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Production, Purification and Characterization of Endoglucanase from Locally Isolated *Aspergillus flavus*

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Abstract. Cellulose is major source of plant biomass and it can be converted into useful products by cellulases. Fungi are considered as good producer of industrially valued enzymes including cellulases. Current study was carried out to enhance production and characterization of endoglucanase by *Aspergillus flavus.* Solid state fermentation of sugarcane bagasse was performed and process was optimized for maximum production. Endoglucanase activity was found maximum after 96 h of incubation. Optimum fermentation conditions for the production of endoglucanase by *A. flavus* were 40 °C temperature, pH 7, 4 mL inoculum size, 50% moisture and 8 g of substrate. There was maximum precipitation of endoglucanase at 70% saturation with ammonium sulphate. The results showed 1.50 fold purification after ammonium sulphate purification and 3.3 folds after gel filtration chromatography. Optimum temperature and pH of endoglucanase were 40 °C and 3 individually. Metal ion effect on endoglucanase activity was observed by using different metals. The endoglucanase activity was repressed by Fe^{3+} and Hg^{2+} and increased by Co^{2+} and Mn^{2+}. The activity of endoglucanase was also found to be enhanced by organic solvents i.e. methanol, glycerol and acetone, while lessened by ethanol and isopropanol. Similarly, SDS stimulated enzyme activity, while Tween-20 decreased it. Increased amount of inhibitor like (EDTA) decreased the endoglucanase activity. The values of K_m and V_{max} were 4.5 mM and 65 µM/mL/min respectively.

Keywords: endoglucanase, *Aspergillus flavus*, optimization, lignocellulose

Introduction

Lignocellulosic materials are important feed stock as renewable and natural source (Perez *et al*., 2002). Cellulose takes universal attention as a renewable source that can easily be converted into bio-based products. Cellulose also produces the biofuels. Lignocellulosic material production is estimated 15×10^{12} tons annually.

Cellulose is crystalline, insoluble and fibrous in nature. Cellulose is major constituent of the plant cell wall that is composed of repeating D-glucose unit which are interconnected through β-1, 4-glycosidic linkages (Alonso *et al*., 2008) and cellulose is the most plentiful carbohydrate polymer present in earth. Cellulases are the important enzymes which convert the cellulose into sugars (Chinedu *et al*., 2005). Cellulases are the type of enzymes that break β-1,4 glycosidic linkage of the cellulose polymer and they also hydrolyse cello-oligosaccharide derivatives. Endoglucanases randomly hydrolyse the internal region of cellulose by hydrolysing β-glucosidic bonds, exoglucanases are act on the terminal region and release cellobiose (disaccharide), while the hydrolysis process is completed by β-glucosidases through converting cellobiose (disaccharide) to glucose (Szijarto *et al*., 2004). Cellulases have many industrial applications and used in paper recycling, cotton processing, textiles, wine and brewer industry, bioethanol industry, food processing, animal feed, detergent, agriculture and waste management. These are also used in the field of research and development (Asgher *et al*., 2013).

For enhanced production of enzymes, solid state fermentation (SSF) is used as one of the important strategies among others, which are working in industries for the improved production of different enzymes. SSF is carried out on the solid substrates in the absence of free water. Now a days SSF is gaining much attention as an appropriate strategy for the use of nutrient rich wastes like lignocelluloses.

A. flavus is a thermophilic fungus classified in the division Ascomycota. *Aspergillus flavus* is best recognized for its establishment in legumes, tree nuts and cereal grains. Postharvest rot characteristically

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grows during the storage, transportation or harvest. *A. flavus* decay dead organic matter and during this process it produces extracellular endoglucanase enzyme.

Materials and Methods

Fermentative organism. *Aspergillus flavus* was used as a fermentative organism for the production of endoglucanase. *A. flavus* is thermophilic fungus which was isolated from the subtropical region of Mirpur Azad Kashmir. It was cultured on potato dextrose agar (PDA) medium and pure cultures were obtained by sub-culturing technique (Sherief *et al*., 2010). The pure cultures were prepared at room temperature and kept in refrigerator at 4 °C for further use.

Preparation of inoculum. Fungal inoculum was prepared for the use in fermentation process. The flask containing the potato dextrose broth (inoculum medium) having pH 5.5 were autoclaved. It was inoculated with loop full of fungal spores from the pure culture and put on shaking incubator at 150 rpm at 37 °C for 96 h.

Preparation of substrate. Sugarcane baggase was used as a lignocellulosic substrate of *A. flavus* for the production of endoglucanase. Sugarcane bagasse was used as substrate because of its high percentage of cellulose, cheap and easily available in the market (Ahmed *et al*., 2009). It was collected from Mirpur Azad Kashmir, air dried and oven dried before grounded to powder. Powder was kept in air tight plastic jars for further use in the SSF process.

Solid state fermentation. Endoglucanase was produced with the SSF of sugarcane baggase by *A. flavus*. The flasks containing 5 g of grounded sugarcane bagasse, 50% moisture level were first autoclaved and then inoculated with 2 mL of fungal inoculum. Flasks were then incubated at 37 °C for specific day.

Harvesting of endoglucanase. After specified day each flask was harvested for the isolation of endoglucanase by contact method (Krishnaiah *et al*., 2007). For this 50 mL distilled water was poured in each flask which is having pH 5.5 and then placed for 30 min in shaking incubator at 150 rpm. These were filtered with filter paper, centrifuged for 10 min at 6000 rpm and stored at 4 °C as crude enzyme before performing assay.

Optimization of endoglucanase production. The enzyme production was optimized to get enhanced production of endoglucanase from *A. flavus*. The following conditions were optimized during the present research.

Fermentation period. The production was studied from 24 h to 96 h to get optimum fermentation period and maximum endoglucanase production.

Incubation temperature. The fermentation process was studied from 25 °C to 45 °C to find the most suitable temperature for endoglucanase production.

Optimization of pH. *A. flavus* was grown at different pH range from 3 to 8 in order to determine the optimum of pH for *A. flavus* growth.

