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Optimization of CMCase production by *Aspergillus niger* BK01 using pretreated rice straw under submerged fermentation

Varsha Goyal[1], Neeraj Kumar Aggarwal*[1], Anish Bhuwal[1], Anita Yadav[2]

1. Department of Microbiology, Kurukshetra University, Kurukshetra-136119, Haryana, India
2. Department of Biotechnology, Kurukshetra University, Kurukshetra-136119, Haryana, India

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Email: neerajkuk26@rediffmail.com

ABSTRACT

Ever increasing use of cellulases in industry has led to the search for new enzymes with high activities. Cellulases play a pivotal role in biomass utilization. The present study was carried out to investigate the potential of a filamentous fungus, *Aspergillus niger* BK01 for hyper-production of carboxymethyl cellulase (CMCase) using alkali assisted acidic pretreated rice straw as growth supporting substrate under submerged fermentation (SmF). An optimization of process parameters is a necessary step to get higher yield of product. The highest CMCase activity was obtained when the fungus was cultivated on (3.5%) pretreated rice straw, pH 5.0 for 72h incubation at 28^{0}C. When CMCase production conditions were optimized with different carbon and nitrogen sources, the highest CMCase production was observed with cellulose and ammonium nitrate respectively.

INTRODUCTION

Cellulose is an unbranched glucose polymer composed of an β-1,4 glucose units linked by a β-1,4-D-glycosidic bond with annual production of 0.85×10^{11} tonnes per annum by both plants and marine algae (Ponnambalam et al., 2011; Acharya et al., 2008). The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest. Cellulose-rich plant biomass is one of the foreseeable and sustainable source of fuel, animal feed and feed stock for chemical synthesis and utilization of cellulosic biomass continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages (Bhat, 2000; Kuhad et al., 1997). Cellulose may be hydrolyzed using enzymes to produce glucose, which can be used for the production of ethanol, organic acids and other chemicals.

Cellulases are inducible enzymes which are synthesized by microbial sources, starting from prokaryotic organisms like bacteria and protozoans to eukaryotic organisms during their growth on cellulosic materials (Lee and Koo, 2001). Cellulase is a synergistic enzyme that is used to break up cellulose into glucose or other oligosaccharide compounds (Chellapandi and Jani, 2008). Cellulases responsible for the hydrolysis of cellulose are composed of three major enzymes i.e. endoglucanase (1,4-β-d-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-β-d-glucan glucohydrolase; EC 3.2.1.74) and β-glucosidase (β-d-glucoside glucohydrolase; EC 3.2.1.21) (Yi et al., 1999). Endoglucanases randomly attack at internal sites in the amorphous regions of cellulose fibre for subsequent attack by cellobiohydrolases. Exoglucanase remove mono- and dimmers from the end of glucose chain and β-glucosidase further hydrolyse these glucose dimmers into glucose units (Sherief et al., 2010).

A large number of bacteria, actinomycetes and filamentous fungi possess the ability to degrade cellulose. These organisms have in common the ability to produce extracellular hydrolytic enzymes that attack the cellulose polymer. Fungi are considered as one of the main microorganisms which can produce cellulase enzymes and use them in secondary metabolite pathways (Leylai et al., 2011). Cellulase production by different cellulolytic microfungi using various waste cellulosic materials is being vigorously studied for cost reduction strategies (Ojumu et al., 2003). Major cellulase producing fungal genera include *Bulgaria, Chaetomium (Ascomycetes), Coriolus, Phaenerochaete, Coriolus, Schizophyllum (Basidiomycetes) Aspergillus, Geotrichum, Penicillium* and T*richoderma (Deuteromycetes)* (Ja'afaru and Fadage, 2010). Cellulases have a wide range of applications include pharmaceutical formulations, detergent preparation and fashion design in textile industry/fabric modification, food processing, brewing and paper and pulp industry. Additionally they can be used in waste management, protoplast production and genetic engineering (Juwaied et al., 2011).

The objective of this study was to investigate the effect of medium component and culture conditions on CMCase production by *Aspergillus niger* BK01 using pretreated rice straw as carbon source. The effect of cultural conditions such as initial pH, temperature, time and use of different types of carbon and nitrogen sources on the enhancement of CMCase production was investigated.

