African Journal of Microbiology Research Vol. 5(27), pp. 4739-4747, 23 November, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR10.833

Full Length Research Paper

Assessment of some wild *Aspergillus* species for cellulase production and characterization

M. F. Begum[1]* and A. R. Alimon[1,2]

[1]Laboratory of animal production, Institute of Tropical Agriculture, University Putra Malaysia 43400-UPM, Serdang, Selangor, Malaysia.
[2]Department of Animal Science, Faculty of Agriculture, University Putra Malaysia, 43300 Serdang, Selangor, Malaysia.

Accepted 19 September, 2011

Fifteen *Aspergillus* species were used to evaluate their potentialities for cellulase production under submerged fermentation. The isolates were grown on carboxymethylcellulose (CMC) agar medium for detection of cellulase activity and *Aspergillus oryzae* S/92Gbr exhibited maximum clear zone (40 mm) around the colony. Protein content and cellulase activity of the filtrates were determined and biomass was harvested from the culture. All the species produced significant ($p<0.05$) amount of reducing sugar and protein; and showed correlation with protein content and reducing sugar level. Biomass did not show correlation with increase of cellulase activity. In all cases maximum β-glucosidase exhibited compare to FPase and isolate *A. oryzae* S/92Gbr showed the highest β-glucosidase (2.5 U/ml), CMCase (2.4 U/ml), Avicilase (2.1 U/ml) and FPase (1.5 U/ml) activity. The optimum pH and temperature of the enzyme estimated as 5 and 45℃, respectively and th e highest activity ranges around at pH 4-6 and 40-50℃. The highest activity was observed after 7 day s of incubation at pH 5.6 and 28℃ when CMC was used as substrate. The enzyme activity strongly inhibited by Cu^{2+}, Hg^{2+}, Fe^{3+}, Zn^{2+}, Ag^{+} and K^{+} and increased by Mg^{2+} and Mn^{2+} about 40%. The Km values estimated as O.44, 0.48, 0.54 and 0.74% by *A. oryzae* S/92Gbr, *Aspergillus flavus* S/23Ogr, *Aspergillus cervinus* S15/Pf and *Aspergillus ochraceus* S/56Y, respectively that revealed the enzyme contained good reducing sugar releasing capacity.

Key words: *Aspergillus* spp., protein, biomass, cellulase activity, Km value.

INTRODUCTION

Cellulase is an inducable enzyme complex (Kocher et al., 2008) consisting of three components viz. exoglucanase, endoglucanase and β-glucosidase (Khan, 1980) which catalyze the cellulose and cellooligosaccharide derivatives (Chinedu et al., 2010). They are widely used for the extraction of valuable compounds from plant cells, improving nutritional values of animal feed and in preparing plant protoplasts for genetic research (Abdul et al., 1999). They are also applied in food, textile, fuel, chemical industries, paper and pulp industry, waste management, medical/pharmaceutical industry, and pollution treatment (Tarek and Nagwa, 2007). Today, these enzymes account for approximately 20% of the world's enzyme market (Jaradat et al., 2008). Indeed, the demand for this enzyme is growing more rapidly than ever before especially in the conversion of lignocelluloses into bulk chemicals and bio-fuels, and this demand has become the driving force for research on cellulases.

A number of bacteria and fungi produce cellulases though species of *Trichoderma* and *Aspergillus* are most commonly reported. Commercial cellulase preparations from *Trichoderma reesei* are popular as it contains high activities of both exo-glucanase and endo-glucanase but low levels of β-glucosidases (Rosgaard, 2006); therefore, attention has recently been diverted to other micro-organisms including the members of genus *Aspergillus*. The genus *Aspergillus* is a group of filamentous fungi with a large number of species and some species have a good fermentation capabilities (Ja'afaru and Fagade, 2007); particularly it produces wide range of enzymes for the degradation of plant cell wall polysaccharides (de Vries and Visser, 2001). However, research is being carried out on isolation of potential cellulase producing

*Corresponding author. E-mail: ferdrita@yahoo.com. Tel: + 603-89471171. Fax: +603-8943 2954.

microorganisms from diverse habitats (Ray et al., 2007). Therefore, the present research was under taken to screen more active strain of *Aspergillus* for cellulose production under submerged fermentation and to analyze some characteristics of the enzyme.

MATERIALS AND METHODS

Microorganisms

To conduct the research fifteen *Aspergillus* species were collected from Mycology and Plant Pathology Laboratory, Department of Botany, Rajshahi University, Bangladesh which were previously isolated from municipal kitchen waste, agriculture residues and soil; identification of the isolates were confirmed by references to Raper and Fennell (1965). The cultures of *Aspergillus* strains were maintained as stock culture in Czapek- Dox agar slants. They were grown at 30℃ for 5 days and stored at 4℃ for regul ar sub culturing.