Optimization of substrate level. Substrate concentration (sugarcane baggase) was adjusted from 2 g to 10 g, to find the best concentration for maximum endoglucanase production.

Optimization of inoculum size. Fungal inoculum was optimized from 1 mL to 5 mL, to check the most suitable inoculum size for endoglucanase production.

Optimization of moisture level. Moisture present in the substrate effect the fungal growth, in current research the effect of moisture contents on enzyme production was monitored from 40%-80%.

Optimization of nutritional conditions. Different carbon and nitrogen sources were used to get a better combination for the enhanced production of endoglucanase from *A. flavus*. Carbon and nitrogen sources were employed in different combination and their effect on the production of endoglucanase was checked.

Effect of carbon sources. Effect of glucose, fructose and sucrose was checked in combination with nitrogen sources to find their combine effect on the production of endoglucanase enzyme.

Effect of nitrogen sources. The effect of ammonium sulphate, urea and ammonium phosphate was monitored on endoglucanase production in combination with above mentioned carbon sources.

Endoglucanase assay. For the estimation of enzyme (endoglucanase) 1 mL of 1% CMC as substrate was picked in test tube. The pH of the mixture was maintained with 1 mL of sodium citrate buffer and its pH was adjusted at 4.8. Test tubes were kept in the incubator for 30 min at 37 °C after adding crude enzyme. The reaction was stopped by the addition of 3 mL of DNS (Dinitrosalicyclic acid) in each test tube and test tubes were then kept in boiling water for 15 min for the development of coloured complexes. The colour complexes developed were checked by taking absorbance at 540 nm in spectrophotometer.

Enzyme activity. One unit enzyme activity was defined as the quantity of enzyme which produces one µ-mole product (glucose) per min.

Estimation of protein. Estimation of protein was performed in crude sample and after each step according to the Bradford method.

Endoglucanase purification. Enzyme was partially purified, which was produced under optimized condition, for further characterization by the following methods.

***Ammonium sulphate precipitations*.** Ammonium sulphate precipitate proteins by decreasing their solubility. Various concentrations of ammonium sulphate were added (30% to 80%) with a 10% gap in 5 mL crude extract separately (Park *et al*., 2015). After precipitation, endoglucanase assay was performed and protein estimation was done by Bradford assay.

***Gel filtration chromatography*.** For further purification, partially purified enzyme was then subjected to gel filtration chromatography. For this purpose sephadex gel column (5%) was prepared in sodium citrate buffer with pH 4.8. The enzyme elutions were maintained at a flow rate of 30 cm/h (Ahmed *et al*., 2009). Enzymes assay was performed for the eluted samples and Bradford assay was carried out to determined protein concentration (Fagain *et al*., 2017).

Characterization of endoglucanase. Endoglucanase was characterized for the determination of following parameters (Ramirez *et al*., 2016; Harshvardhan *et al*., 2013; Balasubramanian *et al*., 2012).

***Determination of optimum pH*.** The endoglucanase was characterized to determine its optimum pH. Enzyme assays were carried out at various pH values (3, 4, 5, 6 and 7) which were maintained with sodium citrate buffer (Ramirez *et al*., 2016; Harshvardhan *et al*., 2013). The activity assays were performed according to procedure discussed earlier.

***Determination of optimum temperature*.** Endoglucanase assays were carried out at various temperatures i.e. 25, 30, 35, 40 and 45 °C (Ramirez *et al*., 2016; Balasubramanian *et al*., 2012). These temperatures were adjusted by placing tubes, having assay mixture, in incubator. The activity assays were performed according to procedure discussed earlier.

***Effect of metal ions*.** The endoglucanase activity assays were performed by adding 10 mM solution of different salt e.g. $CaCO_3$, $CaCl_2$, $MgCl_2$, $FeSo_4$, $Mncl_2$, $HgCl_2$, $CuSo_4$ and $CoCl_2$ to find the effect of metal ions (Ramirez *et al*., 2016).

***Effect of organic solvents*.** Endoglucanase activity assays were performed by incubating enzyme with five different solvents including, ethanol, methanol, acetone, isopropanol and glycerol (1%, 10% and 20% of each) to check their effect on activity (Harshvardhan *et al*., 2013; Balasubramanian *et al*., 2012). The activity of endoglucanase was determined against the control, in which no solvent was included.

***Effect of inhibitor*.** EDTA was used as an inhibitor. The enzyme was incubated for 30 min at optimum temperature and optimum pH with different concentrations of EDTA 0.01%, 0.05% and 0.25%. The activity of endoglucanase was determined against the control, without EDTA (Ramirez *et al*., 2016).

***Effect of non-ionic surfactants on endoglucanase*.** The endoglucanase was incubated at optimum temperature and optimum pH with different surfactants like Triton-X-100, Tween-20, Tween-80 and SDS (0.25%, 0.5% and 1.0%) for 30 min. The endoglucanase activity was determined against the control that was without these surfactants.

***Determination of enzyme kinetics*.** To determine kinetic parameters of endoglucanase, solutions were prepared those contain 2-10 mM of CMC and the reaction was performed for 30 min at 37 °C and 3 pH. The endoglucanase kinetic parameters like K_m and V_{max} were determined by the equation from Line weaver Burk plot (Ramirez *et al*., 2016).

Results and Discussion

Endoglucanase is an important enzyme which have the ability to degrade cellulosic waste material. The optimization of endoglucanase production by *Aspergillus flavus* results in more enzyme production. On the other hand degradation of cellulosic material by endoglucanase also reduces the environmental pollution and will produce valued products from the resultant sugars.

Optimization of fermentation period for endoglucanase production. The results showed that the activity of endoglucanase was increased with increasing fermentation period and it was maximum at 96 h (72.6 IU/mL/min) and then gradually decrease up to 168 h (Fig. 1). This might be due to decrease in concentration of substrate and accumulation of various toxic chemicals.

With the passage of time the concentration of sugarcane bagasse decreased, that's why with the reduction of substrate the activity of enzyme i.e. endoglucanase may also decrease.