MATERIALS AND METHODS

Raw material

Lignocellulosic agro-industrial waste, rice straw was obtained from local fields of Haryana, India. The substrate was crushed into pieces, sun and oven dried (60^{0}C) and ground to 0.5mm mesh size and stored in air tight plastic jars.

Isolation and identification of potential cellulolytic fungal strain

Qualitative screening of CMCase producing fungal strains

Soil samples from different habitats such as sugarcane field, rice field, paper industry, cattle shed, rotten fruits and vegetables, samples of cattle dung were collected in sterile bag and brought to the laboratory. Enrichment was done by adding 1g of soil sample in 25 mL of sterile deionised water having cellulose as carbon source and peptone as nitrogen source at pH 5.0 and incubated under stationary conditions of growth for 5 days at 25^{0}C. Isolation was done by dilution plate method on a carboxymethyl cellulose Agar (CMC) medium (Hi media, India) (g/l) 2.0; $NaNO_3$, 1.0; K_2HPO_4, 0.5; $MgSO_4.7H_2O$, 0.5; KCl, 5.0; CMC, 20.0; agar, pH 5.0. Screening for cellulolytic activity was followed by visualizing the hydrolytic zone, when the plates were flooded with an aqueous solution of 0.1% congo red for 15 min and washed with 1 M NaCl (Apun et al., 2000). The isolated colonies on these plates were maintained on PDA agar slants at 4^{0}C for further analysis.

Quantitative screening of CMCase producing fungal strains

Enzymes were produced under submerged fermentation in 250 ml Erlenmeyer flasks containing 50 ml of CMC supplemented modified mineral salt solution medium (g/l): Yeast extract-2.5, K_2HPO_4–5, NaCl–1, $MgSO_4.7H_2O$ – 0.2, $(NH_4)2SO_4$ – 0.6, Peptone – 2, CMC – 5 (pH 5.0). The flasks were inoculated with 3 days old inoculum and incubated at 25^{0}C for 5 days on a rotating shaker (NSW-256) at 180 rpm. Crude enzymes were harvested by centrifuging at 10,000 g for 20 min at 4 ^{0}C and the clear supernatant was used as the source of enzyme.

Enzyme assay (Endoglucanase activity)

CMCase activity was assayed by the DNS (3, 5-dinitrosalicylic acid) method (Miller, 1959). The reaction mixture contained 900 µl of substrate (CMC in 0.1 M citrate buffer pH 4.8) and 100 µl of crude enzyme was incubated at 50^{0}C for 60 min. An appropriate control which contained 100 µl of distilled water instead of crude enzyme extract was also run along with the test. The reaction was terminated by adding 3 ml of 3, 5-dinitrosalicylic acid reagent. The tubes were incubated for 15 min in a boiling water bath for colour development and were cooled rapidly. The activity of reaction mixture was measured against a reagent blank at 540 nm. The concentration of glucose released by enzyme was determined by comparing against a standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity is defined as the

amount of enzyme that liberates 1 µg of glucose per minute under the assay conditions.

Pretreatment of rice straw

Rice straw was collected from local fields, dried at 60^{0}C in hot air oven and ground to different mesh size. The rice straw was first pretreated with 0.5 M KOH for 4 h at room temperature at the ratio of 1:10 for substrate and KOH solution. The pretreated solid was washed with water till neutrality, filtered and dried. The solid was further treated by 0.1 N H_2SO_4 for 1 h at room temperature and then autoclave at 121^{0}C and 15 psi pressure for 15 minutes. The pulp was washed with water till neutrality, filtered, dried and stored at room temperature for further use. The chemical composition of rice straw was analysed using standard methods (Goerging and van Soest, 1975).

Inoculum development

Spores of *Aspergillus niger* BK01 were grown and maintained on Potato Dextrose Agar (PDA) slants. Spores were cultivated in an Erlenmeyer flask (250 mL) capacity containing 30 mL of Potato Dextrose broth at 28^{0}C for 72h after sterilizing the potato dextrose broth at 15 lbs/inch^2 pressure and 121^{0}C in laboratory scale autoclave for 15 minutes; pH was adjusted before sterilization, and incubated under stationary conditions for the development of fungal spore suspension. The spores were harvested using sterilized water with 0.1% Tween 80 (Smith et al., 1996).

