STUDY ON THERMODYNAMICS AND ADSORPTION KINETICS OF PURIFIED ENDOGLUCANASE (CMCASE) FROM *PENICILLIUM NOTATUM NCIM NO-923* PRODUCED UNDER MIXED SOLID-STATE FERMENTATION OF WASTE CABBAGE AND BAGASSE

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ABSTRACT

In the current study, one thermostable endoglucanase was purified from *Penicillium notatum NCIM NO-923* through mixed solid state fermentation of waste cabbage and bagasse. The molecular weight of the purified enzyme was 55kDa as determined by SDS polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme had low activation energy (E_a) of 36.39KJ mol^{-1} for carboxymethyl cellulose hydrolysis and the enthalpy and entropy for irreversible inactivation was 87 kJ mol^{-1} and 59.3 J mol^{-1} K^{-1} respectively. The enzyme was quite thermostable with a T_m value of 62.2˚C. The pK_{a1} and pK_{a2} of ionizable groups of the active sites were 2.5 and 5.3 respectively. Apparent K_m, V_{max} and K_{cat} of the enzyme were found to be 5.2 mg mL^{-1}, 80 U/gds and 322.4 sec^{-1} respectively. The enzyme showed about 1.4 fold increased activity in presence of 10mM MgSO$_4$. Adsorption of endoglucanase on Avicel at wide pH range was studied at different temperatures. Langmuir type adsorption isotherm at 10˚C showed maximum adsorption strength of enzyme at pH 3.0, which was in a range of optimum pH of the enzyme.

Key words: Endoglucanase, Langmuir adsorption isotherm, *Penicillium notatum,* Solid state fermentation

INTRODUCTION

Cellulose, a homopolymer of anhydro-D-glucose linked by ß-1, 4 bonds, is the most abundant biopolymer on the earth. Enzymatic degradation of cellulose to glucose requires the co-operative action of endo-1,4- ß -glucanase (EC 3.2.1.4), exo-1,4- ß glucanase (EC 3.2.1.91), and ß -glucosidase (EC 3.2.1.21) (1).

Biotechnology of Cellulases and hemicellulases began in early 1980s, first in animal feed, followed by food, brewing and alcohol industries. Subsequently, these were used in textile, laundry and paper industries (2). Although ability to synthesize cellulases and hemicellulases is widely spread among microorganisms, only some are able to synthesize these enzymes in industrially important amount which include fungal

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genera of *Trichoderma, Sporotrichum, Fusarium, Humicola, Talaromyces*, *Penicillium* etc (3). Unlike *Trichoderma*, those of genus *Penicillium* synthesize more balanced cellulolytic complexes, which efficiently degrade cellulose and cellulose-containing residues. In addition, enzymes produced by *Penicillium* fungi were found to be more stable (4). Due to energy and food storage, which is likely to become more predominant in near future, reuse of waste cellulose has become more important. Various agricultural wastes such as wheat straw, saw dust, bagasse, corncob willow, oat bran, wheat bran and rice straw (5, 6) have been used successfully in solid state fermentation (SSF) for cellulase production. Thus the present study was undertaken to assess suitability of waste cabbage and bagasse as substrates of SSF for production and purification of cellulase enzyme, and this is the first report on utilization of cabbage for purification of cellulases from *Penicillium notatum NCIM NO-923.*

Researchers generally use sugar yield or determination of enzyme activity in supernatant of resultant hydrolysate to express efficiency of reused enzyme. It has been difficult to evaluate the reuse and or recycle of cellulases, primarily because our insufficient knowledge on the characteristics of cellulase adsorption onto cellulosic substrates. The aim of the present work was to investigate the physio-chemical characteristics of purified endoglucanase and to study the changes in its adsorption isotherm on microcrystalline cellulose (Avicel PH101) at varying environmental conditions (pH and temperature).

MATERIALS AND METHODS

Microorganism

Penicillium notatum NCIM NO-923 was obtained from the department of Food Technology and Biochemical Engineering, Jadavpur University, India. The sporulated culture was maintained at 4°C on Potato Dextrose Agar slant, with regular subculturing after every 3–4 week. The spores

were harvested by swirling in sterile distilled water with 0.1% Tween 80 and diluted with the same up to 10^7 spores/mL.