Optimization of temperature for endoglucanase production. Temperature plays key role in fungal growth and enzyme production. It has been observed during current study that 40 °C was the optimum temperature for the growth of *Aspergillus flavus* with maximum endoglucanase activity (60.7 IU/mL/min) shown in (Fig. 2).

Different optimum temperatures were shown by many researcher ranges from 40 °C to 70 °C for thermophilic fungus. According to Goa *et al*. (2008) optimum temperature 45 °C was recorded for thermophilic fungus which show resistance up to 70 °C.

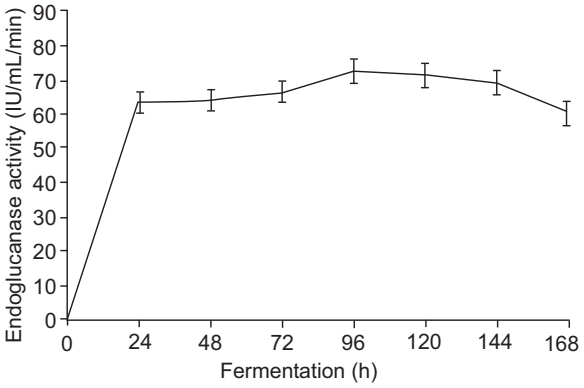

Fig. 1. Optimization of fermentation period for the production of endoglucanase by *A. flavus*.

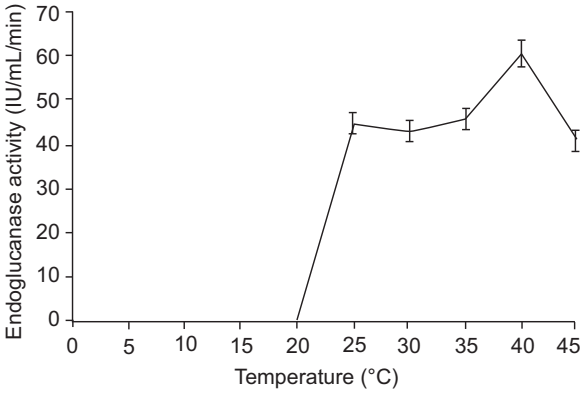

Fig. 2. Optimization of temperature for the production of Endoglucanase by *A. flavus* and positive control was incubated at room temperature.

Optimization of pH for endoglucanase production. The pH of medium was adjusted to find the most suitable pH for endoglucanase production by *A. flavus*, because pH affects the ionic strength of the medium. *A. flavus* was over various pH media and maximum enzyme activity was recorded at 7 pH (69.9 IU/mL/min). The production of endoglucanase was reduced with further increase in the pH of the medium (Fig.3).

The optimum pH reported by Gautam *et al*., (2011) was from 6-7 which shows that current result is in line with previously reported results. The maximum endoglucanase activity was also obtained at the pH 7 and shows good results up to pH 9 has been observed by Goyal and Soni (2011).

Optimization of inoculum size of endoglucanase. Increasing the size of inoculum means more number of spores in the fermentation media. Higher number of spores will be responsible for more fungal growth and enzyme secretion. Enzyme production by *A. flavus* was increased with increasing inoculum size shown in Fig. 4. The maximum endoglucanase activity was observed with 4 mL inoculum (76.1 IU/mL/min), further increase in inoculum leads to decreased production of endoglucanase by *A. flavus*.

The relation between the size of the inoculum and endoglucanase production was reported by Zhang *et al*. (2006). They also narrated that different concentration

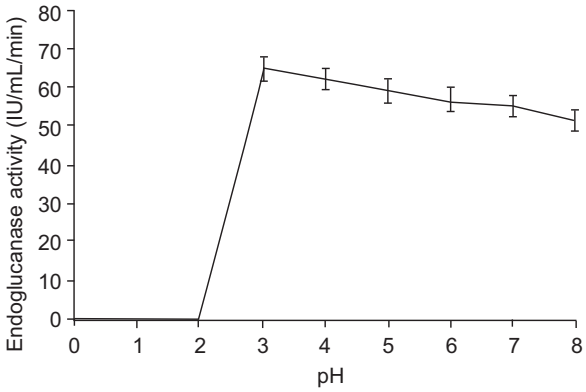

Fig. 3. Endoglucanase activity produced by *A. flavus* under varying pH and positive control was neutral pH.

of inoculum size cause variable degree of production of endoglucanase.

Optimization of moisture level for endoglucanase. Solid state fermentation (SSF) need less moisture as compared to submerged fermentation and have numerous benefits. Moisture of the sugarcane baggase was optimized to obtain maximum endoglucanase activity. The maximum activity of endoglucanase was observed at 50% moisture level (56.0 IU/mL/min). A reduction in the enzyme activity was observed above and below this moisture level described in Fig. 5.

The rational cause in the less production of endoglucanase is due to the fact in the poor aerating condition in the solid-state fermentation.

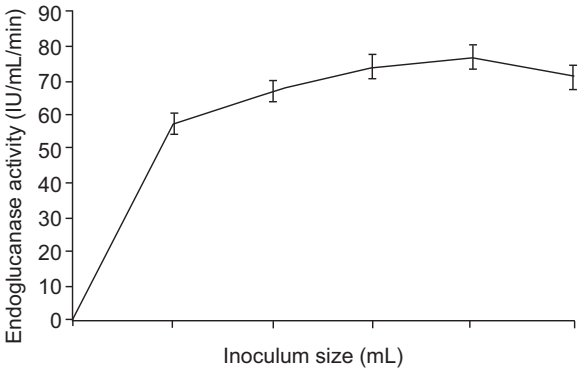

Fig. 4. Endoglucanase activity produced by *A. flavus* under various inoculum size and positive control had 0 mL inoculum size.