Optimization of culture conditions for CMCase production by *Aspergillus niger* BK01

Various optimum parameters required for maximum CMCase production by *Aspergillus niger* BK01 under SmF were determined considering one factor at a time. The various parameters that were optimized were pretreated substrate concentration (1.0-5.0%), incubation temperature (20-40^{0}C), inoculum size (1.0-5.0%), pH (4.0-7.0), incubation time (24-120 h), supplementation of carbon sources (galactose, maltose, carboxymethyl cellulose, sucrose, mannitol, and cellulose powder) at 0.1%, w/v, supplementation of nitrogen sources (ammonium nitrate, ammonium sulphate, ammonium chloride, beef, tryptone, urea, and potassium nitrate) at 0.1%, w/v.

Statistical analysis

The optimization results for different parameters were analyzed using statistical packages system software (SPSS; 16.0). The ANOVA with post hoc analysis was applied for within group comparison. The level of significance was set at 0.05.

RESULTS AND DISCUSSION

Isolation and identification of CMCase producing fungi

Ten fungal isolates from different ecological niches were obtained on CMC agar plates. The simplest first line screening program for CMCase producing fungi is the use of congo red staining. The isolates which were found positive for zone of hydrolysis after staining with congo red were further quantitatively screened for CMCase production and the results obtained (Table 1) showed that the isolate (NVG48, from rice field soil) produced 10.06 U/mL of CMCase. The isolate was further characterised morphologically (Table 2) and was found *Aspergillus sp.* (Figure. 1). The fungal strain was identified as *Aspergillus niger* BK01 by Xcelris Labs Ltd, Ahmedabad, India and has been given National Centre for Biotechnology Information (NCBI) accession No. HM008328.1.

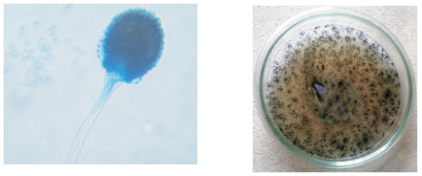

Fig. 1: Colonial characteristics of NVG48 on PDA at 280C after 72 h

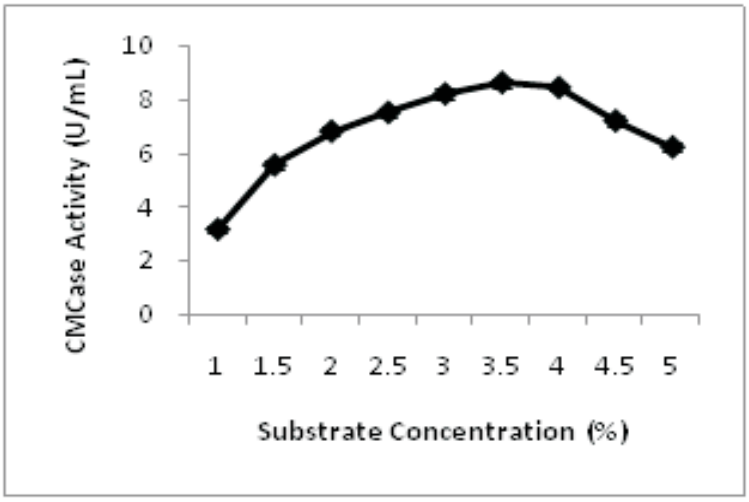

Fig. 2: Effect of substrate concentration on CMCase production by *Aspergillus niger* BK01 under SmF. Values in figure are means of three replicates with standard deviation

