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Production, Purification and Characterization of Endoglucanase from *Aspergillus fumigatus* and Enzymatic Hydrolysis of Lignocellulosic Waste

Zabin K. Bagewadi[1] and Harichandra Z. Ninnekar[2*]

[1,2]Department of Biochemistry, Karnatak University, Dharwad 580 003, Karnataka, India
E-mail: [1]zabinb@gmail.com, [2]hzninnekar@yahoo.com

Abstract—*The present study was focused towards the production of multiple enzymes like endoglucanase, exoglucanase, β-glucosidase , xylanase and FPase comprising the cellulase system from Aspergillus. The strain was induced for the production of cellulase system by solid state fermentation using Sweet Sorghum bagasse and Corn cobs as lignocellulosic substrates. Endoglucanase produced from Sweet Sorghum bagasse was further purified to homogeneity by chromatography on Sephadex G-50 and DEAE-Cellulose with a purification fold of 12. Endoglucanase was characterized and found to produce maximum activity at pH 6 and 55° C. Endoglucanase was stable for 1hour at pH 6 retaining 86% of residual activity and at 55° C retaining 77% activity after 3 hours. Endoglucanase was assessed for stability in the presence of various metal ions like Hg^{+}, K^{+}, Zn^{+}, Ni^{+}, Ca^{2+}, Pb^{2+},Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Na^{+} etc. Enzyme stability in various detergents, organic solvent and additives like SDS, Triton X 100, methanol, ethanol, propanol, acetone, glycerol, EDTA, β-mercaptoethanol, DTT, PMSF, PCMB etc were evaluated. Kinetic parameters suggest high affinity of Endoglucanase towards Sodium Carboxyl Methyl Cellulose (CMC) with K_m of 1.11 mg/ml and V_{max} of 50 U/ml. Endoglucanase is relatively a low molecular weight protein with ~ 20Kd. The purified enzyme was used for enzymatic hydrolysis of lignocellulosic residues like Saw dust after biological pre-treatment with Pleurotus ostretus (a white rot fungi releasing 42 g/100g of reducing sugars. The outcomes reveal the potential application of cellulase system for saccharification of lignocellulosic biomass for bioethanol production.*

1. INTRODUCTION

Lignocellulose is one of the most abundant resources available in nature. Cellulases are capable of hydrolyzing β-1,4-glycosidic bonds in cellulose and have been broadly divided into three classes: endoglucanase, cellobiohydrolase or exoglucanase and β-glucosidase which have been shown to act synergistically to hydrolyze cellulose to glucose unit. Xylanolytic enzyme has the capability of hydrolyze the xylosidic linkages in xylan, which is the major component of hemicelluloses [1]. Solid State Fermentation a better alternative over submerged fermentation for large scale production of fungal metabolites as it resembles the natural environment for fungi to grow and produce the fermented metabolites [2]. Enzyme complexes for the hydrolysis of lignocelluloses have been produced by solid state fermentation (SSF) employing various agricultural residues such as rice straw, wheat bran, corn Stover, sugarcane bagasse, pomace, corncobs, etc. [3]. Sweet sorghum (Sorghum bicolor L. Moench) is a renewable, cheap, widely available substrate. Its high biomass content make it a potential source for ethanol production. It contains soluble (glucose and sucrose) and insoluble carbohydrates (cellulose and hemicellulose) [4].The ability of a particular microorganism to grow on lignocellulosic substrates is related to the production of a broad spectrum of enzymes. Aspergillus and Trichoderma species are well known for efficient production of cellulases. Keeping in view the industrial importance of cellulases, the present study was undertaken to produce cellulase system and xylanase enzyme form sweet sorghum bagasse and corn cobs, further the endoglucanase produced from sweet sorghum bagasse under SSF which purified by chromatographic techniques and characterized. The purified endoglucanase was employed for demonstration of enzymatic saccharification of lignocellulosic substrates.

2. MATERIALS AND METHODS

2.1 Microorganisms, substrates and chemicals

Aspergillus fumigatus strain NCIM 902 was procured from National Collection of Industrial Microorganism (NCIM), Pune. *Aspergillus fumigatus* was cultivated on Potato Dextrose Agar (PDA) and incubated at 30°C for 6-7days until complete sporulation. The spore suspension was used as innoculum. The culture was maintained on PDA slants and frequently sub cultured. *Pleurotus ostretus* MTCC 1804 strain was procured from Microbial Type Culture Collection (MTCC),

Chandigarh. Sweet Sorghum stalks were provided by University of Agricultural sciences (UAS), Dharwad. Corn Cobs were procured from Local market at Hubli. Saw dust was collected from wood cutting mill at Hubli. The substrates were thoroughly washed and dried in oven at 40°C. Substrates were milled to obtained fine powder and sieved with a mesh size of 4mm and stored in a dry air tight container until further use. Sodium salt CMC was purchased from sigma chemicals (Mumbai, India), pNPG (Sigma), Birch Wood Xylan (Himedia), SephadexG-50 (Sigma-Aldrich), DEAE-Cellulose (Himedia) and other analytical grade chemicals were purchased from Merck, SD Fine chemicals and Himedia.

