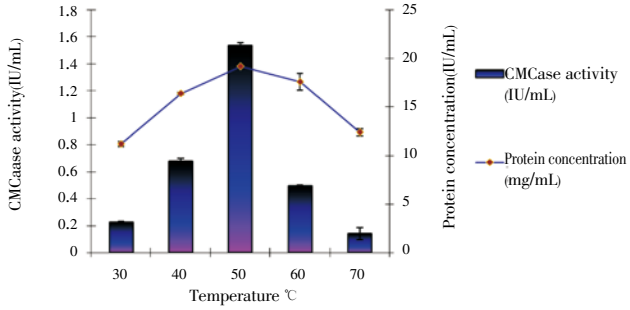

Figure 8. Effect of temperature on CMCase activity. Results are mean of three independent determinations. Bars correspond to standard deviation.

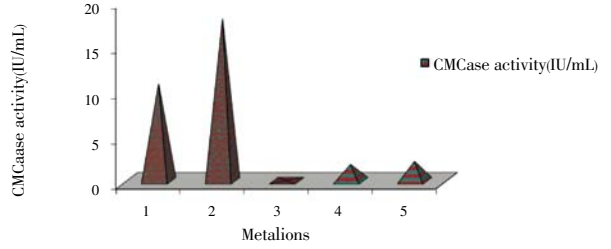

Figure 10. Effect of metalion concentration on CMCase activity. Results are mean of three independent determinations. Bars correspond to standard deviation.

4. Discussion

In the present study, new *A. flavus* strain isolated from the soil samples as the major cellulytic fungal strain for CMCase enzyme activity. Major impediments to exploit the commercial potential of CMCase are the yield stability and cost of cellulase production. Therefore, research should also aim at exploiting the commercial potential of existing and new cellulase in nature[13]. Agricultural residue such as wheat bran is used as substrate for this study.

In our study, the maximum enzyme production is occurred on 3rd day of incubation. This is in line with the findings of Ali *et al.*[14] who reported that the enzyme could be harvested at about 72 hours of fermentation when the activity is highest. It might be due to the depletion of the nutrients and production of other by products in the fermentation medium. Further increase in the incubation period led to a decrease in the production of cellulases by A. flavus. It might be due to the depletion of the nutrients and production of other by products in the fermentation medium[15].

The properties of cellulytic enzyme like those of all proteins are modified by prevailing physical condition such as temperature and pH. Enzyme exhibits its catalytic activity within these ranges of physical conditions. Beside this, concentration, composition and quality of substrate along with enzyme concentration and reaction time are also important factors that determine the rate of hydrolysis and final yield of the product[16]. Enzymes have an optimum pH with in which their activity is maximum and at higher or lower pH values, their activity decreases[5].

The maximum enzyme production was found to be 0.654 IU/ml at pH 6 during 3rd day of incubation. However, Sherief *et al.*[1] reported the initial pH 5-6 for maximum volumetric productivity of cellulases. Our results correlated with Jaradat *et al.*,[9] who reported the CMCase enzyme from the active isolate J2 was found active over a pH range of 4-7 with maximum activity at pH 6. Further, increase or decrease in pH of medium resulted decrease in the production of cellulases because of the organism may require slightly acidic pH for the growth of organism and cellulases production as the productivity of enzyme by mould culture is very specific to pH[17].

The production of CMCase enzyme was maximal at 30 ℃ which was supported by Singh *et al.*[3] and Sarao et al., [18] who reported that the maximum enzyme production was observed at 30℃. As the temperature increased, there was a gradual decrease in the enzyme production because the high temperature of the medium can change membrane composition and can cause the protein catabolism and inhibition of fungal growth.

The maximum enzyme production was found at 4% of inoculam size. These results were almost similar with findings collected by Ray *et al.*[19] elucidated the enzyme production increased gradually up to 3% inoculum size, but decreased thereafter. The enzyme production by both strains Bacillus subtilis and Bacillus circulans in 3% inoculum size was not significantly different ($P< 0.05$) from that in 2% inoculum size. Variation in inoculums size from this optimal range resulted in decreased enzyme production. This decrease in glucose production with further increase in inoculums might be due to clumping of cells which could have reduced sugar and oxygen uptake rate and also, enzyme release[15].