Solid state fermentation

Solid state fermentation was carried out in 250 mL Erlenmeyer flask. 5g of sugar cane bagasse and untreated sun dried cabbage (in a proportion of 2:3 w/w) was mixed with water with a substrate to moisture ratio of 4:3. Following sterilization at 121°C for 20 min, all flasks were inoculated with 10% (w/v) spore suspensions and were incubated at 30°C for 48h.

Enzyme assay

Endoglucanase or CMCase (E.C. 3.2.1.4) activity was determined by incubating 0.5 mL of diluted enzyme with 0.5 mL of 1% carboxymethyl cellulose (high viscosity CMC, SIGMA) in 0.05 M sodium citrate buffer (pH 4.8) at 50°C for 30 min. After incubation the reaction was terminated by adding 3 mL of 3,5-dinitrosalicylic acid (DNS) reagent to 1 mL of the reaction mixture. Reducing sugars were estimated colorimetrically with 3, 5- dinitrosalicylic acid (7) using glucose as standard.

Preparation of the crude enzyme

For harvesting the crude CMCase, 50 mL of distilled water was added in the fermented pith and shaken vigorously on orbital shaker at 120 rpm for 3 h. The crude enzyme was filtered, centrifuged at 10,000×g for 10 min and then concentrated by rotary evaporator (Eyela, Japan).

Ammonium sulfate precipitation

All purification steps were performed at 4°C unless otherwise stated. Crude extract was subjected to 80% ammonium sulfate saturation and was kept overnight at 4°C to precipitate the protein. Resulting precipitate was collected by centrifugation at 10,000×g for 10 min, dissolved in 0.05M acetate buffer (pH 4.8) and dialyzed for 24h against the same buffer with four changes of equal intervals. Total proteins and

CMCase activity was determined before and after dialyzing the sample.

DEAE- ion exchange chromatography

For this, dialyzed enzyme preparation was applied to a DEAE cellulose column (50×1cm) pre equilibrated with elution buffer (50mM sodium acetate buffer; pH 4.8). Elution was achieved with a linear gradient of 0.1–1M NaCl at a flow rate of 0.3 mL/min. The purified fractions were collected using a fraction collector. The absorbance profile of each 5 mL fraction was studied at 280nm using spectrophotometer for the presence of protein and analyzed for CMCase activity. The active fractions were pooled, re-dialyzed and concentrated upto homogeneity level.

Sephadex G100 column chromatography

The enzyme from DEAE column was further purified on Sephadex G-100 (Sigma, St. Louis, MO) column (80×2 cm). The enzyme was eluted with 0.05M acetate buffer (pH 4.8) at a flow rate of 1mL/min. Eluted protein fractions were collected through fraction collector and the active part was pooled and concentrated.

Determination of the molecular weight by polyacrylamide gel electrophoresis

Molecular mass and the purity of the purified CMCase was determined by sodium dodecyl-sulphate polyacrylamide gel electrophoresis (10% SDS-PAGE), using a BioRad gel electrophoresis apparatus. After electrophoresis, gels were stained by Coomassie blue R-250. Molecular mass was determined against standard markers proteins (Sigma).

Zymogram analysis

For zymogram analysis, purified endoglucanase was subjected to 10% polyacrylamide gel electrophoresis with 0.1% carboxymethyl cellulose. After electrophoresis, gel was incubated in 0.05M acetate buffer (pH-4.0) for 1h. The clear hydrolysis zone that corresponded to enzyme activity was visualized using 0.5% (w/v) Congo red (8).

Determination of optimum pH, temperature and Thermostability

The optimum pH was determined by measuring the activity at 50°C using the following buffers: 50 mM citrate–phosphate (pH 2.0–4.0), 50 mM sodium acetate (pH 4.0–6.0) and 50 mM phosphate (pH 6.0–7.0). Temperature optimum was determined by assaying the enzyme activity at several temperatures (30 to 70°C) at pH 4.0.