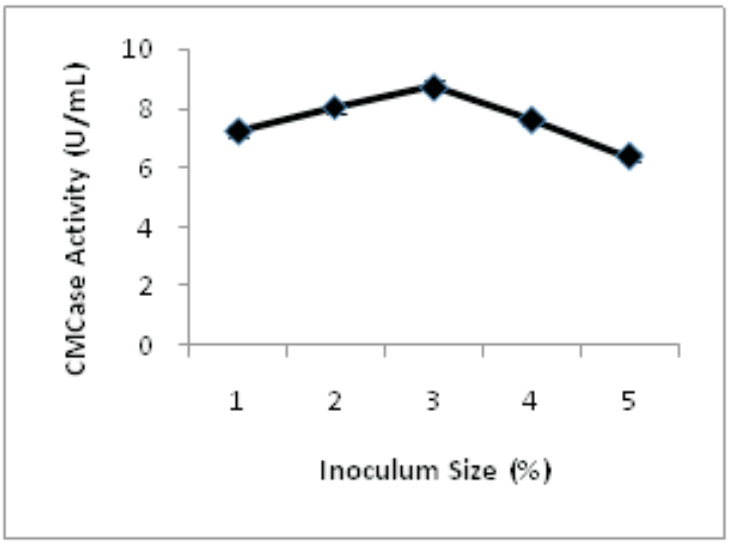

Fig. 3: Effect of inoculum size on CMCase production by *Aspergillus niger* BK01 under SmF. Values in figure are means of three replicates with standard deviation

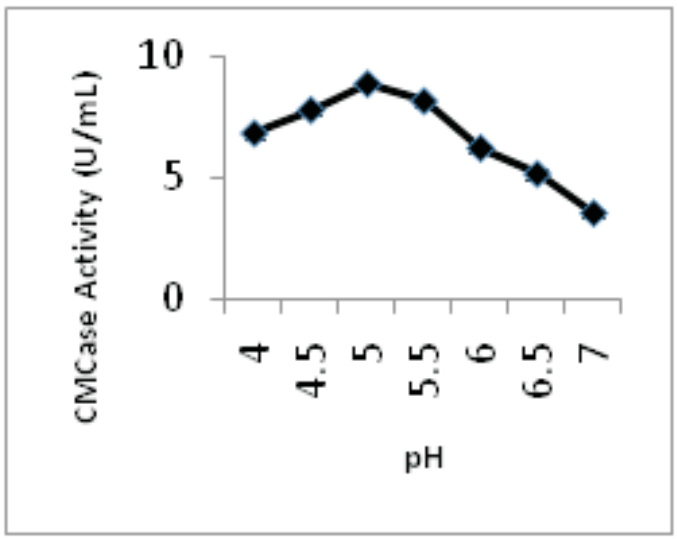

Fig. 4: Effect of pH on CMCase production by *Aspergillus niger* BK01 under SmF. Values in figure are means of three replicates with standard deviation

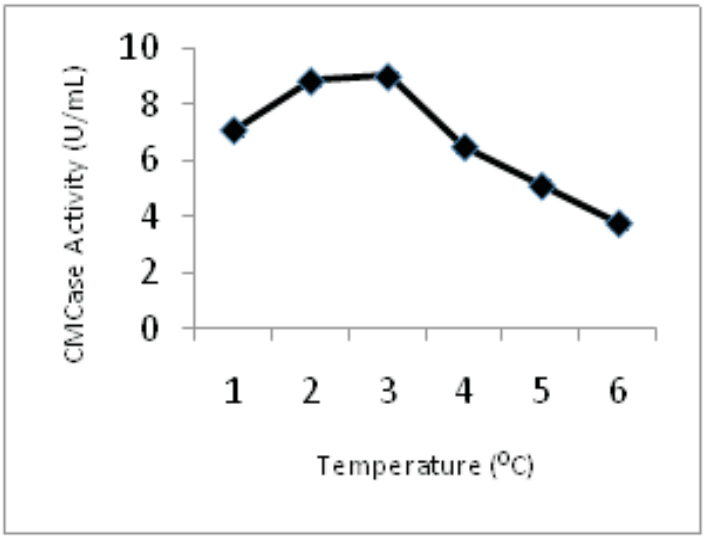

Fig. 5: Effect of incubation temperature on CMCase production by *Aspergillus niger* BK01 under SmF. Values in figure are means of three replicates with standard deviation

Table1: Qualitative and Quantitative screening of different CMCase producing fungal isolates from different habitats.

Isolates	Source	Diameter (mm) of zone of clearance	Enzyme Activity (U/mL)[a]
NVG7	Rotten banana	23±1	5.79±0.10
NVG14	Cow dung	34±1	6.57±0.15
NVG22	Cattle shed	31±3	2.40±0.12
NVG26	Rice field	15±1	5.30±0.10
NVG48	Rice field	38±1	10.06±0.17
NVG58	Rhizospheric soil	14±2	1.46±0.10
NVG64	Cow dung	29±2	4.89±0.16
NVG71	Cow dung	27±3	3.82±0.12
NVG78	Cattle shed	30±1	2.54±0.21
NVG79	Wheat field	28±2	7.30±0.23

Results presented are the mean of three independent experiments with standard deviation values. aUnder unoptimized conditions.