The carbon sources induce production of cellulase, but amount produced is variable. This is because of the influence of substrate (carbon source) on the growth of cellulolytic organisms[20]. In our study, wheat bran produces higher cellulase production when compared with other substrates. Wheat bran is a cheap agricultural by-product which is a rich nutrient and it can be stimulate fungal growth[21]. Haq *et al.*[22] and Abo-State *et al.*[23] also reported the wheat bran as best source of carbon and nitrogen for cellulase production. It might be due to the fact that wheat bran contains adequate amount of nutrient like proteins 1.32%, carbohydrates 69%, fats 1.9%, fiber 2.6%, ash 1.8%, Ca

0.05%, Mg 0.17%, Po 35%, K 0.45%, S 0.12%, various amino acids and porosity for oxygen supply. All these nutrients are necessary for the production of enzymes as well as cell biomass formation[24]. CMCase production was high when 4.0 g of wheat bran is used as substrate. It might be due to the fact that increase level of substrates decreases the aeration and porosity of the medium, which were very essential for the proper growth of the organism. As the growth of organism was not proper therefore the production of cellulases was significantly decreased[10]. In contrast Haq *et al.*[22] reported the cellulase production was found to be maximal when 10g of wheat bran was used as substrate in fermentation medium.

All the nitrogen sources used in the study favored the synthesis of cellulases complex but there was slight differences in the yield of enzymes. Peptones were found to be most suitable nitrogen source, especially soy peptone[25]. The various concentrations of the peptone were also investigated in the production of CMCase. In that 3% of peptone gave the best result for the production of CMCase. It was reported that good cellulase yield can be obtained with the addition of organic nitrogen source like peptone which showed increase in the growth and enzyme production [26].
The optimum pH values for carboxy methyl cellulase in this study were 7.0 for A. flavus. This is similar to the result of Bakare *et al.*[27]. These results showed that near neutral pH value was more favourable to cellulase activity in this organism. It was reported that the optimal pH for a CMCase from A. niger was found between 6.0 and 7.0[28]. Gautam *et al.*[29] reported that the pH range over which the CMCase were highly active at 7.0.

The maximum enzyme activity was found to be 50℃. These results are in agreement with results reported by Jang and Chen[30] and Ravindran *et al.*[31] who reported that the cellulase activity was higher at 50 ℃ for a Streptomyces T3-1 and Chaetomium species.

Km value of purified extracellular cellulase was determined by Lineweaver-Burk double reciprocal plot (Figure 9) and was found to be 5,0% using wheat bran as substrate. The Km value of CMCase from A. flavus was 0.5 mg/mL and Vmax value was 40 U/mL. Das and Ghose[32], who reported the Km value of 0.0033M/I and Vmax value of 80 IU/gds for Penicillum notatam NCIM NO-923. The results are in conformity with these of Ekperigin[33], who reported the Km value of cellobiose as substrate were 0.32 and 2.50 mM for *A. anitratus* and *Branhamella* sp., respectively.

Metal ions have been reported to influence enzyme production by increasing their activity in microorganisms[34,35]. Zn^{2+}, Cu^{2+}, Na^{+} and Ca^{2+} were found to increase the activity of the enzyme (Figure 10). These cat ions might be probably involved in the protection of the enzyme or strengthening of the active site thereby maintaining the conformation of the enzyme in active state, whilst $Mg^{2+}$was found to inhibit the activity. Cellulase activity by *A. terreus* was enhanced with the addition of calcium chloride (3 mM) and magnesium sulfate (5 mM) in the medium was reported by Shahriarinour *et al.*[36].

In this study, CMCase was produced from *A. flavus* using wheat bran as substrate. The optimal pH, temperature and incubation time for cellulases production was found to be 5.5, 28 ℃ and 3rd day of incubation respectively. To promote CMCases production, the evaluation of carbon and nitrogen sources was studied and it was found that wheat bran and peptone when the medium was supplemented with 4% and 1% respectively. The optimization study of fermentation conditions would enhance the production of CMCase at different levels using wheat bran as substrate. It may be concluded from this work *A. flavus* has the potential to produce highly thermostable CMCase which could have potential application for wide range of industries.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgement

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References

[1] Sherief AA, El-Tanash, Atia N. Cellulase production by Aspergillus fumigatus grown on mixed substrate of rice straw and wheat bran. *Res J Microbiol* 2010; **5**(3): 199-211.