Plate screening

Carboxymethylcellulose-agar (CMC-agar) medium was used for screening. This medium consisted of CMC (1 g), sucrose (20 g), $NaNO_3$ (2 g), KH_2PO_4 (1 g), $MgSO_4.7H_2O$ (0.5 g), KCl (0.5 g), $FeSO_4.7H_2O$ (0.01 g), agar (20 g) and distilled water (1000 ml). Conidia from one-weekend Czapek's plates were suspended in sterile H_2O. A small well created in the middle of the screening plates and 100 µl of conidial suspension (1×106) of each strain was inoculated into the wells plates and incubated at 28℃ for three days followed by 18 h at 50℃. For cellulolytic acti vity, observation plates were stained with 1% Congo red dye for 30 min followed by distaining with 1 N NaCl solution for 15-20 min.

Cellulase production

Czapek-Dox broth medium was used which consists (in gL^{-1}) of sucrose (20.0 g), $NaNO_3$ (2.0 g), KH_2PO_4 (1.0 g), KCl (0.5 g), $MgSO_4.7H_2O$(0.5 g) and $FeSO_4.7H_2O$ (0.01 g) and 1.0 ml of trace solution (containing 1.0 g $ZnSO_4$ and 0.5 g $CuSO_4$. $5H_2O$ L^{-1}) with 1% of carboxylmethyl cellulose powder (CMC). The pH of the medium was adjusted to 5.6. Then 100 ml of the liquid medium was placed in 250 ml Erlenmeyer flask and sterilized by autoclaving 121℃ for 15 min. This was cooled and inoculated wi th 1x106 spores/cells of each culture and allowed to grow at 28℃ on a Gallenkamp (England) rotary shaker at 100 rpm for 5 days. Fungal mat was removed by filtering and the filtrate was centrifuged at 10,000 rpm for 10 min at 4℃ using refrigerated ultrace ntrifuge (REMI, k-70) and stored at 4℃ with few drops of tolue ne to avoid bacterial contamination.

Determination of reducing sugar, protein and biomass

The amount of reducing sugar in culture filtrate was measured by Miller (1959) method using DNS reagent and measuring the absorbance at 550 nm in a spectrophotometer (Spectronic 21).

Protein contents were determined in the filtrate by the method of Lowery et al. (1951) using bovine serum albumin as protein standard.

Biomass produced by fungi was filtered and residue was dried in oven at 80℃ for a constant weight and amount of bi omass was calculated by subtracting the weight of filter paper.

Enzyme assays

Endoglucanase (CMCase) activity was determined by using 1% CMC as the substrate in 0.02 M acetate buffer pH 5.2. The reaction mixture containing 1 ml of 1% CMC and 0.5 ml of enzyme extract which were incubated for 2 h at 45℃, after this the release of reducing sugar was measured by Miller (1959) method using DNS reagent.

Avicelase activity was determined by mixing 0.5 ml of enzyme extract with 1 ml of 1% avicel (microcrystalline cellulose) in 0.02 M acetate buffer (pH 5). The mixture was incubated at 45℃ for 2 h. Then 3 ml DNS reagent was added and the mixture boiled for 15 min to terminate the reaction and absorbance read at 540 nm.

β-glucosidase activity was estimated by mixing of 0.5 ml of enzyme extract and 1 ml of 1% salicin (DIFCO lab) prepared in 0.02 M acetate buffer (pH 5.2) and incubated for 2 h at 45℃ after which the release of reducing sugar was assayed by using DNS reagent.

For assay of FPase activity 0.5 ml of enzyme extract was added to 1 ml of 0.02 M acetate buffer, pH 5.2 belong with 50 mg filter paper strip (Whatman no. 1, 1×6 cm) in a test tube and incubated at 45℃ for two hours in a water bath on shaking cond ition, after which the release of reducing groups were assayed by using DNS reagent.

Enzyme activity was measured as the amount µmol glucose released min^{-1} ml^{-1} of culture filtrate as enzyme solution and was expressed as U/ml.

Characterization

Effect of varying pH

Activity of cellulase enzyme obtained from four *Aspergillus* strains were determined in different pH values ranging from 2 to 10 following the procedure as same described before.

Effect of varying temperature

Activity of cellulase was determined in different temperatures ranging from 10-80℃. Temperature below 30℃ was main tained by using the thermostat water bath (GFL-1083).

Effect of varying time

In order to find out the optimum time course for enzyme activity, *Aspergillus* species were grown on Czapek's medium containing 1% CMC as carbon source at 28℃. The culture filtrates were analyzed for enzyme activity from 3 to 11 days of incubation.

Effect of various metallic ions and salts

The effect of different metalic ions and salts on enzyme activities was tested by pre-incubating enzymes solutions (0.5 ml) with specified concentrations of different reagents (0.25 mg/ml) for 15 min at 37℃ and the activities were assayed.