Table 2: Colonial and sporulating features of strain NVG48

Culture medium	PDA
Colony characteristics	Blackish brown to black
Reverse side of colony	Creamish
Microscopic features	Hyphae septate and hyaline; Conidiophores 2.5 mm long, 15 -20 µm thick, smooth walled each arise from the foot cell; Vesicles globose with 20-50 µm diameter, whole vesicle fertile bearing two series of sterigmata; Catenate conidia arranged in basipetal manner, unicellular globose, black and 2-5 µm in diameter.

Table 3: Optimized conditions for maximum CMCase production by *Aspergillus niger* BK01 under submerged fermentation conditions using pretreated rice straw

S. No.	Parameters	SmF range	Optimum
1	Substrate concentration (%)	1.0 - 5.0	3.5
2	Inoculum size (%)	1.0 - 5.0	3.0
3	Initial pH	4.0 - 7.0	5.0
4	Temperature (^{0}C)	20 - 40	28
5	Incubation time (h)	24 - 120	72
6	Supplementations: -		
	a) Carbon Source (0.1% w/v)	Galactose, maltose, sucrose, cellulose powder, carboxymethyl cellulose (CMC)	Cellulose powder
	b) Nitrogen Source (0.1% w/v)	$(NH_4)_2NO_3$,$(NH_4)_2Cl$, $(NH_4)_3PO_4$, KNO_3 beef, tryptone, urea	$(NH_4)_2NO_3$
7	CMCase Activity (U/mL)		9.67

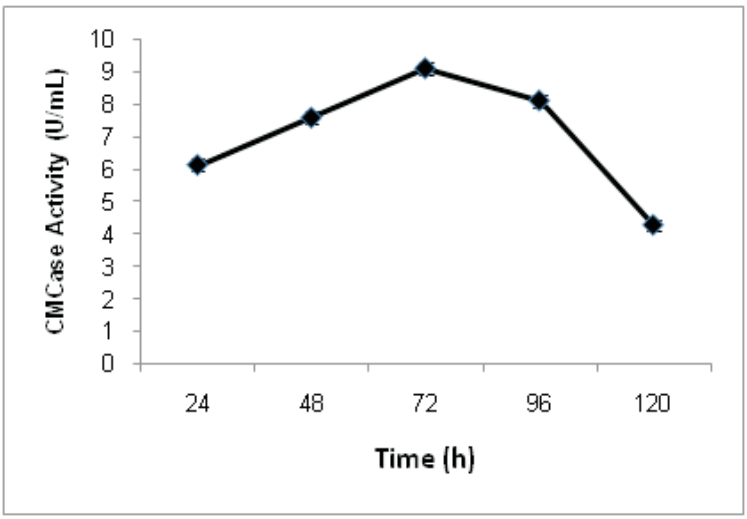

Fig. 6: Effect of incubation time on CMCase production by *Aspergillus niger* BK01 under SmF. Values in figure are means of three replicates with standard deviation

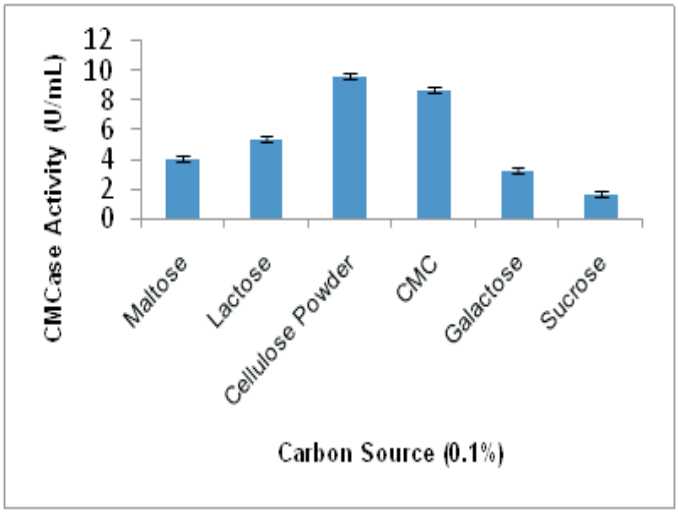

Fig. 7: Effect of carbon sources on CMCase production by *Aspergillus niger* BK01 under SmF. Values in figure are means of three replicates with standard deviation