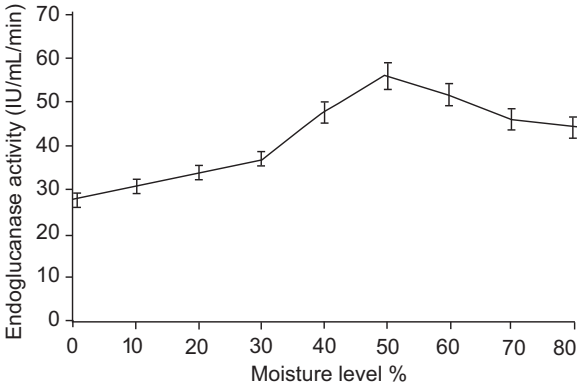

Fig. 5. Endoglucanase activity produced by *A. flavus* at different moisture level and positive control was 0% moisture level.

Optimization of substrate size for endoglucanase. Solid state fermentation (SSF) takes place on a solid medium as compared to liquid state fermentation. The solid medium act as support with little moisture, on which the process of fermentation takes place. Substrate size for solid state fermentation was optimized to find the most suitable amount for endoglucanase production by *A. flavus*. The maximum activity of endoglucanase was observed at 8 g substrate size which was 73.4 IU/mL/min. Above and below this size there was reduction in the enzyme activity reported in Fig. 6.

Optimization of carbon-nitrogen sources for endoglucanase. Carbon and nitrogen are necessary elements, required for the growth and metabolism of the fungus (*A. flavus*). Varying concentration of carbon and nitrogen concentrations were used simultaneously in order to obtain the optimum condition at which *A. flavus* showed maximum growth in the form of maximum enzyme production i.e. endoglucanase. The maximum production of endoglucanase was on the combination of 0.5% sucrose and 0.25% urea (C_3N_1) which was 65.23 IU/mL/min shown in Fig. 7.

Optimized conditions for endoglucanase production. After optimizing all the parameters, these were used for the growth of *Aspergillus flavus*. Thereafter the production of endoglucanase was estimated and its enzyme assay activity was monitored. The optimized conditions were, 40 °C temperature, 96 h period of incubation, pH 7.0, 4 mL inoculum size, carbon-nitrogen sources (sucrose and urea). The results which were deduced from the current study showed that endoglucanase production at optimized condition was

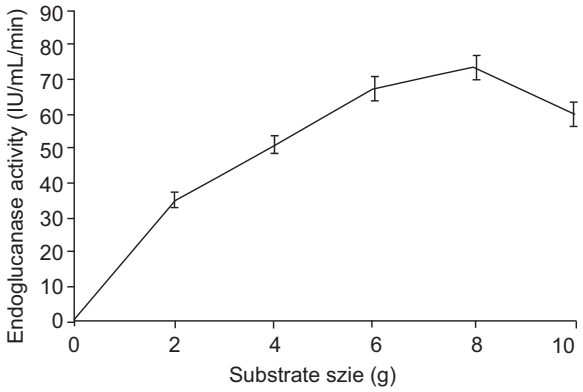

Fig. 6. Endoglucanase activity produced by *A. flavus* at different substrate level.

more with higher activity (64.5 IU/mL/min) shown in Fig. 8.

Ammonium sulphate purification of endoglucanase. Optimized conditions were used for the production of endoglucanase and then it was purified by ammonium sulphate precipitation. Ammonium sulphate changes the ionic strength of the solution and reduce solubility of proteins. Varying concentration of ammonium sulphate (i.e. 30%, 40%, 50%, 60%, 70% and 80%) was added in 5 mL of enzyme solution separately. Sample was left for overnight and was subjected to centrifugation for 30 min at 6000 rpm before performing enzyme. The results showed that at 70% saturation with ammonium sulphate there was maximum endoglucanase activity (20.3 IU/mL/min) by further increasing the concentration there was decrease in enzyme activity in Fig. 9.

Gel filtration chromatography of endoglucanase. Partially purified endoglucanase was subjected to further purification though gel filtration chromatography. Enzyme was added in 5% silica gel column followed by citrate buffer whose pH was adjusted at 4.8 in order to obtain different elution. Endoglucanase activity assay was performed for these elutions. Twenty-five elutions were collected and results revealed that elution number eighteen showed maximum activity (14.6 IU/mL/min) and then gradually a decrease in activity was observed in Fig. 10. Ahmed *et al*. (2009) also observed increase enzyme activity with each elution and after optimum value a decreased in enzyme activity was seen. The elution with maximum enzyme activity was used for protein estimation and characterization of enzyme.

The use of gel filtration chromatography and ammonium sulphate purification for the purification of enzymes reported by (Noronha *et al*., 2009; Jabbar *et al*., 2008; Piston *et al*., 1997).

Estimation of protein by Bradford assay. Bradford assay was performed for protein estimation after partial purification by ammonium sulphate and purification by gel filtration chromatography. The amount of protein was decreased after each purification step because nonspecific proteins were removed during purification. Endoglucanase activity was increased after each purification step compared to crude enzyme which confirm the increase in endoglucanase concentration.

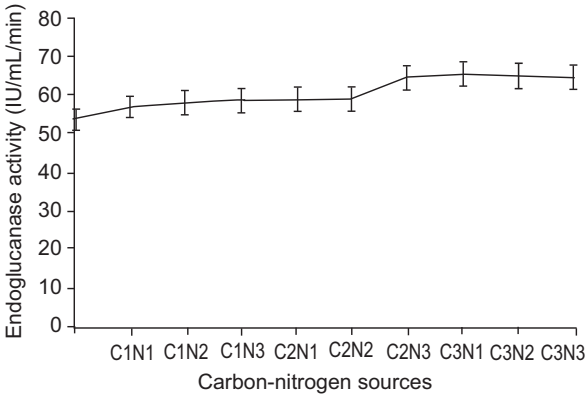

Fig. 7. Activity of endoglucanase produced by *A. flavus* at carbon-nitrogen sources and positive control is without carbon nitrogen sources.