Thermostability of enzymes was determined by pre incubation of the enzyme solutions at varying temperature range 30-70°C (in 0.05 M sodium acetate buffer, pH 4.0) in the absence of substrates. Aliquots were removed at different times between 0 and 2 h and immediately cooled on ice. The treated enzyme solutions were then assayed for residual enzymatic activities using standard assay conditions.

Effect of metallic ions on the enzyme activity

The enzyme activity was measured in the presence of some metal ions at 5 and 10 mM concentrations, and the residual activity was determined. The stock ionic solutions used in this study were prepared in distilled water at the concentration of 1 M: $BaCl_2$, $CoSO_4$, $CuSO_4$, $CaCl_2$, $ZnCl_2$, and $FeSO_4$.

Adsorption studies

During adsorption study of the purified enzyme, Avicel pH 101 was used as the adsorbing substrate. 20 mg Avicel was mixed with 2 mL buffer solution of various pH (4.0-8.0) values with a purified enduglucanase load of 0–1 mg/mL. After incubation for 45min at 10°C with shaking, reaction mixture was centrifuged for 5 min at 5,000 ×*g*. The amount of protein in the supernatant was determined by the Lowry et.al method (9) using bovine serum albumin as standard.

RESULTS AND DISCUSSION

Enzyme purification

The purification profile of endoglucanase from Sephadex G100 column showed sharp peak of endoglucanase activity

obtained from fractions 35–40 using an NaCl concentration of 0.2–0.3M (Fig. 1).The purified endoglucanase had a specific activity of 50.2 U/mg with a 28.99% recovery. The results are summarized in Table 1.

The active fraction showed a single band on SDS-PAGE analysis, having a mass of ~55 kDa (Fig. 2). The zymogram analysis of the corresponding band exhibited CMCase activity when observed after staining with Congo red.

Table 1. Profile of step wise endoglucanase (CMCase) purification from *Penicillium notatum NCIM NO*-923 under mixed solid state fermentation of waste cabbage and bagasse.

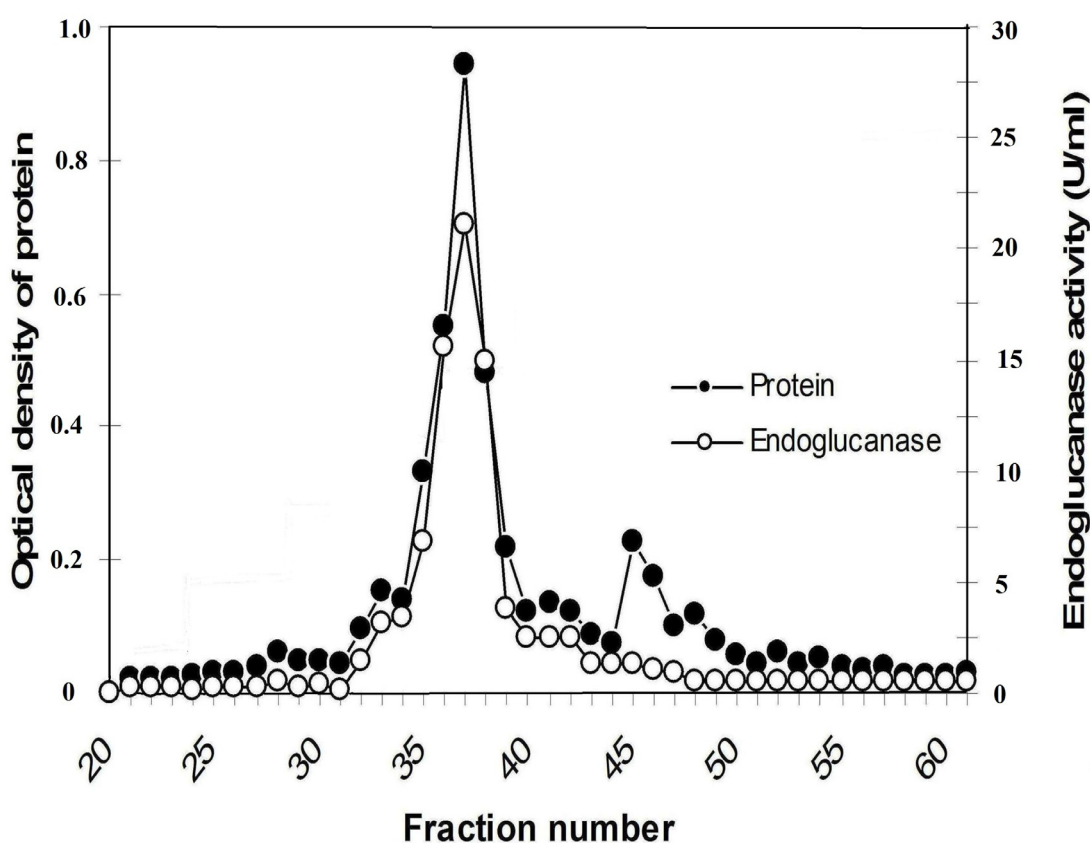| Treatment	Total enzyme (U)	Total protein (mg)	Specific activity (U/mg)	Purification factor	% Recovery
Crude enzyme	3325	1269	2.62	1	100
(NH4)$_2$ SO$_4$ precipitation	2581	637	4.05	1.54	77.62
DEAE ion exchange chromatography	1513	69.0	21.92	8.36	45.5
Sephadex G100 column chromatography	964	19.2	50.2	19.16	28.99