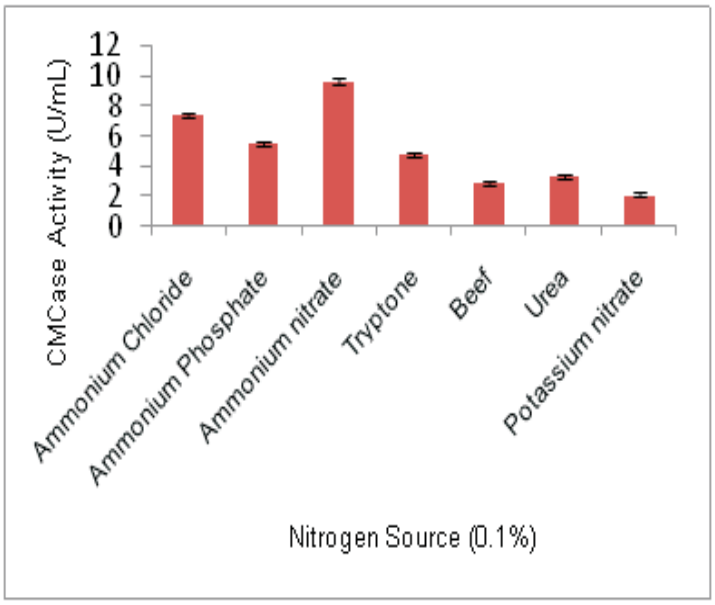

Fig. 8: Effect of nitrogen sources on CMCase production by *Aspergillus niger* BK01 under SmF. Values in figure are means of three replicates with standard deviation

The fungal strain was identified as Aspergillus niger BK01 by Xcelris Labs Ltd, Ahmedabad, India and has been given National Centre for Biotechnology Information (NCBI) accession No. HM008328.1.

Alkali assisted acidic pretreatment of rice straw.

The effect of alkali assisted acidic pretreatment on chemical composition of rice straw such as cellulose, hemicellulose and lignin was analyzed. It was determined that cellulose, hemicellulose and lignin content of obtained alkali assisted acidic pretreated rice straw were 59.5%, 8.26% and 5.17%, respectively. Pretreatment of lignocelluloses with alkali overcomes the lignin barrier, by dissolving the lignin caused by the breakdown of ether linkages (Lee, 1997). Lu et al. (2007) have examined that hemicelluloses can effectively solubilise and hydrolyze into monomeric sugars and soluble oligomers by dilute sulphuric acid pretreatment. Zhao et al. (2011) reported Alkali-PAA pretreatment of sugarcane bagasse with NaOH and PAA. Zhang et al. (2010) examined the combined pretreatment effect of dilute sulphuric acid and NaOH on corncob and found 66.8% decrease in hemicellulosic content and 81.0% decrease in lignin content.

Effect of substrate concentration

The effect of different concentrations (1.0-5.0%) of pretreated rice straw on enzyme production by *Aspergillus niger* BK01 was evaluated. The results revealed that 3.5% pretreated rice straw was found to be best under submerged fermentation with 8.63±0.08 U/ml of CMCase activity (Figure. 2). The ANOVA for the data on CMCase as a function of variation due to different substrate concentration is statistically significant ($F = 2.34$; $P < 0.0001$).

Substrate concentration is a dynamic influencing feature that affects the yield and initial hydrolysis rate of cellulose (Raghavarao et al., 2003). Similar to our results, Kumar et al. (2012) reported maximum CMCase activity at 7% substrate concentration with mango peel by *Paenibacillus polymyxa*. Mushimiyimana and Tallapragada (2013) used substrate concentration of 5.8% (w/v) for CMCase production by *Cladosporium cladosporioides* while Kiranmayi et al. (2011) used 2% banana peel powder and coir powder for CMCase production by *Aspergillus niger*.

Effect of Inoculum size

The size of inoculum plays an important role in the production of metabolites under submerged fermentation. The effect of inoculum size (1.0-5.0%) on CMCase production *Aspergillus niger* BK01 under submerged fermentation was evaluated. It was found that 3.0% inoculum resulted in maximum CMCase activity i.e., 8.74±0.05 U/mL (Figure. 3). The ANOVA for the data on CMCase as a function of variation due to different inoculum size is statistically significant ($F = 9.58$; $P < 0.0001$).