Determination of Km value

Michaelis constant (Km) of crude cellulase was determined by Lineweaver-Burk double reciprocal plot. The initial velocity was equal to the amount of product formed per unit time. The initial velocity (Vi) is determined by quantitatively measuring the amount of one of the product at various time intervals (Robyt and White, 1990).

Table 1. Diameter of clear zone, reducing sugar, protein and biomass by fifteen strains of *Aspergillus* grown on broth Czapek's medium (with 1% CMC) at 28°C and pH 5.6 .

Isolates	Diameter of clearing zone (mm)	Reducing sugar (µg/ml)	Protein (µg/ml)	Biomass (mg)
A. flavus S/23OGr	37 ±0.577 [b]	492 ±1.154 [b]	395 ±0.577 [b]	564 ±0.721 [b]
A. fumigatus S/77Blgr	28 ±0.235 [f]	386 ±0.577 [g]	290 ±0.317 [e]	396 ±0.289 [l]
A. oryzae S/92Gbr	40 ±0.471 [a]	520 ±0.471 [a]	485 ±0.866 [a]	557 ±0.433 [c]
*A.parasiticus*S/3Dygr	25 ±0.259 [g]	360 ±0.585 [h]	235 ±0.577 [i]	463 ±0.490 [j]
*A.tubengensis*S/84Blb	21 ±0.471 [i]	326 ±0.545 [l]	196 ±0.433 [l]	497 ±0.577 [g]
A. niger S/33Blc	30 ±0.707 [e]	397 ±0.490 [e]	287 ±0.288 [f]	565 ±0.202 [b]
A. ficuum S/55Blpb	17 ±0.471 [j]	298 ±0.283 [m]	198 ±0.144 [k]	521 ±0.433 [e]
A. terreus S/95By	25 ±0.188 [g]	352 ±0.863 [i]	272 ±0.375 [g]	486 ±0.259 [h]
A. awamori S/42Blcb	30 ±0.136 [e]	390 ±0.433 [f]	256 ±0.317 [h]	578 ±0.577 [a]
A. ochraceus S/56Y	35 ±0.471 [c]	415±0.144 [d]	325 ±0.433 [c]	504 ±0.433 [f]
A. foetidus S/52Bbl	15 ±0.235 [k]	242 ±0.317 [n]	186 ±0.144 [m]	543 ±0.288 [d]
A. japonicus S/50Pbl	13 ±0.256 [l]	234 ±0.656 [o]	155 ±0.721 [n]	253 ±0.866 [n]
A. cervinus S/15Pf	33 ±0.942 [d]	423 ±0.011 [c]	305 ±0.228 [d]	481 ±0.317 [i]
A. aculeatus S/40Pbl	23 ±0.378 [h]	340 ±0.325 [j]	212 ±0.744 [j]	420 ±0.490 [k]
A. crustosus S/90Blbr	21 ±0.356 [i]	329 ±0.866 [k]	185 ±0.490 [m]	345 ±0.404 [m]

Values are presented as mean ± SEM of triplicate experiments. Values within column with different subscripts are significantly different at $p<0.05$.

Statistical analysis

Data were recorded and statistically analyzed with the help of computer package program SPSS (software version 10.0, Chicago IL, USA) for DMRT test. The results of all analysis were judged for its significant at $P < 0.5$.

RESULTS AND DISCUSSION

A total of fifteen different *Aspergillus* species were used to screen for cellulase production which were previously isolated from differentcellulosic waste substrates. Upon initial screening, it appeared that all of the isolates were able to produce cellulase enzyme (Table 1). Cellulase producing isolates were categorized into 3 groups according to the width of clear zones; very strong (30-40 mm), strong (20-30 mm) and moderate (10-20 mm). Among the isolates, *A. oryzae* S/92 Gbr strain showed the highest clear zone (40 mm) and next of *A. flavus* S/23 Ogr (37 mm), *A. ochraceus* S/56Y (35 mm) and *A. cervinus* S/15 Pf (33 mm). This results support the work of Onsori et al. (2005) who obtained the highest clear zone 40 mm in *A. niger*.

Further screening was conducted by assessment of reducing sugar, protein and biomass production (Table 1). From the results it was observed that *A. oryzae* S/92Gbr exhibit maximum protein (485 µg/ml), reducing sugar (520 µg/ml) and biomass (557 mg). Protein level and reducing sugar of the culture filtrate support that the organisms secrete extracellular proteins which have cellulolytic activity. Most cellulolytic fungi secrete hydrolytic enzymes for the breakdown of the polymers into their growth media; this largely accounts for the protein contents of the cell-free filtrates (Chinedu et al., 2008). Biomass production of individual strain responded differently based on their different growth behaviour. Statistical analysis results using Duncan's Multiple Range Test (DMRT) showed that most of the species produced significant ($p<0.05$) amount of protein, reducing sugar and biomass and *A. oryzae* S/92Gbr was the best.