2.2 Screening for Cellulase and xylanase production

Aspergillus fumigatus was screened for cellulase and xylanase production. It was screened on selective carboxymethyl cellulose (CMC) agar containing 2.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 10.0 g carboxymethyl cellulose sodium salt 10.0 g or Birch wood Xylan 5.0 g , 0.2 g peptone and 17.0 g agar in 1L distilled water (pH 5.5-6.0). Plates were spot inoculated with spore suspension of *Aspergillus fumigatus* and incubated at 30ºC. After 3 days, the plates were flooded with 1% Congo red solution for 15 minutes and then destained with 1M NaCl solution. The diameter of zone of decolorization around the colony was measured. Cellulolytic index (CI) was determined and expressed by the ratio of the diameter of the degradation halo and the diameter of the colony [5].

2.3 Pretreatment of Substrates

Pretreatment decreases the crystallinity of Sweet-

Sorghum bagasse and Corn cobs thereby removing the lignin and other inhibitors enabling the enzymatic hydrolysis. 50 g of the Sweet Sorghum bagasse and Corn cobs were treated separately with 1L of 4% NaOH and autoclaved at 121°C for 30 minutes. It was then filtered, washed with distilled water and excess alkali present was neutralized with 1N phosphoric acid. Again it was filtered and the residual material was dried at 65°C in a hot air oven to a constant weight. To the cellulosic material obtained, the same volume of distilled water was added and heated at 121°C for 30 minutes. The suspension was filtered and the solid material was dried at 65°C to constant weight in hot air oven. The pretreated lignocellulosic substrates were used as the sole source of carbon to produce cellulase system by *Aspergillus fumigatus* [6].

2.4 Solid State Fermentation for Production of Cellulase system

The cellulase system production was carried out in 250 ml Erlenmeyer flasks containing 10 g of dried substrates (Sorghum bagasse and Corn cobs) moistened with 20 ml of Mandels-Weber medium containing the following (g/L): urea, 0.3; ammonium sulfate, 1.4; KH_2PO_4, 0.3; $CaCl_2$, 0.3; $MgSO_4.7H_2O$, 0.3; proteose peptone, 1.0 and (mg/L) $MnSO_4.7H_2O$, 1.6; $ZnSO_4.7H_2O$, 1.4; $CoCl_2$, 2; Tween 80, 0.1%; and initial pH 5.3. The medium and the substrate were sterilized at 121°C for 15 min at 15 lbs. The inoculum was prepared with 6 days old *Aspergillus fumigates* strain. The spore suspension was prepared by adding 5 ml of 1% Tween 80 to the culture slant. A spore suspension approx. 10^8 spore/ml was inoculated into the flasks and incubated at 30°C under static condition. The contents of the flasks were mixed intermittently (twice a day), and the crude enzyme was extracted from each flask at regular interval of time (at every 24 h) for a period of 10 days.

2.5 Harvesting of Cellulase System

The enzyme was extracted by a simple contact method [7]. After incubation the fermented samples were left for shaking on a rotary shaker at 150 rpm for 1 hour with 0.05 M citrate buffer (pH 5) by applying substrate:buffer (S:L 1:10) concentration for 1 h and filtered through Whatman No. 1 filter paper. The filtrates were centrifuged for 20 min at 10,000 rpm and 4°C to remove spores and the supernatant of crude enzyme extract was used for enzyme assay. The supernatants were stored at 4˚C until further use

2.6 Analytical Measurements

2.6.1 Enzyme assays

Multiple enzymes like Total cellulase (FPA), endoglucanase, and exoglucanase activities were determined using Whatman no. 1 filter paper, Carboxymethyl cellulose (CMC, low viscosity), and microcrystalline cellulose as substrates, respectively, according to standard conditions described by Ghose [8]. Reducing sugars produced were expressed as glucose liberated during reactions and were estimated by the DNS method [9]. Xylanase activity was determined using xylan from birch wood as substrate according to methods described by Bailey [10]. For all enzymes one enzyme unit (U) was defined as the amount of enzyme that liberates 1 µmol of the product (glucose for all cellulases and xylose for xylanase) per minute under the assay conditions (30 min incubation time at 50°C with 50 mM citrate buffer pH 5.0). β-Glucosidase activity was assayed in a 100 µL reaction mixture containing 2mM ρ-nitophenyl-β-D-glucopyranoside (pNPG), 50 mM citrate buffer (pH 5.0), and an appropriate dilution of enzyme preparation. After 10 min incubation at 50°C, the reaction was stopped by adding 200 µL of 1M Na_2CO_3, and ρ-nitrophenol (ρNP) release was monitored at A_{410}nm. Enzyme unit was defined as the amount of enzyme that releases 1 µmol ρ-nitrophenol per minute under the standard assay conditions. All analyses were done in triplicate.