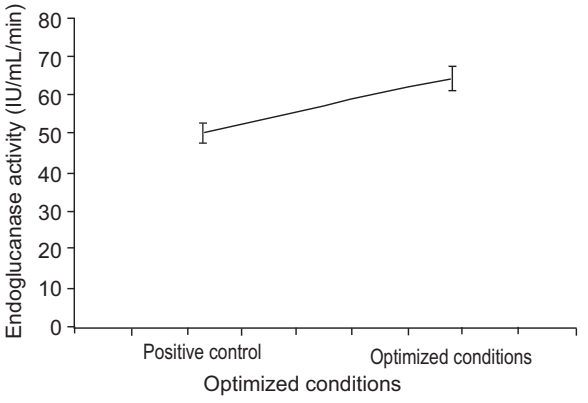

Fig. 8. Activity of endoglucanase produced by *A. flavus* at all optimized conditions, positive was control without optimization.

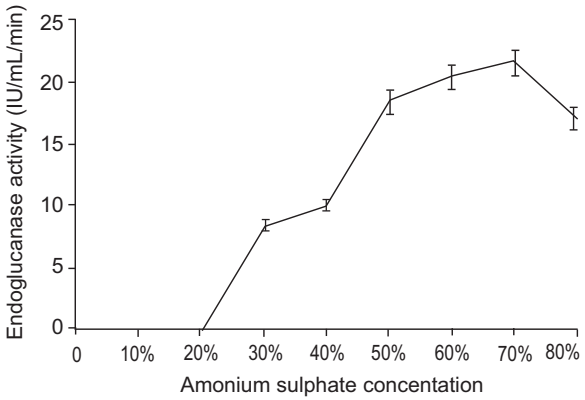

Fig. 9. Activity of endoglucanase with varying concentrations of ammonium sulphate.

Bovine albumin serum was used as standard in Bradford assay shown in Fig. 11. A graph was plotted between concentration of protein and absorbance at 595 nm and regression equation was used for the determination of protein concentration in the sample after ammonium sulphate purification and gel filtration chromatography.

Protein concentration increased upto 1.50 folds after ammonium sulphate purification and 3.3 folds gel filtration chromatography in Table 1 reported by Jabbar *et al*. (2008). Enzyme was subjected to characterization after gel filtration chromatography (Ahmed *et al*., 2009). 2.53 folds increase in concentration of cellulases after purification by ammonium sulphate and gel filtration was also reported by Irfan *et al*. (2011).

Characteriation of endoglucanase. ***Effect of temperature on endoglucanase.*** The effect of temperature on the activity of the endoglucanase is shown in Fig. 12. Endoglucanase remain functional from 30 °C to 40 °C after that temperature the activity of endoglucanase drop due to denaturation. Maximum enzyme activity (74.92 IU/mL/min) was observed at 40 °C which is its optimum temperature.

Devanathan *et al*. (2007) and Kathiresan and Manivannan (2006) confirmed the optimum temperature of endoglucanase as 37 °C for *Aspergillus* sp.

Effect of pH on endoglucanase. The effect of pH on endoglucanase activity is shown in Fig.13. Endoglucanase perform good activity from pH 3-5 after that the activity of endoglucanase began to drop. The maximum activity (65.26 IU/mL/min) was observed at pH 3 and it is its optimum pH.

(Falkoski *et al*., 2012; Almeida *et al*., 2011) reported that the optimum pH of the endoglucanase enzyme is in the range from pH 3.0 to 6.0, which is not dependent on the cultivation system.

Effect of metal ions on endoglucanase. The activity of endoglucanase was increased by addition of 10 Mm Mn^{2+}, Cu^{+2}, Co^{+2}, Ca^{+2}, Mg^{+2} and this is well in line with already reported by Nazir *et al*. (2009). Zn^{+2} caused slight decrease in endoglucanase activity which was shown in the studies of Wang *et al*. (2009). Furthermore, endoglucanase activity was inhabited by Hg^{+2} and Fe^{3+} ion. A similar inhibitory effect of these metals was also

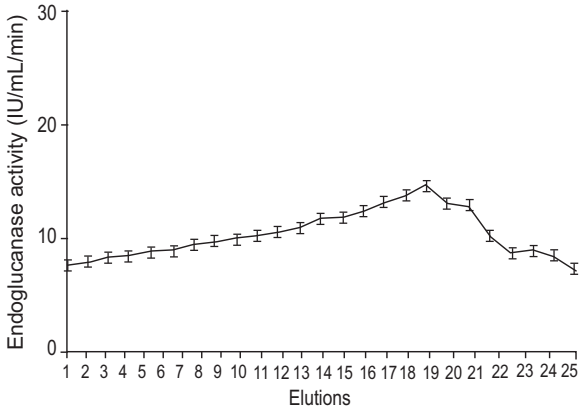

Fig. 10. Endoglucanase activity with different elutions after gel filtration chromatography.

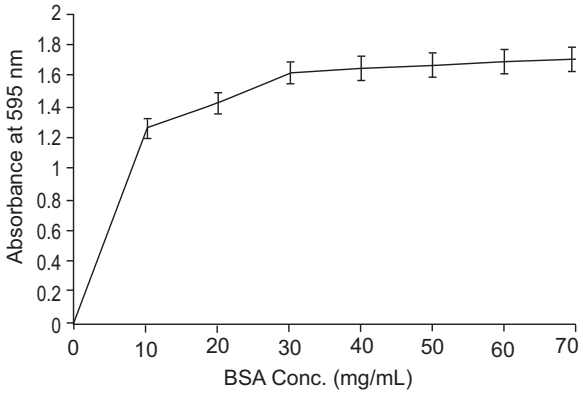

Fig. 11. Bovine serum albumin standard curve.