Depending on the growth vigour, zone of clearance, accumulation of reducing sugar, protein and biomass production only four *Aspergillus* species viz. A. oryzae S/92Gbr, *A. flavus* S/23Ogr, *A. ochraceus* S/56Y and *A. cervinus* S/15Pf were selected for further study. Comparative analysis of different cellulase enzymes production by *Aspergillus* species showed that all the strains contained higher amount of β-glucosidase, followed by CMC ase, Avicelase and FPase activities (Figure 1). In all the cases the FPase activity of the crude enzyme was much less. In previous study the values of CMC activity were always higher than that of filter paper activity (Singh et al., 2009). This hold true for all the studies of fungi and used different substrates for fermentation (Pandey et al., 1999).

Figure 2 shows that *Aspergillus* species produced moderate to high cellulase activity at pH 4 to 6 with relative activities of 50-75% whereas optimum activity was observed at pH 5 with relative activity of 100% in *A. oryzae* S/92Gbr. From the results, it was also noted that the enzymes are more active in moderate acetic region than alkaline. Fungal cellulases with pH values of 4.5 to

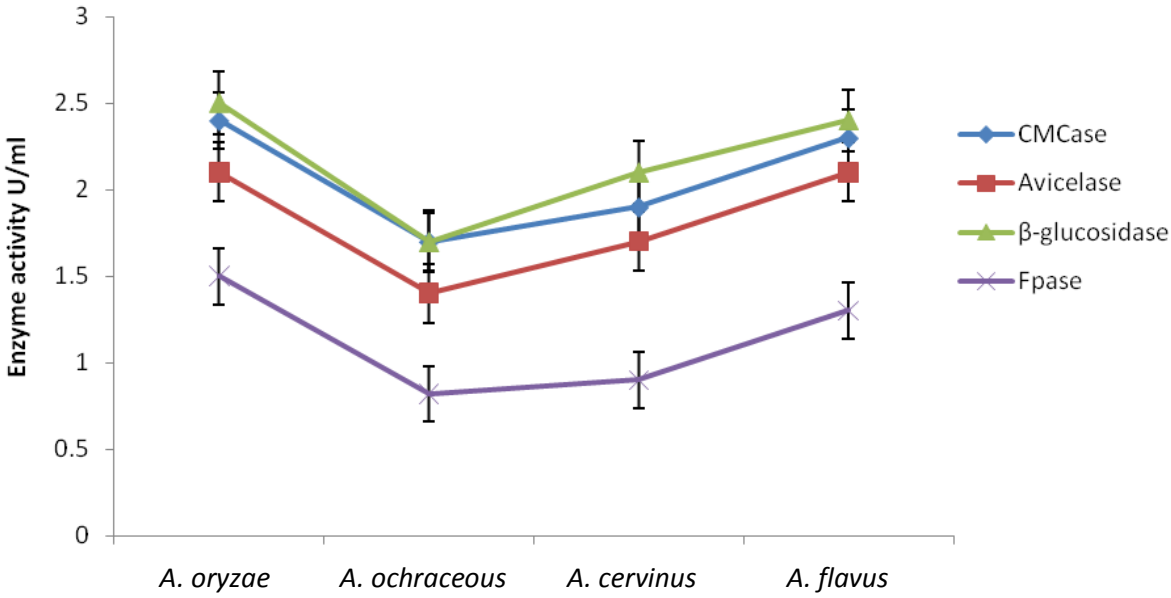

Figure 1. Cellulase activities of four *Aspergillus* species on pure cellulosic substrates. Bars represent the standard errors for means at $p < 0.05$.