2.6.2 Determination of Protein content

The concentration of soluble protein was determined by using bovine serum albumin as the standard by standard procedures described by Lowry [11].

2.6.3 Biomass Determination

Mycelial dry biomass was collected on a pre-weighed Whatmann filter paper no. 5, dried to a constant weight at 60°C and reweighed. The difference in weight denoted the mycelial growth of fungus [12].

2.7 Purification of Endoglucanase

All purification steps were performed at 4˚C. Crude extract of endoglucanse obtained from Solid state fermentation process using Sweet sorghum bagasse substrate from *Aspergillus fumigates* was centrifuged (10,000g) for 20 min. The cell debris was discarded and the cell free supernatant was collected and used as a source of crude enzyme for further purification. An initial step in protein purification, which attempts to precipitate the proteins in the solution, was ammonium sulphate precipitation with 70% saturation as described by [13]. Total proteins and activity of partially purified endoglucanse were determined before and after dialysis with 0.05 M phosphate buffer pH 6 for 24 hours of ammonium sulfate precipitation. The desalted ammonium sulfate fraction was lyophilized for further purification by chromatography. The lyophilized enzyme was redissolved in 10 ml of phosphate buffer (pH 6.0) and loaded on to a DEAE-cellulose chromatographic column (2.5×40 cm) that was equilibrated and eluted with 50 mM phosphate buffer containing 0.5 M NaCl, at a flow rate of 0.5ml/min. The endoglucanase fractions were pooled, concentrated, dialyzed against the same buffer once again to remove Na^+ and Cl^-, lyophilized, redissolved in 5 ml of the same buffer and loaded into a SephadexG-50 column (2.5×40 cm) that was equilibrated and eluted with 50 mM phosphate buffer (pH 6.0). Fractions of 1ml were collected at a flow rate of 0.5ml/min. The fractions were analyzed for protein by A_{280} method and endoglucanase activities were determined. The resulting concentrated active endoglucanse fractions were pooled and used as the purified endoglucanse enzyme [14, 15].

2.8 Characterization of Purified Endoglucanase

2.8.1 Effect of pH and pH stability

Optimum pH for the purified enzyme was determined using 0.1 M buffers of different pH values such as citrate (pH 3.0–5.0), sodium phosphate (pH 6.0-7.0), Tris-HCl (pH 8.0–9.0), and glycine-NaOH (pH 10.0) and its pH stability was determined by incubating the enzyme in the pH range 4–7 for 3 hours and the residual activity was calculated.

2.8.2 Effect of temperature and temperature stability

The optimum temperature for endoglucanase activity was determined by measuring the activity at various temperatures (25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80 and 85 °C) at optimum pH and its thermal stability was determined by incubating the enzyme at different temperature range of 50-65°C for 3h and the residual activity was calculated.

2.8.3 Effect of Metal ions, Additives and Solvents

The purified endoglucanase was incubated with 10 mM of different salt solutions (Mn^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Fe^{2+}, K^+, Na^+, Hg^{+2}, Co^{+2}, Cd^{2+}, Pb^{+2}, Ca^{2+} and Ni^+), Effect of Different additives (Triton X-100, sodium dodecyl sulphate, Tween 20, Tween 80, phenyl methyl sulphonyl fluoride (PMSF), dithiotheitol (DTT), p-chloromercuribenzoate (PCMB), EDTA and Dimethyl sulpfoxide (DMSO) at a concentration of 10 and 50 mM were analyzed. Effect of various organic solvents (Glycerol, ethanol, acetone, methanol, petroleum ether, 1-propanol, benzene, toluene, n-hexane, decanol, and ethyl acetate) at 50% (v/v) for 1 h were determined and the residual activity of the purified endoglucanase with such chemical additives was determined and compared with the control (without inhibitors as 100%).

2.8.4 Determination of Substrate specificity

The purified endoglucanase was evaluated for substrate specificity toward 1% CMC, microcrystalline cellulose, Filter paper, Soluble starch, birchwood xylan and xylan oat splet. The relative activity was calculated.