Table 1. Purification table for endoglucanase by *Aspergillus flavus*

Samples	Volume (mL)	Activity (IU/mL/min)	Protein (mg/mL)	Total activity	Total protein	Specific activity (IU/mg)	Purification fold
Crude enzyme	50	61.3	3.47	3,065	173.5	17.6	1
Ammonium sulphate purified	5	64.4	2.43	322.0	12.15	26.50	1.5
Gel filtration chromatography	2	70.1	1.19	140.2	2.38	58.9	3.3

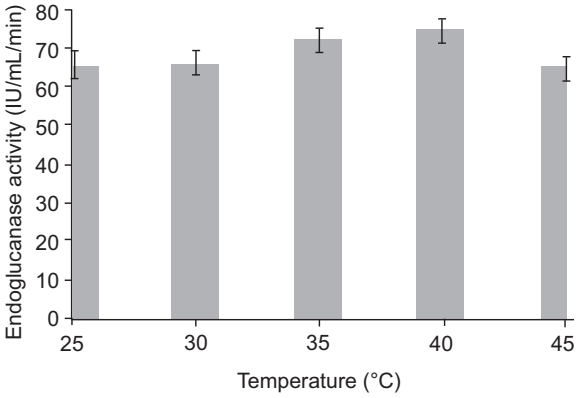

Fig. 12. Effect of varying temperature on endoglucanase activity.

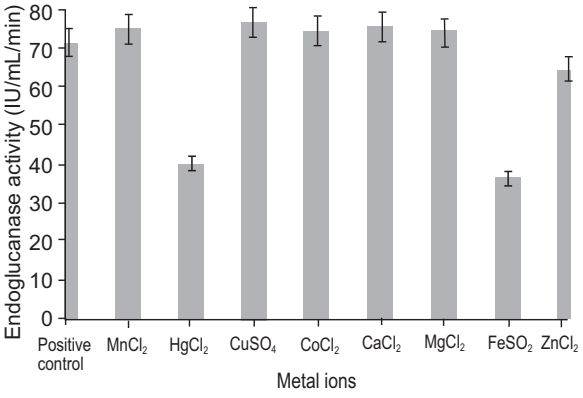

Fig. 14. Effect of metal ions on endoglucanase activity, positive control is without metal ions.

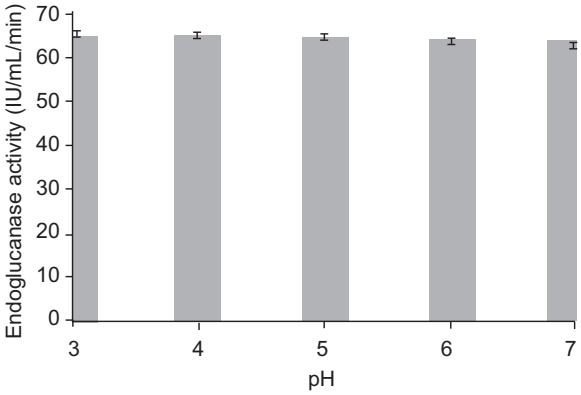

Fig. 13. Effect of pH on endoglucanase activity.

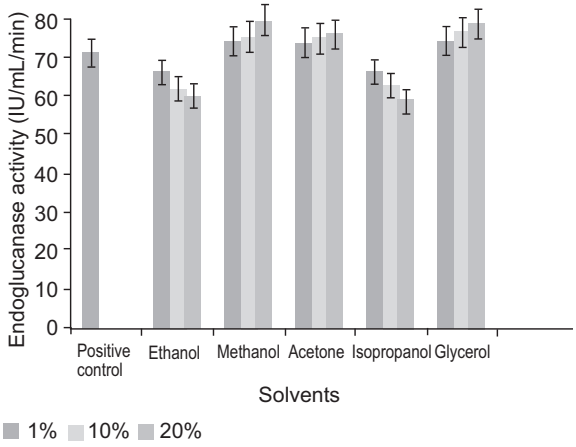

Fig. 15. Effect of different solvents on endoglucanase activity, positive was control is without solvents.

shown by Tejirian *et al*. (2010). It was published that endoglucanase activity was inhibited by Hg^{+2} due to binding of mercury ions with the thiol group, tryptophan residues and carboxyl group of the amino acid remains in the cellulase enzyme (Lusteri *et al*., 1992). Effect of metal ions on enzyme activity is shown in Fig. 14.

Effect of solvents on endoglucanase. The effect of various solvents on endoglucanase activity is shown in Fig. 15. Endoglucanase activity was increased by the addition of solvents e.g. methanol, acetone and glycerol. Further, enzyme activity was increased with increasing concentration of these solvents (1%, 10% and 20%). Similar results were also reported by Zaks and Klibanov (1998). The addition of ethanol and isopropanol during assay slightly decreases the endoglucanase activity. Reduction of enzyme activity in presence of ethanol and isopropanol was also observed in the studies of (Annamalai *et al*., 2013).

Effect of inhibitors on endoglucanase. A decrease was observed in endoglucanase activity by addition of EDTA (ethylene diamine tetra acetic acid) during assay (Fig. 16) possibly due to instability of enzyme. The decrease in endoglucanase activity in the presence of EDTA is in line with (Yin *et al*., 2010; Wang *et al*., 2009).

Effect of non-ionic surfactants on endoglucanase. Endoglucanase showed a slight increase in the activity with low concentration (0.25%) of surfactants and reduced rapidly in the higher concentration (1%) of non-ionic surfactant *viz*., Tween-20, Tween-80 and Triton x 100. The endoglucanase activity was also

increase with SDS at 0.25% and 0.5% and its activity was slightly decreased at 1%. Effect of non-ionic surfactant on endoglucanase activity is shown in Fig. 17. The same results were also reported by Rajeeva Gaur and Soni Tiwari (2015).

Effect of substrate on endoglucanase. To determine the effect of substrate concentration on endoglucanase activity and to determine the values of K_m and V_{max}, solutions of substrate having different concentration were prepared. Velocity of enzyme increased with the increasing concentration of substrate but its rate become constant after certain concentration. It happens because binding sites of the enzyme become saturated. To determine V_{max} and K_{max}, Line-Weaver Burk plot was drawn between 1/[S] on X-axis and 1/ [V] on Y-axis. V_{max} was calculated as 65 µM/mL/min and K_m was 4.5 mM (Fig. 18).