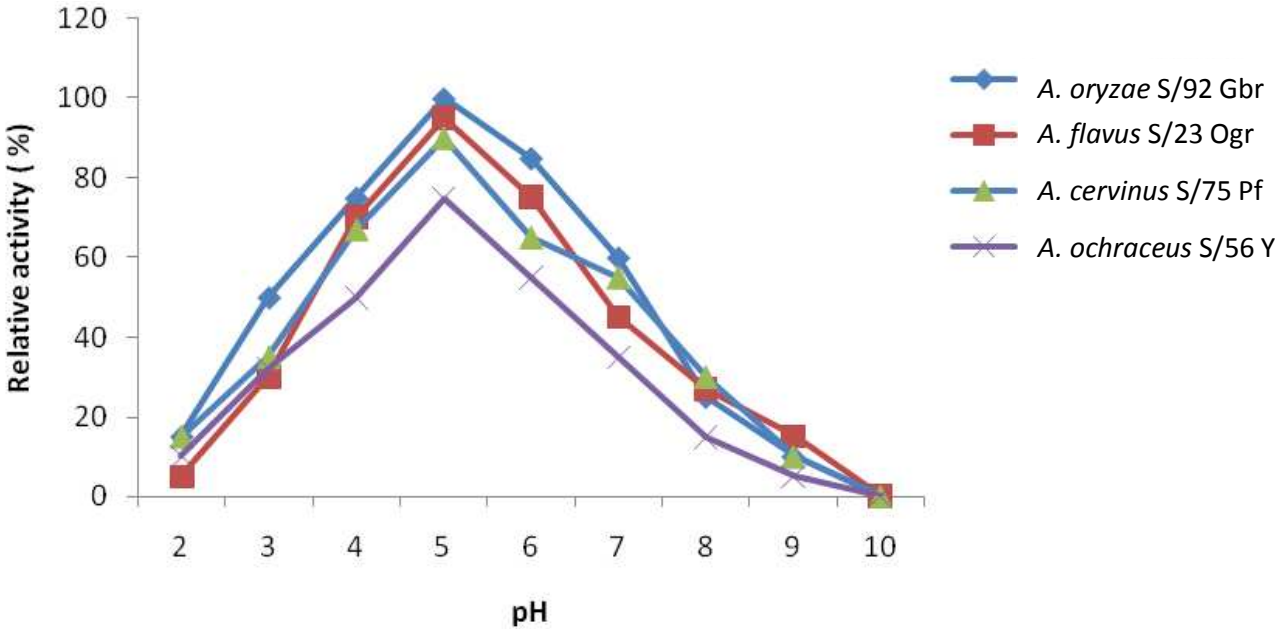

Figure 2. Effect of different pH on cellulase activity of four *Aspergillus* strains. Enzyme activities at different pH were compared to the highest value, considered as 100%.

6.0 are common and have been obtained from *Aspergillus niger* and *A. terreus* (Goma et al.,1982); *Rhizopus oryzae* (Amadioha, 1993); *Volvariella diplasia* (Bhadauria et al., 1997); and *Trichoderma reesei* QM 9414 (Wang, 1999).

The enzyme exhibited high activity at 40 to 60℃ and maximum activity measured at 50℃ with 100% relative activity which decreased considerably at 80℃ reaching only 15% (Figure 3). This decrease in activities of enzymes at higher temperature might be due to destruction

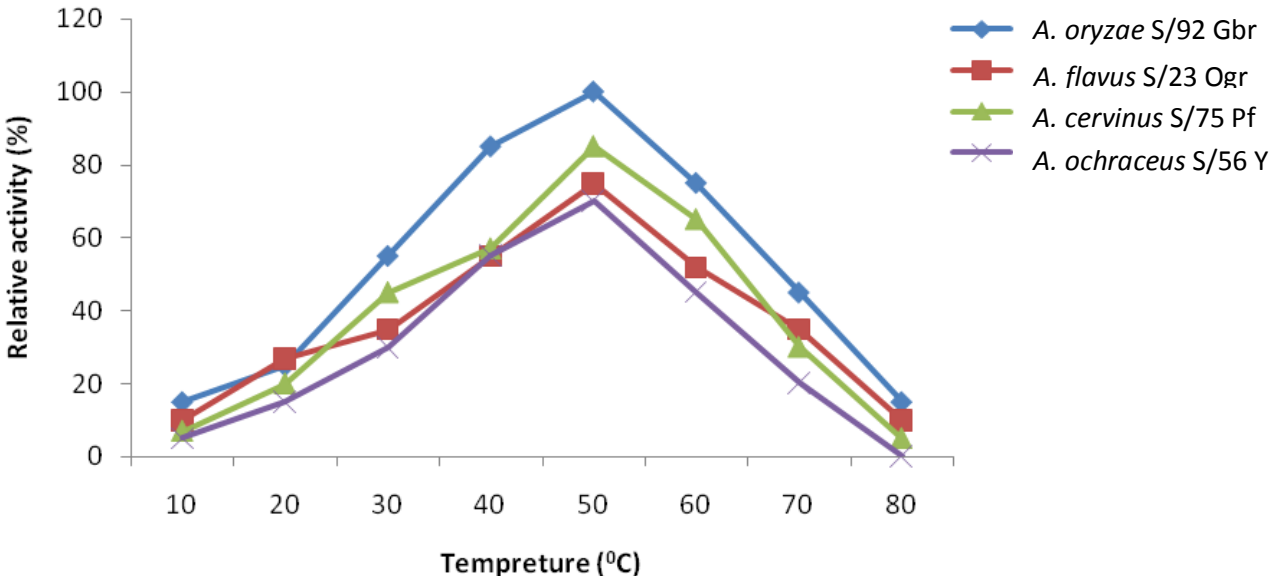

Figure 3. Effect of different temperature on cellulase activity of four *Aspergillus* species. Enzyme activities at different temperatures were compared to the highest value, considered as 100%.