2.8.5 Determination of Enzyme Kinetics

For the determination of endoglucanase kinetic parameters, solutions containing 2.5-25mg/ml of CMC was prepared and the reaction was carried out at 55 ◦ C, pH 6 for 30 minutes. Enzyme saturation was observed by Michaelis Menten model and Kinetic Parameters K_m and V_{max} were estimated using linear transformations of Lineweaver Burk plots.

2.8.6 Molecular Mass Estimation

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was performed on 5% stacking gel and a 15% separating gel, according to the method described by Laemmli [16] to determine the molecular weight of Endoglucanase. Separated Protein bands were visualized by staining with Coomassie Brilliant Blue R-250. The approximate molecular weight of the purified endoglucanase was determined from the medium range protein molecular weight markers.

2.9 Enzymatic saccharification of Saw dust

The enzymatic saccharification of saw dust was carried out. Alkali pretreated and combination of alkali pretreatment and biological pretreatment with *Pleurotus ostretus* strain for 8 days and untreated Saw dust were used as substrates for saccharification by purified endoglucanase from *Aspergillus fumigates*. The enzymatic hydrolysis was performed in 150-ml screw cap Erlenmeyer flasks containing 2.5% different substrates, as mentioned earlier, in 50 mM sodium phosphate buffer (pH 6) containing 0.1% Tween 80. Controls were kept for each reaction in which the active enzyme was replaced with heat-inactivated enzyme. The reaction system was fortified with 10 mg % of sodium azide to prevent microbial

contamination. The reaction was carried out at 50°C in water bath. The samples were withdrawn every 6 h for a period of 72 hours and incubated in boiling water bath (100°C) to inactivate the enzyme; the reaction mixture was then filtered through the muslin cloth and centrifuged at 10,000g to collect the clear supernatant. This supernatant was analyzed for reducing sugars by DNS method [17].

3. RESULTS AND DISCUSSIONS

3.1 Production of cellulase system by Solid state fermentation

Aspergillus fumigatus showed a significant CMC hydrolysis halo when subjected to screening for cellulolytic activity on Congo red plate assay with CI of 8.2 and also produced distinct zones in xylan supplemented plates indicating good xylanase production. Comparatively *Aspergillus fumigates* produced better Cellulase system and xylanase from Sweet sorghum (Fig. 1) than Corn cobs (Fig. 2) by solid state fermentation.

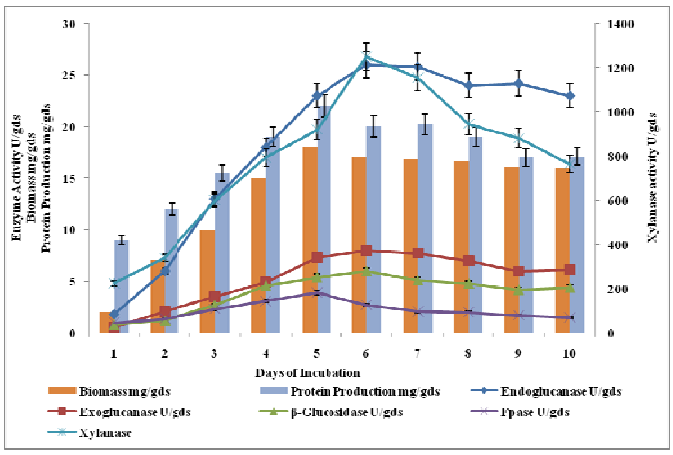

Fig. 1: Production Profile of cellulase system, xylanase, Biomass and Protein by *Aspergillus fumigates* from Sweet sorghum bagasse under solid state fermentation.

Maximum enzyme production was found between 5-6th day of incubation which is evidenced in both the substrates. The protein production profile also depicts the multi enzyme production capability of the strain. Multiple enzyme production has been also reported in *P. betulinus* [18]. Growth profiling illustrates the production of primary metabolites.

Maximum Endoglucanase, Exoglucanase, β-glucosidase, FPase and xylanase enzyme activity from sweet sorghum were found to be 26 U/gds, 8 U/gds, 6 U/gds, 3.9 U/gds and 1250 U/gds respectively and from corn cobs were 24 U/gds, 9.2 U/gds, 4.8 U/gds, 2.4 U/gds and 1105 U/gds respectively. Both the substrates proved to be potential feedstocks for the production multi enzyme system. Even though the strain had a huge potential for xylanase production the focus was to purify endoglucanase component of the multi enzyme system.