Figure 1. Sephadex G150 column chromatography of endoglucanase from *Penicillium notatum NCIM NO-923*. Active fraction was collected from fraction no.35-40.
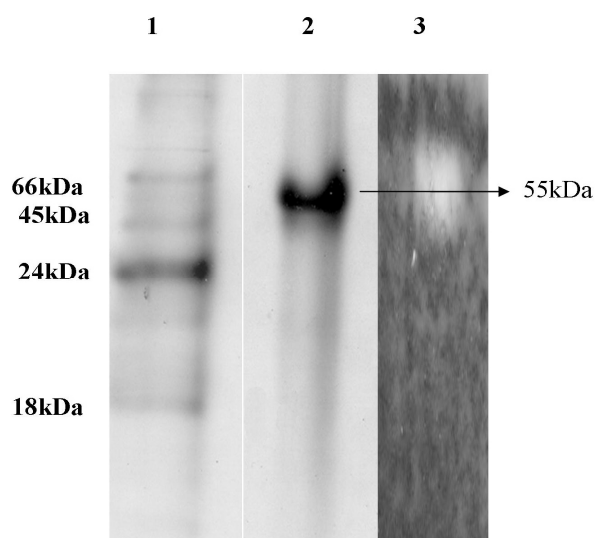
Figure 2. Polyacrylamide gel electrophoresis of endoglucanase from *Penicillium notatum NCIM NO-923*. Lane 1 Molecular marker proteins, Lane 2 purified endoglucanase, Lane 3 zymogram band of purified endoglucanase after staining with 0.1% congo red.