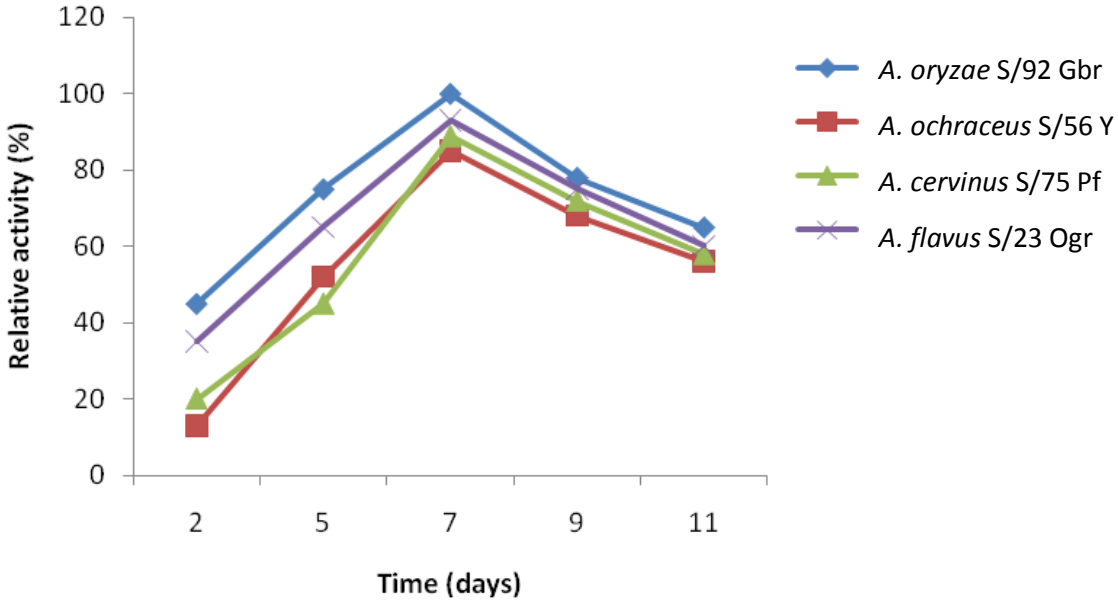

Figure 4. Effect of different time course on cellulase activity of four *Aspergillus* species. Enzyme activities at different days were compared to the highest value, considered as 100%.

of secondary or tertiary structure of enzyme. In case of celulase activity the optimum temperature 40 to 50°C are obtained from *Aspergillus* aureoles and *A. clavatus* (Mishra, 1988); *Trichodrma viridie* (Sandhya, 1992) and *Morchella conica* (Cavazzoni and Manzoni, 1994); these temperatures nearly coinciding with the charecteristics of mesophiles (Baig et al., 2004).

Incubation period is an important factor for enzyme production. In the present study cellulase activity increased rapidly up to incubation periods of 7 days with 100% relative activity and thereafter the activities were decreased gradually (Figure 4). In an earlier study, Sakamoto et al. (1982) found maximum cellulase activity on 4th day; Singh et al. (2009) obtained on 5th day and

Table 2. Effect of various metallic salts on the relative activity (%) of cellulases for *Aspergillus* spp.

Metalic salts	Concentrations (M)	*A. oryzae* S/92Gbr	*A. ochraceous* S/56Y	*A. cervinus* S/75Pf	*A. flavus* S/23Ogr
None	-	100.00	100.00	100.00	100.00
	0.001	119.56	106.25	102.85	110.66
$MgCl_2$	0.002	128.68	115.06	112.24	121.24
	0.005	142.23	127.35	121.05	136.78
	0.001	86.26	79.45	77.84	83.68
$ZnCl_2$	0.002	78.62	70.16	70.88	75.25
	0.005	68.25	62.45	60.27	66.58
	0.001	78.28	72.78	72.00	75.89
$CuCl_2$	0.002	69.56	61.00	60.15	68.08
	0.005	50.00	52.45	53.56	66.55
	0.001	87.14	88.66	87.06	89.25
$HgCl_2$	0.002	76.07	78.25	73.88	71.88
	0.005	57.02	55.66	51.75	54.26
	0.001	106.46	104.05	100.77	102.88
$MnCl_2$	0.002	118.64	116.55	110.42	112.78
	0.005	139.88	135.12	132.04	131.06
	0.001	84.34	81.35	79.00	79.02
$FeCl_3$	0.002	79.00	70.55	72.65	69.35
	0.005	66.58	65.71	60.12	62.55
NaCl	0.001	100.00	100.00	100.00	100.00
	0.002	98.09	95.42	97.06	96.46
	0.005	92.02	90.15	90.44	89.33
	0.001	97.87	96.96	98.85	95.41
KCl	0.002	96.62	93.70	95.00	92.52
	0.005	92.10	90.04	91.65	89.50
	0.001	92.87	90.65	87.87	88.41
AgCl	0.002	88.07	86.05	82.56	83.21
	0.005	75.85	79.00	73.14	71.05

Omajasola et al. (2008) reported on 5th day in *Aspergillus aculeatus, A. heteromorphus* and *A. niger*, respectively. The observations in the present work differ from the earlier ones due to the different organisms, which were isolated from different types of cellulolysic materials as well as climatic conditions.