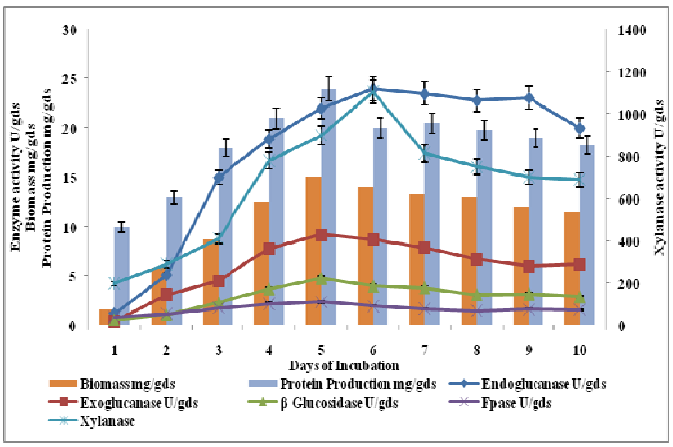

Fig. 2: Production Profile of cellulase system, xylanase, Biomass and Protein by *Aspergillus fumigates* from Corn cobs under solid state fermentation.

3.2 Purification strategy of Endoglucanase

The purification of endoglucanase involved the crude enzyme (12 U/ml) to be initially precipitated by Ammonium sulphate 70 % saturation (38 U/ml) followed by dialysis.

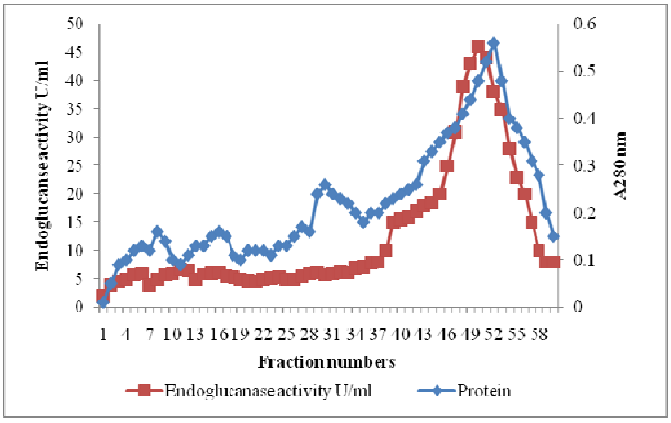

Fig. 3: Exchange chromatography Elution Profile of endoglucanase on DEAE-Cellulose matrix.

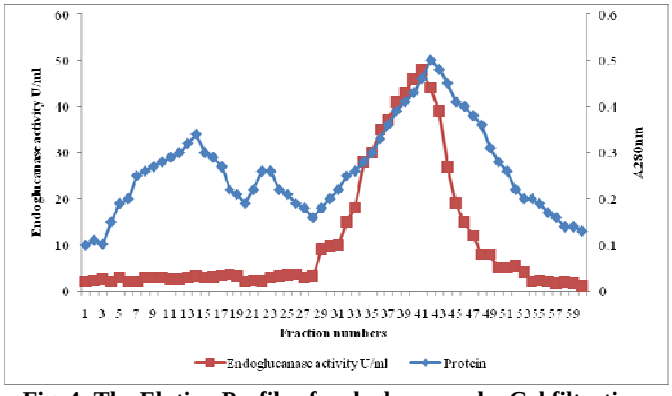

Fig. 4: The Elution Profile of endoglucanase by Gel filtration chromatography using SephadexG-50 matrix.

The partially purified endoglucanase was subjected to ion exchange chromatography with DEAE-cellulose matrix. The elution profile (Fig. 3) indicates one pool of endoglucanase activity depicting the absence of isozymes. The active fractions were further subjected to gel filtration chromatography employing SephadexG-50 matrix. The elution profile (Fig. 4) indicates a good pool of low molecular mass proteins.

Table 1: Purification summary of endoglucanase from sweet sorghum bagasse produced by *Aspergillus fumigates* under solid state fermentation

Steps Crude	Total Protein (mg) 180	Total activity (U) 1800	Specific activity (U/mg) 10	Yield % 100	Fold 1
(NH4)2 SO4 Fractionation	56	760	13.571	42.22	1.36
DEAE-Cellulose	5.6	322	57.5	17.88	5.75
SephadexG-50	1.2	144	120	8	12

The summary of the purification strategy is presented (Table 1) which shows the attainment of 12 purification folds with specific activity of 120 U/mg after chromatography with 8% recovery of endoglucanase. A purification fold of 15.9 with a yield of 27.7% was achieved for endocellulase from endophytic *Fusarium oxysporum* [19].

3.3 Characterization of purified Endoglucanase

3.3.1 Effect of pH and pH stability

Endoglucanase exhibited maximum activity at pH 6 (Fig. 5) retaining 86% relative activity after 3 hours.