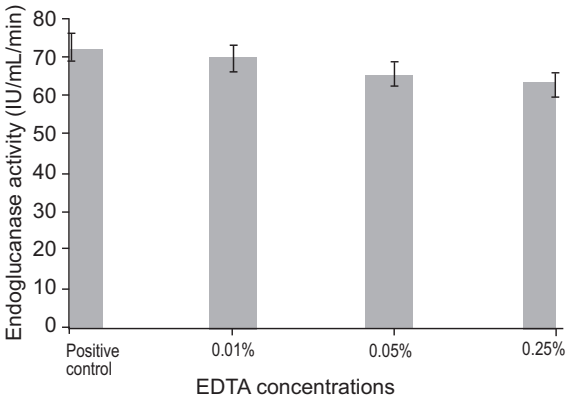

Fig. 16. Effect of different concentrations of EDTA (inhibitor) on endoglucanase activity, positive was control is without EDTA.

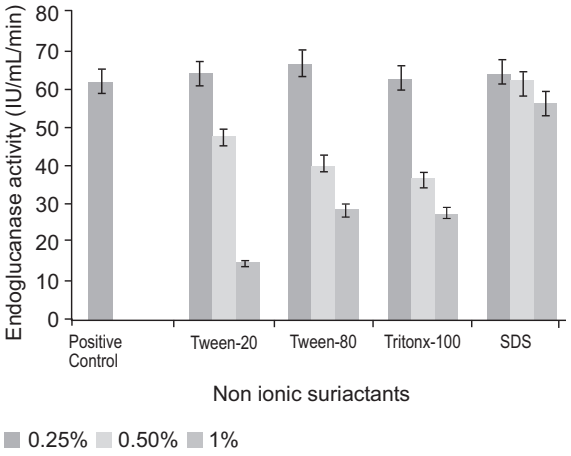

Fig. 17. Effect of non-ionic surfactants on endoglucanase activity, positive is control was without non-ionic surfactants.

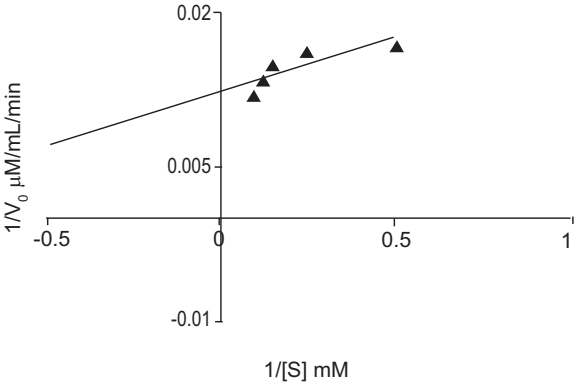

Fig. 18. Effect of substrate concentration on endoglucanase for determination of K_m and V_{max}.

Conclusions

The current study was carried to enhance production and purification of endoglucanase by *A. flavus*. Maximum enzyme production was observed after 96 h, 40 °C, pH 7, inoculum size of 4 mL and 8 g substrate level with 50% moisture contents. The 70% ammonium sulphate saturation resulted in maximum activity of enzyme, there was 1.50 fold purification observed after ammonium sulphate purification and 3.3 folds after gel filtration chromatography.

The most suitable temperature and pH for endoglucanase were 40 °C and 3 respectively. The enzyme activity was inhibited by Fe^{3+}, Hg^{2+} ions, ethanol and isopropanol. In contrast activity was stimulated by Co^{2+}, Mn^{2+} methanol, glycerol and acetone. The enzyme activity was also stimulated by SDS, while Tween-20 decreased its activity. Endoglucanase activity was rescued with higher concentration EDTA inhibitor. The values of kinetic parameters i.e. V_{max} and K_m calculated by Line-Weaver Burk plot and were 65 µM/mL/min and 4.5 mM respectively. Further, the endoglucanase molecular weight can be determined by SDS-PAGE. Characterization of endoglucanase gene in *A. flavus* may aid in additional enhance endoglucanase production. Targeted mutation in promoter site or some other regions of genes may further enhance the production.

Conflict of Interest. The authors declare no conflict of interest.

References

Ahmed, S., Bashir, A., Saleem, H., Saadia, M., Jamil, A. 2009. Production and purification of cellulose degrading enzymes from a filamentous fungus *Trichoderma harzianum*. *Pakistan Journal of Botany*, **41:** 1411-1419.

Almeida, M.N., Guimaraes, V.M., Bischoff, K.M. 2011. Cellulases and hemicellulases from endophytic *Acremonium* species and its application on sugarcane bagasse hydrolysis. *Applied Biochemistry and Biotechnology*, **165:** 594-610.

Alonso-Diaz, M.A., Torres-Acosta, U.J., Sandoval-Castro, C.A., Hoste, H., Aguilar-Caballer, A.J., Capetillo-Leal, C.M. 2008. Is goats' preference of forage trees affected by their tannin or fiber content when offered in cafeteria experiments? *Animal Feed Science and Technology*, **141:** 46-48.

Annamalai, N., Rajeswari M.V., Elayaraja, S., Balasubramanian, T. 2013. Thermostable, halo-alkaline cellulase from *Bacillus halodurans* CAS 1 by conversion of ligno-cellulosic wastes. *Carbohydrate Polymers*, **94:** 409-15.

Asgher, M., Ahmad, Z., Iqbal, H.M.N. 2013. Alkali and enzymatic delignification of sugarcane bagasse to expose cellulose polymers for saccharification and bio-ethanol production. *Industrial Crops* and *Products*, **44:** 488-495.

Balasubramanian, N., Toubarro, D., Teixeira, M., Simos, N. 2012. Purification and biochemical characterization of a novel thermo-stable carboxy-methyl cellulase from Azorean isolate *Bacillus mycoides* S122C. *Applied Biochemistry and Biotechnology*, **168:** 2191-204.

Chinedu, S.N., Okochi, V.I., Smith, H.A., Omidiji, O. 2005. Isolation of cellulolytic microfungi involved in wood-waste decomposition: prospects for enzymatic hydrolysis of cellulosic wastes. *International Journal of Biomedical and Health Sciences*, **1:** 20-28.