A balance between the proliferating bacterial biomass and substrate material would yield maximum enzyme. Above 3.0% inoculum, the decrease in the CMCase production may be due to depletion of nutrients as a result of enhanced biomass production thereby decreasing the metabolic activity. Doppelbauer et al. (1987); Sekar and Balaraman (1998); Ariffin et al. (2008) reported that the fungal growth was increased as the inoculum size increased until it achieved the optimal inoculum size then, the growth decreased sharply by the increase of the inoculum size over the optimal one. It was proven that after the optimal growth, an increase in inoculum size will lead to the low enzyme productivity due to the fast growing rate of the microorganism that will increase the competition for the nutrient and space. This at the same time will affect the stationary phase length which in the end affects the enzyme productivity. Pervez (2011) reported maximum CMCase production at 15% inoculum size by *Rhizopus sp*. JBT.

Effect of pH

The pH of the production medium plays a significant role in production of different metabolites. In the present study, various pH (4.0-7.0) were tested in order to enhance CMCase yield. It was observed that fungal strain gave maximum CMCase activity at pH 5.0 i.e. 8.86±0.06 U/mL under submerged fermentation (Figure. 4). The ANOVA for the data on CMCase as a function of variation due to different pH is statistically significant ($F = 3.11$; $P < 0.0001$).

Similar to our findings, Ja'afaru and Fagade (2010) reported maximum cellulase production by *A. niger* YL128 at pH 5.0. Shahriarinour et al. (2011) reported maximum CMCase production by *A. terrus* at pH 5.5.The optimum pH for the growth of fungi has been reported to vary from one organism to another. The maximum CMCase production was reported at different pH e.g. at pH of 4.0 to 5.5 in *A. terrus* (Garg and Neelakantan, 1981; Pushelkar et al.,1995), at pH of 4.5 to 5.0 in *Sclerotium rolfsii* (Darmwal,1986) and at pH of 5.0 to 6.0 in *Rhizopus oryzae* (IMI 298280) (Amadioha, 1993). Steiner et al. (1994) observed that cellulase synthesis is maximum at pH 3.0 and 9.0 because low or high pH values inactivates the enzyme and may affect its production.

Effect of incubation temperature

Incubation temperature affects various metabolic processes such as protein denaturation, enzymatic inhibition, promotion or inhibition of production of a particular metabolite, cell death, etc. So, the effect of temperature ranging from 20-40^{0}C on CMCase production was studied. Maximum CMCase activity i.e. 9.03±0.06 U/ml was observed at 28^{0}C (Figure. 5). The ANOVA for the data on CMCase as a function of variation due to different incubation temperature is statistically significant ($F = 3.27$; $P < 0.0001$).

Medium temperature plays an important role in cellulase production. Our observations are in agreement with CMCase produced from *Aspergillus terrus* which has an optimum temperature of 28^{0}C for maximum CMCase production (Shahriarinour et al., 2011). Similarly, Juhasz et al. (2004) reported optimum temperature of 28^{0}C for growth and cellulase production in *T. ressei* RUT C30. Some other workers reported the maximum production of CMCase at 300C by A. niger YL128 (Ja'afaru and Fagade, 2010) and by *Aspergillus terreus* (Garg and Neelakantan, 1981), at 25 to 28^{0}C by T. ressei (Sibtain et al., 2005) while at 28^{0}C by *Trichoderma harzianum* Rut-C 8230 (Kocher et al., 2008). Many workers have reported different temperatures for maximum cellulase production using *Aspergillus sp*. suggesting that the optimal temperature for cellulase production also depends on the strain variation of the microorganism (Murao et al., 1988; Lu et al., 2003).

Effect of incubation time

Optimization of incubation period was carried out for harvesting CMCase enzyme from production medium. In our study, time course for enzyme production was monitored up to 120h under submerged fermentation. The enzyme production was started from early growth and reached at the highest level after 72 h, thereafter it started decreasing. It might be due to the depletion of nutrients in the medium which stressed the fungal physiology resulting in the inactivation of secretary machinery of the enzymes (Nochur et al., 1993). Our results revealed that maximum CMCase activity of 9.11±0.05 U/ml was observed at 72 h (Figure. 6). The ANOVA for the data on CMCase as a function of variation due to different incubation time is statistically significant ($F = 3.59$; $P < 0.0001$).