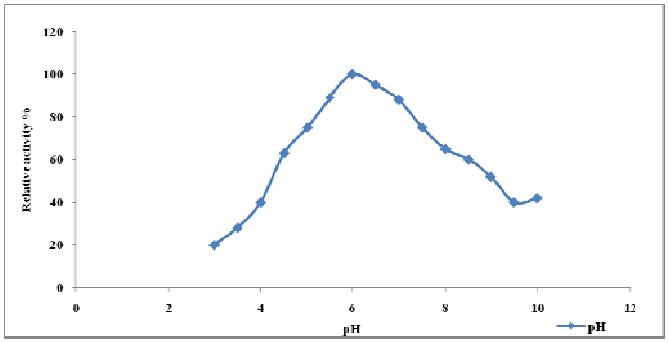

Fig. 5: Effect of pH on Endoglucanase activity from *Aspergillus fumigates*

It showed 57%, 71%, 68% of relative activity at pH 4, 5 and 7 respectively after 3 hours (Fig. 6). The results were similar to other endoglucanases from *T. viride* HG 623 which reported to be optimum at 55°C at pH 5 and stable for 1 h from 35 to 55°C between 3.0 to 7.5 pH [20].

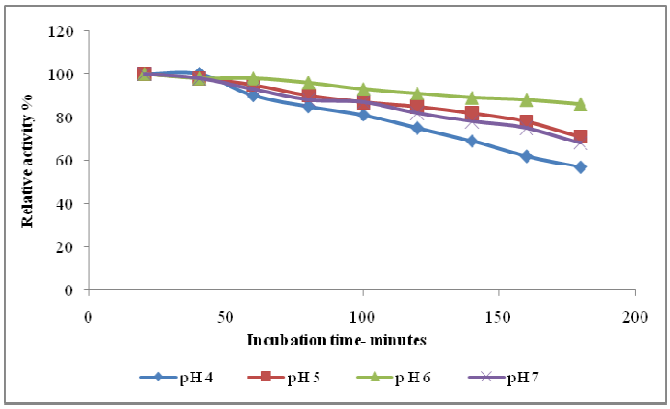

Fig. 6: Effect of pH stability on Endoglucanase activity from *Aspergillus fumigates*

3.3.2. Effect of Temperature and temperature stability

Temperature has an influence on the enzyme. 55° C was found to be optimum for endoglucanase activity from *Aspergillus fumigate* (Fig. 7).

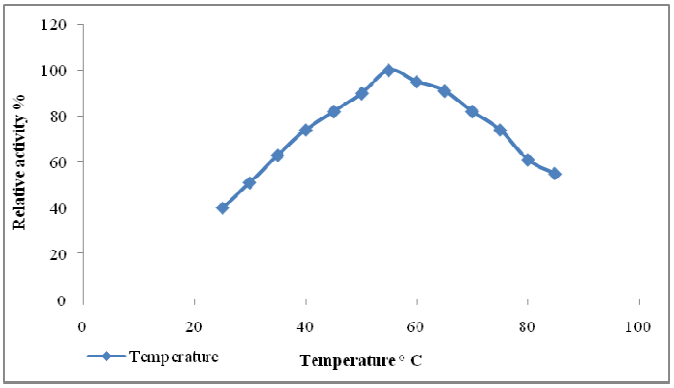

Fig. 7: Effect of temperature on Endoglucanase activity from *Aspergillus fumigates*

77% of the activity was retained after incubating the enzyme at 55° C for 3 hours. 68%, 71% and 66% of residual activity was recorded at 50° C, 60° C and 65° C respectively (Fig. 8). These findings indicate the thermostable nature of enzyme. [21] found that cellulase activity was dropped by 50% at 60°C after 6 h and 20–24 % activity was lost at 55°C after incubation of 5 h.

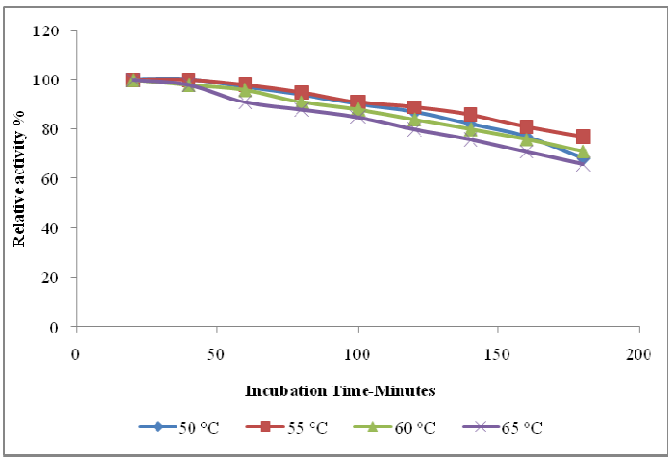

Fig. 8. Effect of temperature stability on Endoglucanase activity from *Aspergillus fumigates*

3.3.3. Effect of Metal ions, Additives and organic Solvents

Effect of various metal ions at 10mM concentration was studied (Fig. 9). Enzyme was incubated with different metal ions for 1 hour at pH 6 and 55°C.