From the results it was also observed that presence of Cu^{2+}, Hg^{2+}, Fe^{3+}, Zn^{2+}, Ag^{+} and K^{+} strongly reduced the activities of cellulases, while the presence of Mg^{2+} and Mn2+ increased the activities about 40% (Table 2). On the other hand, Na^{+} and K^{+} had little inhibitory effect on the activities whereas Zn^{2+} and Ag^{+} decreased the cellulase activity moderately. Gupta and Gupta (1979) reported that Ag^{2+}, Hg^{2+}, Zn^{2+}, Cu^{2+} and N^{-3} were inhibitory for ecllulase production in *Trichoderma viridie*. Mishra (1988) reported that Cu^{2+}, Zn^{2+}, Mn^{2+} and Fe^{3+} inhibited the cellulase activities of *Aspergillus clavatus*. Murushima et al. (2002) observed that Cu^{2+}, Zn^{2+}, Co^{2+} and Pb^{2+} inhibited the cellulase activity of *Rhizopus oryzae*. The present observation was partially resembles with the work done earlier.

The Km value, as determined by Linweaver-Burk double reciprocal plots of crude cellulase enzyme which was estimated to be O.44, 0.48, 0.54 and 0.74% (Figures 5 to 8) by *A. oryzae* S/92Gbr, *A. flavus* S/23Ogr, *A. cervinus* S/15Pf and *A. ochraceus* S/56Y, respectively

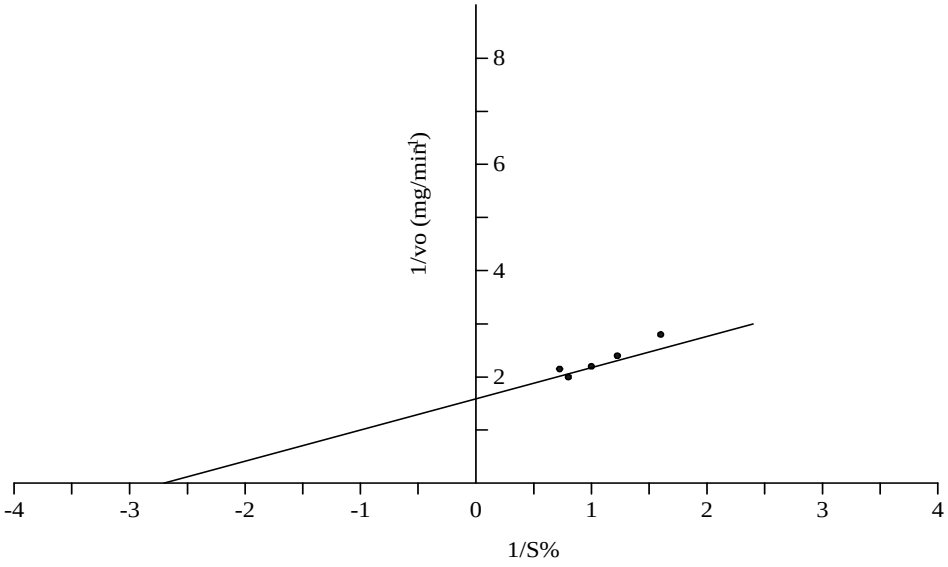

Figure 5. A Lineweaver-Burk double reciprocal plot for the determination of Km value of cellulase of *A. cervinus S/15*Pf.

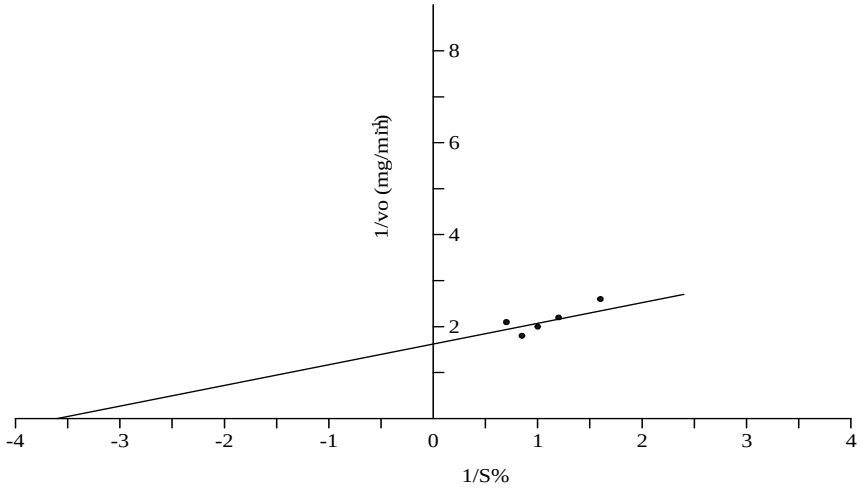

Figure 6. A Lineweaver-Burk double reciprocal plot for the determination of Km value of cellulase of *A. ochraceus* S/56Y.

against cellulose (CMC) as substrates. Further, the Km values of crude cellulase showed good correlation with the data of their reducing sugar releasing capacity. In previous work the Km value of cellulase from *Favouls arcularicas* was 0.28% (Enokibara et al., 1991) and 1.32% in *T. reesei* (Busto et al., 1996).