Effect of pH on endoglucanase activity

The effect of pH on enzymatic activities was tested at pH values from 2.0 to 7.0 using different buffers under standard assay conditions as described before. CMCase had pH optima in a range of 3-5. A Dixon plot (10) (Fig. 3) was applied to determine the pK_a values of the active site residues that control V_{max} of the enzyme. Generally two carboxyls are involved in the catalytic mechanism of most hydrolases; one donates protons to the substrate while the other stabilizes it (11). The pK_{a1} for proton donating ionizable group and proton receiving residues were 2.5 and 5.3 respectively. Comparison of the pK_a values of the ionizable groups of amino acids revealed that α-carboxyl might be acting as proton donor during catalysis. On the other hand, the pK_{a2} of ionizable groups involved in catalysis as proton receiver was comparable with the imidazole group. The active site residues for A. niger CMCases have been determined containing carboxyl as ionizable group with pK_a values of 3.5 and 5.5, respectively (12). Endoglucanases from *Schizophyllum commune* also involve with the amino acid residues with pKa values of 3.7 and 6.1 for CMC catalysis (13). The presence of histidine residues in the active site domain of *A. niger* and *A. awamori* glucoamylase has also previously been reported (14).
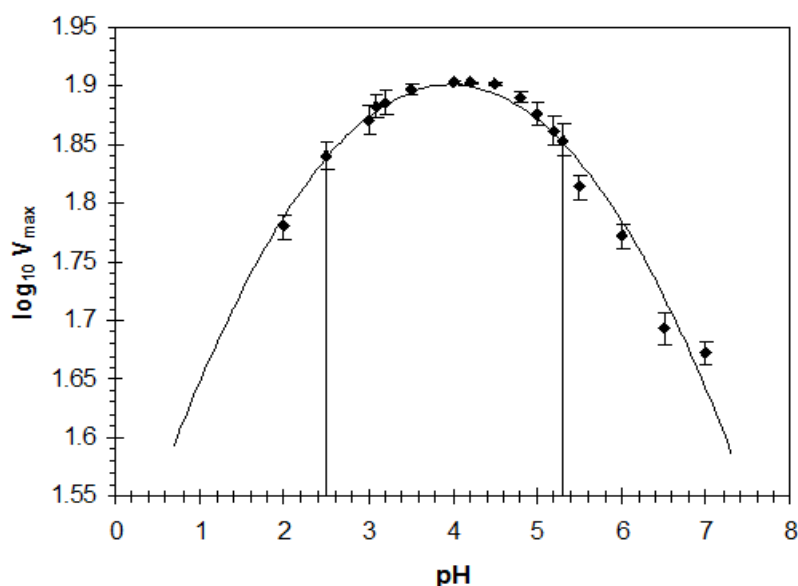

Figure 3. Dixon plot for the effect of pH on endoglucanase activity and determination of pK_a of ionizeable groups of active side residue. Data presented are average value +/- SD of n= 3 experiments.

Effect of temperature on endoglucanase activity

To determine the optimal temperature, the reaction mixtures were incubated at temperature ranging from 30 to 70°C at pH 4.0. Then the enzymatic activities were assayed using standard conditions. Temperature optimum for CMCase activity was found to be 50°C. An Arrhenius plot (15) for activation energy (E_a) was calculated from the curve (Fig. 4) and found to be 36.39 kJ mol^{-1}. During thermostability study, the denaturation midpoint temperature (T_m) was determined and found to be 62.2°C (Table 2). The thermodynamic parameters were calculated by rearranging the Eyring absolute rate equation (16) to study the overall thermodynamic parameters in the range 30-70°C.

Table 2. Kinetics and thermodynamics property of purified endoglucanase (CMCase) from *Penicillium notatum NCIM NO*-923.

Parameters	Endoglucanase
T_m (°C)	62.2
K_m (mg mL^{-1})	5.2
V_{max} (U/gds)	80
K_{cat} (sec^{-1})	322.4
K_{cat}/K_m	62
pK_{a1}(acid limb)[a]	2.5
pK_{a2}(basic limb)[b]	5.3
E_a (KJ mol^{-1})[c]	36.39
$\Delta H*$ (kJ mol -1)	87
$\Delta S*$(J mol -1 K-1)	59.3
$\Delta G^{\#}_{E-T}$(KJ mol^{-1})[d]	-21.5
$\Delta G^{\#}_{E-S}$ (KJ mol^{-1})[e]	3.8