The incubation time for cultivation depends on the type of microorganisms used for CMCase production. Different workers reported different incubation time for maximum CMCase production in yeast. Our results are in agreement with results of Gori and Malana (2010) who reported an incubation period of 72 h for maximum CMCase production by *Aspergillus sp.* and Gautam et al. (2011) reported an incubation period of 4 days for maximum CMCase production by *Aspergillus niger.* An incubation period of 144 h was reported for maximum CMCase production by *Aspergillus terrus* under shake flask conditions (Shahriarinour et al., 2011). Devi and Kumar (2012) reported maximum CMCase production by *Aspergillus niger* after 7th day of incubation.

Effect of carbon sources

Carbon sources play a vital role in the cell metabolism and synthesis of cellulases. It is one of the most important energy sources that is essential for the growth of the microorganism. It could be a pure monosaccharide compound such as glucose or complex molecules such as cellulose or starch. The effect of different carbon sources (0.1% w/v) on the production of CMCase was evaluated under submerged

CMCase i.e. 9.58±0.04 U/ml when supplemented with cellulose powder as a carbon source (Figure. 7). The ANOVA for the data on CMCase as a function of variation due to different carbon sources is statistically significant ($F = 8.92$; $P < 0.0001$).

Similar to our results, Gautam et al. (2011) reported cellulose as best carbon source for CMCase production by *Aspergillus niger*. Ja'afaru and Fagade (2010) reported highest CMCase activity by *A. niger* YL128 when grown on 1.0% CMC as carbon source and Pushelkar et al. (1995) reported maximum cellulase production by *A. terrus* when grown at 1% cellulose concentration.

Effect of nitrogen sources

Nitrogen source is very essential component for growth and enzyme production by the microorganisms. Different nitrogen sources (0.1%w/v) like ammonium nitrate, ammonium chloride, ammonium phosphate, beef, tryptone, urea and potassium nitrate were evaluated for their effect on CMCase production. It may be depicted from the results that in *Aspergillus niger* BK01, ammonium nitrate showed stimulatory effect on CMCase production i.e. 9.67±0.07 U/mL under submerged fermentation (Figure. 8), while all other nitrogen sources tested were found to be inhibitory for CMCase production. The ANOVA for the data on CMCase as a function of variation due to different nitrogen sources is statistically significant ($F = 4.83$; $P < 0.0001$).

Ammonium compounds are reported to be the most favourable nitrogen compounds for protein and enzyme synthesis (Rajoka, 2004). Shahriarinour et al. (2011) reported maximum CMCase production in the presence of yeast extract whereas other workers reported maximum CMCase production in the presence of (1.5%) ammonium sulphate by *Paenibacillus polymyxa* (Kumar et al., 2012) and (0.1%) ammonium sulphate by *A. niger* YL128 (Ja'afaru and Fagade, 2010). Peptone (1%) was found to be the best nitrogen source for the production of maximum CMCase by *Aspergillus niger* (Gautam et al., 2011). The effectiveness of nitrogen source in supporting CMCase production along with growth and secretion of extracellular protein has also been reported in many microorganisms (Narasimha et al., 2006).
Under optimized submerged fermentation conditions, enzyme production of 9.67 U/mL using pretreated rice straw as substrate was obtained (Table 3).

CONCLUSION

Results of this present study indicate the remarkable CMCase production potential of *Aspergillus niger* BK01 from agro-industrial residue rice straw. Classical method of optimization was used to evaluate the physico-chemical factors for hyper-production of CMCase. Substrate amended with 0.1% cellulose as carbon source, 0.1% ammonium nitrate as nitrogen source at 28^{0}C, pH 5.0 after incubation of 72 h produced maximum CMCase. The *Aspergillus niger* BK01 isolate thus showed the ability of producing cellulolytic enzyme, which makes it a suitable candidate for large scale production of the enzyme for possible industrial application including production of food and medicines and help to breakdown the waste plants materials which helps in their usage up gradation such as in bioethanol production and hence eventually to clean up the environment.

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