[2] Okemoto K, Uekita T, Tsumuraya Y, Hashimoto Y, Kasama T. Purification and characterization of an endo-beta-(1>6)-galactanase from Trichoderma viride. *Carbohydrate Res* 2003; **338**: 219-230.

[3] Singh A, Singh N, Bishnoi NR. Production of cellulases by *Aspergillus* heteromorphus from wheat straw under submerged fermentation. *Int J Environ Sci Engineering* 2009;**1**:20.

[4] Sukumaran KR, Singhania RR, Pandey A. Microbial cellulases-production, applications and challenges. *J Scientific Indus Res* 2005; **64**: 832-844.

[5] Jahangeer S, Khan N, Jahangeer S, Sohail M, Shahzad S, Ahmad A, Khan SA. Screening and characterization of fungal cellulases isolated from the native environmental source. *Pak J Bot* 2005; **37**(3): 739-748.

[6] Miller GL. Use of DNS reagent for the measurement of reducing sugar. *Anal Chem* 1959;**31**:426-428.

[7] Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurements with the folin phenol reagent. *J Biochem* 1951; **193**: 265-275.

[8] Lineweaver H, Burk D. The determination of enzyme dissociation constants. *J Am Chem Soc* 1934; **56**: 658-666.

[9] Jaradat Z, Dawagreh A, Ababneh Q, Saadoun I. Influence of

Culture Conditions on Cellulase Production by Streptomyces Sp. (Strain J2). *Jordan J Biol Sci* 2008; **1**(4): 141-146.

[10] Bigelow P, Wyman E. Production of cellulolytic on bagasse pretreated with chemicals. *Appl Biochem Biotechnol* 2004; **102**: 78-82.

[11] Smiths JP, Rinzema A, Tramper J, Van-sonsbeek HM, Knol W. Solid-state fermentation of wheat bran by Trichoderma reesei QM9414.: substrate composition changes, C balance, enzyme production, growth and kinetics. *Appl Microbiol Biotechnol* 2003; **46**: 489-496.

[12] Haq IU, Javed MM, Khan TS, Siddiq, Z. Cotton saccharifying activity of cellulases production by Co-culture of Aspergillus niger and Trichoderma viride. *Res J Agr Biological Sci* 2005; **1**: 241-245.

[13] Aboul-Enein A, Abou elalla F, Serour E, Hussien T. Purification and characterization of a novel thermoactive cellulase from thermophilic actinomycetes isolated from soil sample of Egypt. *Int J Acad Res* 2010; **2**: 81-86.

[14] Ali FU, El-Den SSH. Production and partial purification of cellulase complex by *Aspergillus niger* and *A. nidulans* grown on water Hyacinth Blend. *J Applied Sci Res* 2008; **4**(7): 875-891.

[15] Haq IU, Hameed U, Shahzadi K, Javed M, Ali S, Qadeer MA. Cotton saccharifying activity of cellulases by Trichoderma harzianum UM-11 in shake flask. *Int J Bot* 2005b; **1**: 19-22.

[16] Godfrey T. Textiles: Baking and key characteristics of enzymes. In:T Godfrey, S West (eds). *Industrial enzymology*. 2nd ed. London: Macmillan Press Ltd. ;1996.

[17] Puntambekar US. Cellulase production by the edible mushroom volvariella diplasis. *World J Microbiol Biotechnol* 1995; **11**: 695.

[18] Saro LK, Arora M, Sehgal VK. Use of Scopulariopsis acremonium for the production of cellulose and xylanase through submerged fermentation. *Afr J Microbiol Res* 2010;**4**(14). 1506-1510.

[19] Ray AK, Bairagi A, Ghosh KS, Sen SK. Optimization of fermentation conditions for cellulase production by Bacillus subtilis CY5 and Bacillus circulans TP3 isolated from fish gut. *Acta Ichthyologica ET Piscatoria* 2007; **37**: 47-53.