[a]Nucleophelic carboxyl group
[b]Proton donating carboxyl group
[c]Activation energy
[d]Free energy of transition state binding
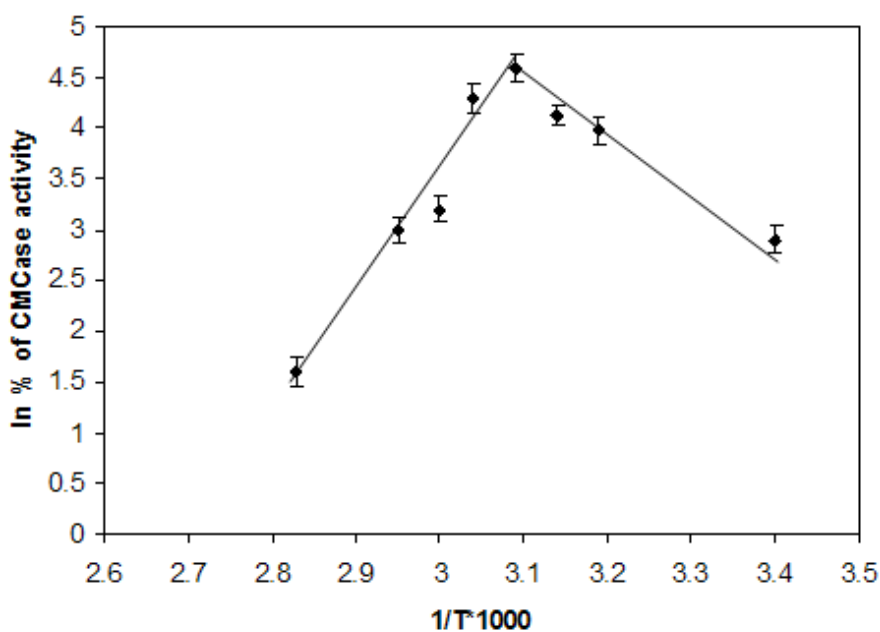[e]Free energy of substrate binding were calculated according to Rashid &Siddiqui 1998).

Figure 4. Arrhenius plot for effect of temperature and determination of activation energy for carboxy methyl cellulose hydrolysis. Data presented are average value +/- SD of n= 3 experiments.

Enzyme kinetics and thermodynamics study

Classical Michaelis–Menten kinetics were observed with CMC as the substrate for CMCase, and the apparent K_m and V_{max} values calculated from a Lineweaver–Burk plot were 5.2 mg mL^{-1} and 80 U/gds. While Turn over number (K_{cat}) and catalytic efficiency (K_{cat}/K_m) were determined to be 322.4s^{-1} and 62 respectively. The data on free energy for substrate binding ($\Delta G*_{E-S}$) and formation of activated complex ($\Delta G*_{E-T}$) showed that the affinity to carboxymethyl cellulose and the formation of transition complex was high (Table 2) for

endoglucanase from *Penicillium notatum NCIM NO-923.*

In thermal inactivation, native enzyme becomes unfolded which could be reversibly refolded upon cooling or is permanently inactivated after prolonged exposure to heat. The disintegration of the enzyme structure is accompanied by an increase in randomness or entropy of activation (15). The enzyme had a lower enthalpy of deactivation ($\Delta H*$; 87 kJ mol -1) and entropy of deactivation ($\Delta S*$;59.3 J mol -1 K-1) in the temperature range 30–70˚C. The result indicates that stabilization of enzymes is mostly accompanied by a decrease in $\Delta S*$ and $\Delta H*$ (12, 17).

Effects of metal ions on endoglucanase activity

Table 3 shows the results of activity in presence of various divalent ions added at 5 or 10 mM. Magnesium at 10 mM greatly activates the enzyme ending up with 138.8% of relative activity, followed by Ca^{2+}, Zn^{2+} and Ba^{2+}ions. Inversely, Fe^{2+} decreased the activity to 66.8%. Cobalt has no effect on enzyme activity.

Table 3. Effect of metal ions on endoglucanase activity from Penicillium notatum NCIM NO-923.

Cataion	Residual endoglucanase activity	
	5mM	10mM
Ca^{2+}	115.4	128.4
Zn^{2+}	112.4	121.8
Cu^{2+}	72.8	85.9
Co^{2+}	95.8	98.5
Ba^{2+}	103.9	112.8
Mg^{2+}	127.4	138.8
Fe^{2+}	85.4	66.8
Control	100	100

Adsorption study

The adsorption kinetic results of endoglucanase from *Penicillium notatum NCIM NO-923* showed that the % of adsorption of endoglucanase on Avicel was maximum at 10°C while beyond 30°C it decreased (data not shown). With the increase in temperature, endoglucanase bound on the surface of Avicel became more accessible to the substrate and as a result due to hydrolysis of cellulose at these higher temperatures the

adsorption behavior of endoglucanase was affected.