The recent thrust in bioconversion of lignocellulosic biomass to chemical feedstock has led to extensive studies on cellulolytic enzymes produced by bacteria and fungi (Singh et al., 2009). In the present study *A. oryzae* S/92Gbr cultivated in Czapek's Dox medium containing CMC as sole carbon source has been shown as more active to produce extracellular protein with significant cellulase activity. Further, this enzyme more active in acidic pH and moderate temperature; and posses potential reducing sugar releasing capacity. Thus this filamentous fungus may be served as potential tools in the industrial saccharification of cellulose.

ACKNOWLEDGEMENT

The authors are grateful to Prof. Nurul Abser of the Department of Biochemistry and Molecular Biology,

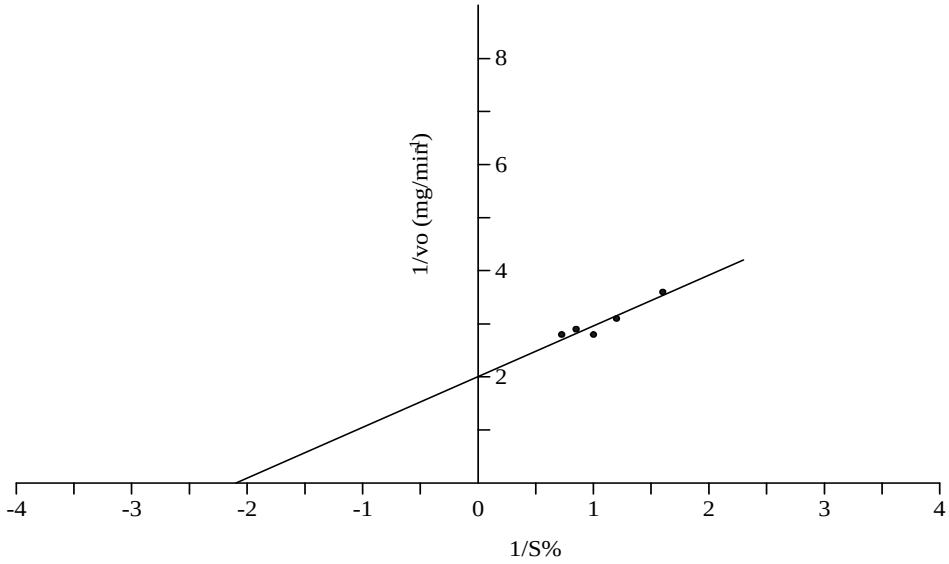

Figure 7. A Lineweaver-Burk double reciprocal plot for the determination of Km value of cellulase of *A. oryzae* S/92Gbr.

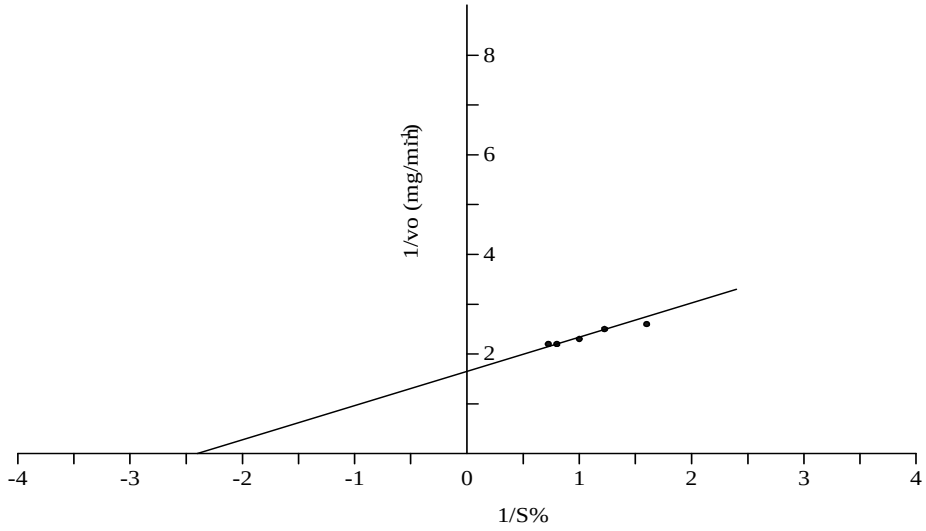

Figure 8. A Lineweaver-Burk double reciprocal plot for the determination of Km value of cellulase of *A. flavus* S/23Ogr.

University of Rajshahi, Rajshahi-6205, Bangladesh for his kind assistance.

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