[20] Immanuel G, Bhagavath C, Raj IP, Esakkiraj P, Palavesam A. Production and partial purification of cellulase by *Aspergillus niger* and *A. fumigatus* fermented in Coir waste and Sawdust. *Int J Micro* 2007; 3(1):200.

[21] Yu XB, Nam JH, Yun HS, Koo YM. Optimization of Cellulase Production in Batch Fermentation by Trichoderma reesei. Biotechnol. *Bioprocess Eng* 1998; **3**: 44-47.

[22] Haq IU, Shahzadi K, Hameed U, Javed MM, Quadeer MA. Solid-state fermentation of cellulase by locally isolate T. harzianum for the exploitation of agricultural byproducts. *Pak J Biol Sci* 2006; **9**(9): 1779-1782.

[23] Abo-State MAM, Hammad AL, Serlim M, Gannam RB. Enhanced production of cellulases by Aspergillus spp. Isolated from agricultural wastes by solid state fermentation. *American-Eurasian J Agric Environ Sci* 2010; **8**(4): 402-410.

[24] Bakri Y, Jacques P, Thonary P. Xylanase production by Penicillium canescens 10-10c in solid-state fermentation. Applied Biochem *Biotechnol* 2003; **108**: 737-748.

[25] Baig MMV. Cellulolytic enzymes of *Trichoderma lignorum* produced on banana agro-waste: optimization of culture medium and conditions. *J Scientific Indus Res* 2005; **64**: 57-60.

[26] Gautam SP, Bandela PS, Pandey AK, Khan J, Awasthi MK, Sarsaiya S. optimization for the production of cellulose enzyme from municipal solid waste residue by two novel cellulolytic fungi. *Biotechnol Res Int* 2011; **1**:1-8

[27] Bakare MK, Adewale IO, Ajayi A, Shonukan OO. Purification and characterization of cellulase from the wild-type and two improved mutants of Pseudomonas fluorescens. *Afr J Biotechnol* 1998; **4** (9): 898-904.

[28] Akiba S, Kimura Y, Yamamoto K, Kumagap H. Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. *J Fermen Bioengin* 1995; **79**: 125-132.

[29] Gautam SP, Bandela PS, Pandey AK, Jamaluddin, Awasthi MK, Sarsaiya S. Cellulase production by *Pseudomonas* sp. Isolated from municipal solid waste compost. *Int J Acade Res* 2010; **2**(6): 330-333.

[30] Jang HD, Chen KS. Production and characterization of thermostable cellulases from Streptomyces transformant T 3-1. *World J Microbiol Biotechnol* 2003; **19**: 263-268.

[31] Ravindran C, Naveenan T, Varatharajan G. Optimisation of alkaline cellulase production from marine derived fungi, *Chaetomium* sp., using agricultural and industrial wastes as substrates. *Bot Mar* 2010; **53**(3): 275-282.

[32] Das A, Ghosh U. Solid-state fermentation of waste cabbage by Penicillum notatum NCIM NO-923 for production and characterization of cellulases. *J Scientific Indus Res* 2009; **68**: 714-718.

[33] Ekperigin MM. Preliminary studies of cellulase production by *Acinetobacter anitratus* and *Branhamella* sp. *Afr J Biotechnol* 2007; **6** (1):28-33.

[34] Rani DS, Thirumade S NK. Production of cellulase by Clostridium papyrosolvens CFR-703. *World J Microbiol. Biotechnol* 2004; **20**: 629-32.

[35] Kotchoni SO, Gachomo EW, Omafuvbe BO, Shonukan OO. Purification and biochemical characterization of carboxymethyl cellulase (CMCase) from a catabolite repression insensitive mutant of Bacillus pumilus. *Int J Agri Biol* 2006; **2**: 286-392.

[36] Shahriarinour M, Wahan MNA, Mohamad R, Mustafa S , Ariff AB. Effect of medium and cultural condition on cellulase production by Aspergillus terreus. *Afr J Biotechnol* 2011; **10**(38): 7459-7467.