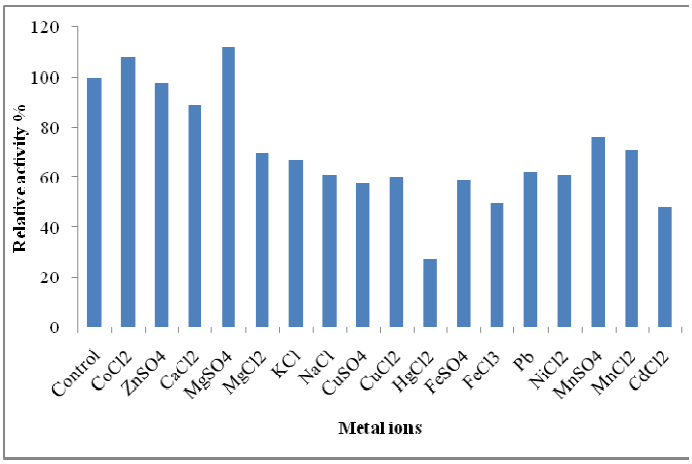

Fig. 9. Effect of Metal ions on Endoglucanase activity from *Aspergillus fumigates*

$CoCl_2$ and $MgSO_4$ acted as activators for endoglucanase. These metal ions were reported to enhance the enzyme activity in *Fusarium oxysporum* [19]. Significant activity was retained in the presence of $ZnSO_4$ and $CaCl_2$. Enzyme activity was moderately lost in the presence of $MgCl_2$, KCl, $MnSO_4$, $MnCl_2$.Enzyme was inhibited in the presence NaCl, $CuSO_4$, $CuCl_2$, $FeSO_4$, $FeCl_3$, Pb, $NiCl_2$ and $CdCl_2$. $HgCl_2$ strongly inhibited endoglucanase activity.

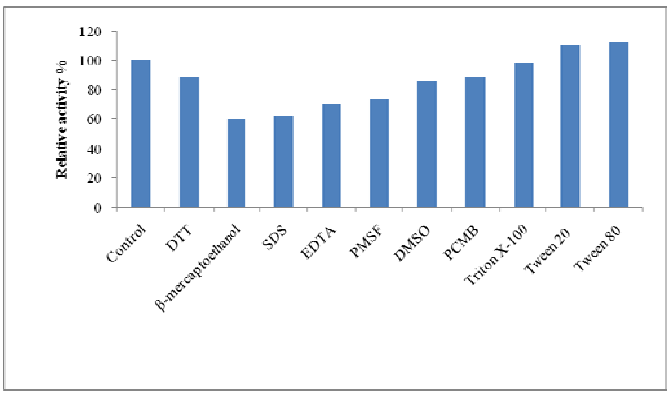

Fig. 10. Effect of Additives on Endoglucanase activity from *Aspergillus fumigates*

Surfactants enhanced the activity of enzyme. Endoglucanase showed significant stability in the presence of various additives and detergents (Fig. 10).

Glycerol and n-hexane was found to enhance the endoglucanase activity. Enzyme showed good stability in the presence of different organic solvents. Propanol and ethyl acetate were found to denature the enzyme drastically with 52% and 57% of relative activity (Fig. 11) Enzyme stability from *Streptomyces* sp. in the presence of various additives and solvents has been reported [22].

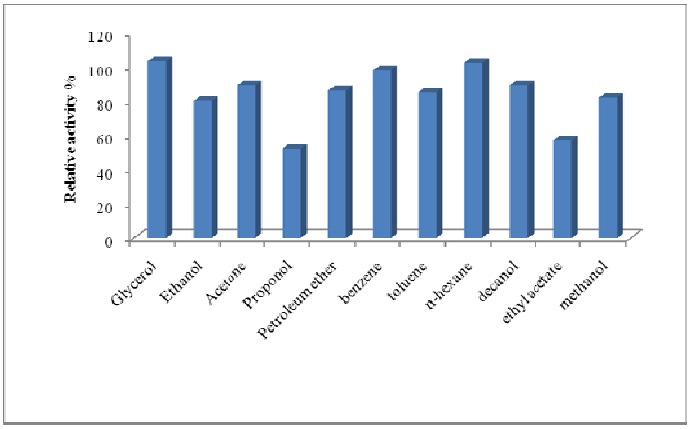

Fig. 11. Effect of organic solvents on Endoglucanase activity from *Aspergillus fumigates*

3.3.4. Determination of Substrate specificity

Endoglucanase showed 100% activity in presence of CMC (Table 2). 10%, 13%, 8% of relative activity for microcrystalline cellulose, pNPG and Filter paper substrates was seen. No activity was found for birchwood xylan, oat

splet xylan and soluble starch. This indicated the specificity of purified endoglucanase for CMC. Endoglucanse1 from *P. betulinus* exhibited the highest substrate specificity for CMC [18] which is in agreement with our findings.