Therefore this incubation time was chosen for the isotherm experiments. The adsorption parameter values were determined using the Langmuir adsorption (18) isotherm equation in order to obtain a quantitative analysis of the effect of pH on endoglucanase adsorption. From the Langmuir adsorption isotherm, the adsorption of cellulase can be described as follows:

(A)= {(A$_{max}$) K$_{ad}$ (E)}/1+ K$_{ad}$(E) ……………………(1)

where A_{max} and K_{ad} are the maximum amount of enzyme adsorbed per unit weight of cellulose and the adsorption equilibrium constant, respectively. (E) is the concentration of enzyme in liquid phase at the adsorption equilibrium. Fig. 5 represents the adsorption isotherm of purified endoglucanase at varying pH values and enzyme concentrations onto Avicel at 10°C. The result indicates a rapid adsorption of enzyme on Avicel at lower enzyme concentrations, and then remains in equilibrium.

The adsorption isotherm, Equation (1), can be rearranged as follows:

(E)/(A)=1/{K$_{ad}$ (A$_{max}$)}+1/ (A$_{max}$)(E) ……………..(2)

The adsorption equilibrium constant (K_{ad}) and amount of maximum adsorption (A_{max}) were determined from the slope of plots of (E) / (A) vs. (E) using a scattered plot analysis. The adsorption strength of the enzyme is represented by R (=K$_{ad}$ × A$_{max}$) which is the slope of the adsorption isotherm (18) near P =0, As shown in Fig. 6 the plot (E)/(A) vs (E) gave a fairly straight line which indicates the adsorption of endoglucanase on Avicel follows the langmuir isotherm.

From the results it was found that maximum adsorption of the enzyme was favorable at nearly optimum pH values of the enzyme, while at higher pH values adsorption strength of the enzyme decreased (Table 4) which may be due to structural change in substrate binding site of the enzyme at higher pH values.
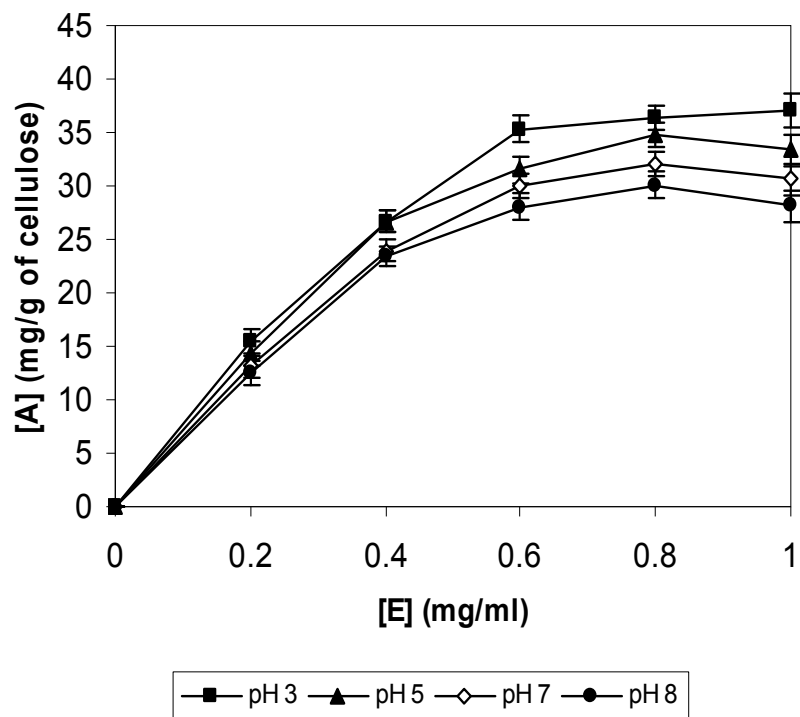
Figure 5. Adsorption isotherm of endoglucanase purified from *Penicillium notatum NCIM NO- 923* on Avicel at 10°C. Data presented are average value +/- SD of n= 3 experiments.
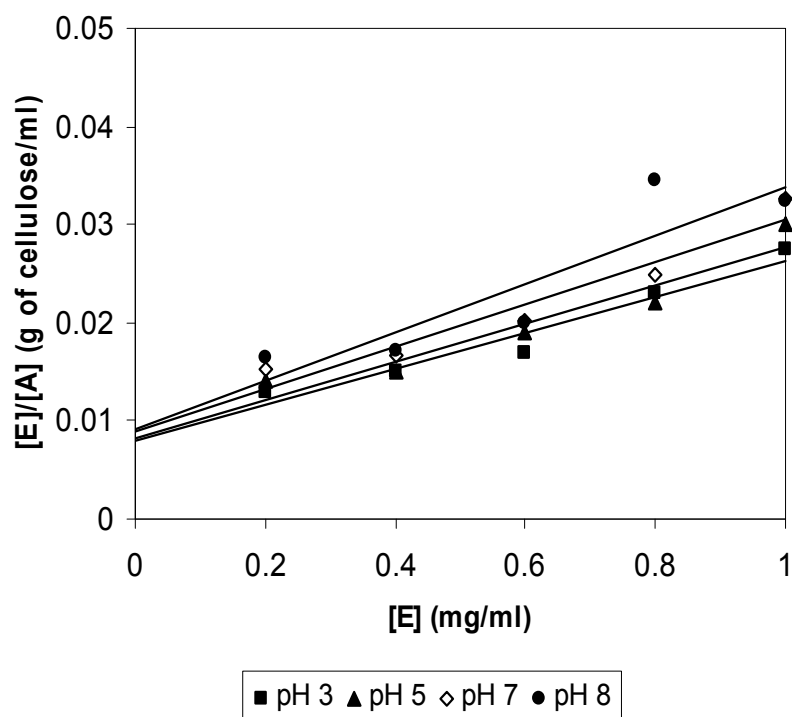