Devanathan, A., Shanmugan, T., Subramanian, B., Manivannan, S. 2007. Cellulase production by *Aspergillus niger* isolated from coastal mangrove debris. *Applied Scientific Research*, **2:** 23-27.

Fagain, C.O., Cummins, P.M., Connor, B.F.O. 2017. Gel filtration chromatography. *Protein Chromatography*, **1485:** 15-25.

Falkoski, D.L., Guimaraes, V.M., de Almeida, M.N., Alfenas, A.C., Colodette, J.L., de Rezende, S.T. 2012. Characterization of cellulolytic extract from *Pycnoporus sanguineus* PF-2 and its application in biomass saccharification. *Biotechnology and Applied Biochemistry*, **166:** 1586-1603.

Goyal, M., Soni, G. 2011. Production and characteriztion of cellolytic enzymes by *Pleurotus florida*. *African Journal of Microbiology Research*, **5:** 1131-1136.

Harshvardhan, K., Mishra, A., Jha, B. 2013. Purification and characterization of cellulase from a marine *Bacillus* sp. H1666: a potential agent for single step saccharification of seaweed biomass. *Journal* of *Molecular Catalysis B*: *Enzymatic*, **93:** 51-6.

Irfan, M., Gulsher, M., Abbas, S., Syed, Q., Nadeem, M., Baig, S. 2011. Effect of various pre-treatment conditions on enzymatic saccharification. *Songklanakarin Journal of Science and Technology*, **33:** 397-404.

Jabbar, A., Rashid, M.H., Javed, M.R., Perveen, R., Malana, M.A. 2008. Kinetics and thermodynameics of a novel endoglucanase (CMCase) form *Gymnoascella citrine* produced under solid state condition. *Journal of Industrial Microbiology and Biotechnology*, **33:** 515-524.

Kathiresan, K., Manivannan, S. 2006. Cellulase production by *Penicillium fellutanum* isolated from coastal mangrove rhizosphere soil. *Research Journal of Microbiology*, **1:** 438-442.

Krishnaiah, D., Sarbatly, R., Bono, A. 2007. Phytochemical antioxidants for health and medicine a move towards nature. *Biotechnology and Molecular Biology Reviews*, **1:** 097-104.

Lusteri, D.D., Suizo, F.G., Labunos, N.M., Valledor, M.N., Ueda, S., Kawai, S. 1992. Alkali-resistant, alkaline endo-1,4-β-glucanase produced by *Bacillus* sp. *Bioscience*, *Biotechnology and Biochemistry*, **56:** 1671-2.

Nazir, A., Soni, R., Saini, H.S., Manhas, R.K., Chadha, B.S. 2009. Purification and characterization of an endoglucanase from *Aspergillus terreus* highly active against barley β-glucan and xyloglucan. *Journal of Microbiology and Biotechnology*, **25:** 1189-1197.

Noronha, E., Kipnis, F., Kipinis, A.P.J., Ulhoa, C.J. 2009. Regulaltion of 36 kDa 1-3 gulcanase synthesis in *Trichoderma harzianum*. *FEMS Microbiology Letters*, **188:** 19-22.

Park, S., Lim, C.Y., Kim, D.S., Ko, K. 2015. Optimization of ammonium sulfate concentration for purification of colorectal cancer vaccine

candidate recombinant protein GA733-FcK isolated from plants. *Frontiers in Plant Science*, **6,** 1040.

Perez, J., Munoz-Dorado, J., de la Rubia, T., Martinez, J. 2002. Biodegradation and biological treatments of cellulose, hemicellulose and lignin an overview. *International Microbiology*, **5:** 53-63.

Pitson, S.M., Seviour, R.J., Modougall, M.B. 1997. Purification and characterization of an extracellular β-glucosidase form the filamentous fungus *Acremonium persicinum* and its probable role in β-glucan degradation. *Enzyme and Microbial Technology*, **21:** 182-190.

Gaur, R., Tiwari, S. 2015. Isolation, production, purification and characterization of an organic-solvent-thermostable alkalophilic cellulase from Bacillus vallismortis RG-07. *BMC Biotechnology*, **15:** 1-12.

Ramirez, C.C., Hernandez, A.S., Orduna, F.N.R., Huante, Y.G., Zuniga, G., Lara, M.E.H. 2016. Expression, purification and characterization of an endoglucanase from *Serratia proteamaculans* CDBB-1961, isolated from the gut of *Dendroctonus adjunctus* (Coleoptera: Scolytinae). *AMB Express*, **6,** 13 pp.

Sherief, A.A., El-Tanash, B.A., Atia, N. 2010. Cellulase production by *Aspergillus fumigates* on mixed substrate of rice straw and wheat bran. *Research Journal of Microbiology*, **5:** 199-211.

Szijarto, N., Szengyel, Z., Liden, G., Reczey, K. 2004. Dynamics of cellulase production by glucose grown cultures of *Trichoderma reesei* Rut-C30 as a response to addition of cellulose. *Applied Biochemistry and Biotechnology*, **113-116:** 115-24.

Tejirian, A., Xu, F. 2010. Inhibition of cellulase catalyzed lignocellulosic hydrolysis by iron and oxidative metal ions and complexes. *Applied and Environmental Microbiology*, **76:** 7673-7682.

Wang, X., Yu, X., Xu, Y. 2009. Homologous expression, purification and characterization of a novel high-alkaline and thermal stable lipase from *Burkholderia cepacia* ATCC 25416. *Enzyme and Microbial Technology*, **45:** 94-102.

Yin, L.J., Lin, H.H., Xiao, Z.R. 2010. Purification and characterization of a cellulase from *Bacillus subtilis* YJ1. *Journal of Marine Science and Technology*, **18:** 466-471.

Zaks, A., Klibanov, A.M. 1998. Enzymatic catalysis in nonaqueous solvents. *Journal of Biological Chemistry*, **263:** 3194-201.

Zhang, Y.H.P., Michael, E., Himmel, E.M., Mielenz, R.J. 2006. Outlook for cellulose improvement: Screening and selection strategies. *Biotechnology Advances*, **24:** 452-481.