Table 2. substrate specificity of purified endoglucanase from *Aspergillus fumigates*

Substrates	Relative activity %
CMC	100%
Microcrystalline Cellulose	10%
pNPG	13%
Filter paper	8%
Birchwood xylan	0%
Oat splet xylan	0%
Soluble starch	0%

3.3.5. Kinetic parameters of endoglucanase

The enzyme was analyzed for the effect of CMC concentration with 2.5-25mg/ml substrate concentration. The enzyme showed exhibited the Michaelis Menten saturation curve. From the Lineweaver Burk plots K_m and V_{max} were found to be 1.11mg/ml and 50U/ml respectively (Fig. 12). Fungal β-glucosidase K_m value ranged from 0.47 to 719 µM , while V_{max} ranged from 0.21 $\mu g \cdot min^{-1}$ to 9.63 $\mu g \cdot min^{-1}$ [23].

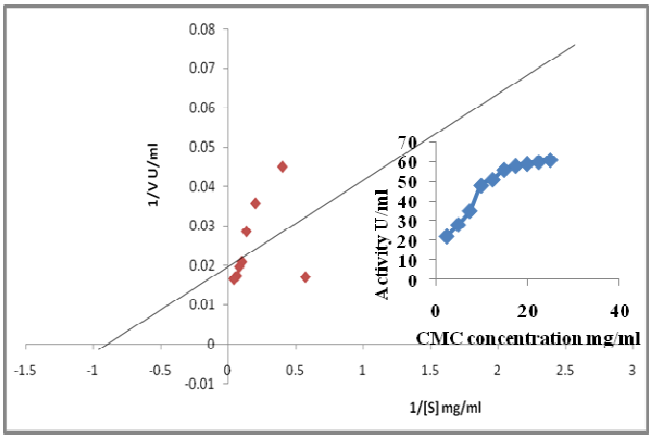

Fig. 12. Kinetics of Endoglucanase enzyme from *Aspergillus fumigates*

3.3.6. Molecular mass determination

Purified endoglucanase exhibited a molecular mass around 20kD indicating to be a low molecular mass protein (Fig. 13). Cellulolytic protein molecular mass of ~50 kDa and 20 kDa was reported from *Trichoderma harzianum* [24].

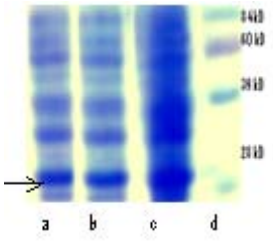

Fig. 13. SDS-PAGE with a-SephadexG-50. b-DEAE-cellulose c-Crude enzyme d-Protein ladder

1.1. Enzymatic hydrolysis of saw dust

Saw dust was pretreated with alkali and also a combinational pretreatment was employed where the alkali pretreated saw dust was further subjected to biological pretreatment with *Pleurotus ostretus* strain for 8 days.

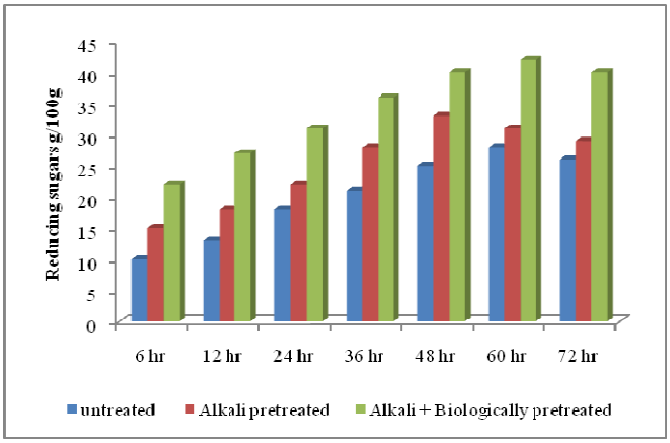

Fig. 14: Enzymatic saccharification of saw dust by purified Endoglucanase from *Aspergillus fumigates*

The alkali and combinational pretreated substrates were hydrolyzed with purified enzyme. 42 g/100g of reducing sugars were released from combinational pretreated saw dust at 60 hours of reaction and 31 g/100g of reducing sugars were released from alkali pretreated saw dust at 60 hours of reaction (Fig. 14). A combinational pretreatment and enzymatic saccharification has found to produce better reducing sugar yield. A similar observation was reported by [17].

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