Figure 6. Langmuir-type Adsorption isotherm of endoglucanase purified from *Penicillium notatum NCIM NO-923* on Avicel at 10°C.

Table 4. Adsorption parameters of purified endoglucanase (CMCase) from Penicillium notatum NCIM NO-923 on Avicel at varying pH and temperatures.

Temperature (°C)	pH 3			pH 5			pH 7			pH 8		
	A_{max}	K_{ad}	R	A_{max}	K_{ad}	R	A_{max}	K_{ad}	R	A_{max}	K_{ad}	R
10	54.94	2.36	129.65	50.00	2.50	125	47.61	2.33	110.93	40.48	2.71	109.70
20	53.16	2.30	122.27	50.69	2.36	119.62	46.46	2.11	98.03	40.53	2.13	86.32
30	50.64	2.14	108.36	49.51	2.10	103.97	45.19	2.10	94.89	39.59	1.95	77.20
40	49.00	1.89	92.61	48.05	1.81	86.97	43.30	1.78	77.07	39.61	1.68	66.54

CONCLUSION

This analytical study revealed that the fungi *Penicillium notatum NCIM NO-923* produced significantly high amount of endoglucanase under mixed fermentation using cabbage and bagasse which were too inexpensive and easily available substrates for enzyme production. The 55kDa enzyme exhibited low activation energy for carboxymethyl cellulose hydrolysis; low K_m values while high V_{max} and K_{cat} values, which indicate a high efficiency of the enzyme towards cellulose hydrolysis.

Apart from these, the purified endoglucanase was quite thermostable which is a unique property because elevated temperatures are used in cellulose saccharification process to protect both substrate and products of the enzymatic reaction from microbial contamination and deterioration. Study on the enzyme adsorption at varying pH and temperature was also important from industrial view point because a large amount of bound enzymes are unutilized due to lack of the knowledge of adsorption kinetics. All these characteristics make the endoglucanase promising for application in industries especially from textile and paper industries. Furthermore this is the first report on purification and thermodynamics study of endoglucanase from *Penicillium notatum NCIM NO-923* under solid state fermentation of waste cabbage.

ACKNOWLEDGEMENTS

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