Purification and characterization of carboxymethyl cellulase (CMCase) produced by *Cladosporium sp. NCIM 901*
5.0 Introduction

Cellulose, a linear polymer of D-glucose units linked by 1. 4-β-D-glucosidic bonds, is found in nature almost exclusively in plant cell walls and is the most abundant renewable biomass available on earth (Jeya et al., 2010). Cellulases are the hydrolytic enzymes which are responsible for the decomposition of the natural cellulose polymer (cotton, filter paper or lignocellulosic biomass) by acting at 1. 4-β-D-glucosidic linkages thus finally converting into glucose monomer, which can be used for production of ethanol, organic acids and other chemicals (Sternberg et al., 2000).

Cellulase production in fungi is found to be extracellular and has three components such as endoglucanase (EC.3.2.1.4.), exoglucanase (EC.3.2.1.91) and β-glucosidases (EC.3.2.1.21) (Breznak and Brune, 1994; Yi et al., 1999). These enzymes act together synergistically and cooperatively to convert native crystalline cellulose to oligosaccharides and glucose. Endo-β-glucanase (1. 4-β-D glucanhydrolyase or CMCase) attacks randomly on internal glycosidic bonds of cellulose chain resulting in a rapid scission to yield oligosaccharides and glucose (Wood, 1985). Exo-β-glucanase (1. 4-β-D-glucanocellobiohydrolyase or cellohydrolyase) hydrolyzes highly crystalline cellulose attaching on newly generated ends (Hoshino, 1997). The enzyme β-glucosidase hydrolyzes the aryl- and alkyl-glucoside as well as cellbiose and cello-dextrin to glucose (Kubicck, 1994). The enzymatic saccharification of lignocellulosic materials for the production of ethanol was performed by commercial cellulases, in which the major cellulase was carboxymethyl cellulase (Ballesteros et al., 2004; Tomas-Pejo et al., 2009). Until now cellulolytic enzymes have been isolated from bacteria and fungi (Tomme et al., 1995), plants (Brummell et al., 1994), molds (Blume and Ehnis, 1991), microbes from animal intestines (Moriya et al., 1998) and herbivorous invertebrates such as arthropods (Watanabe et al., 1997; Watanabe et al., 1998), nematodes (Smart et al., 1998) and mollusks (Yokoe and Yasumasu, 1964).

Majority of industrial enzymes are produced by large-scale submerged fermentation. Agro-industrial wastes can eventually be used as substrate and act as good sources of carbon and nitrogen. This involves growing selected microorganism in closed vessels in which all the conditions critical for growth are carefully
controlled. Selected microorganisms are either bacteria (Bacillus species) or fungi (Aspergillus or Trichoderma species) that have been carefully chosen and optimized. Generally, these organisms secrete the enzymes directly into the growth medium from where they can be recovered by filtration and centrifugation (Ali et al., 2002; Skowronek and Fiedurek, 2006). Carboxymethyl cellulase was produced by several fungi such as Humicola insolens (Subrahmanyeswara et al., 1988), Cryptococcus sp. (Thongekkaew et al., 2008), Mucor circinelloides (Saha, 2004), Melanocarpus sp. (Kaur et al., 2007), Chaetomium thermophile var. coprophile (Ganju et al., 1990) and Myceliophthora thermophila D-14 (Roy et al., 1990).

Carboxymethyl cellulase have versatile applications in various fields; widely used in textile industry and in laundry detergents; used in the pulp and paper industry for various purposes; facilitates fermentation of biomass into biofuels and even to treat Phytobezoars, a form of cellulose bezoar found in human stomach. It has great potential for utilization in the food industry; for coffee processing, combination of CMCase, hemicellulases and pectinases is used in the extraction and clarification of fruit and vegetable juices (Galante et al., 1998; Grassin and Fauquembergue, 1996a; Uhlig, 1998), extraction of oils from the oilseeds and olive plant (Dominguez et al., 1995; Sosulski and Sosulski, 1993), bread production, brewery and wine biotechnology (Harada et al., 2005; Uhlig, 1998; Galante et al., 1998; Grassin and Fauquembergue, 1996b; Caldini et al., 1994; Gunata et al., 1990) production of fruit nectars and purees and to alter the sensory properties of fruits and vegetables (Humpf and Schrier, 1991; Marlatt et al., 1992; Pabst et al., 1991).

The purification of protein is an essential first step for the study of its molecular and biological properties in order to understand its biological function. There are several properties (such as molecular weight, charge, hydrophobicity, etc.) that can be exploited to purify or single out a protein from a mixture. The production of microbial enzymes is generally achieved through either aerobic submerged culture or solid state fermentation. A number of industrial enzymes such as amylases, tannase, cellulase and lipase have been purified using series of purification steps from a number of microbial sources. Most of these purification protocols involve ammonium sulfate precipitation as the first step of purification followed by ion exchange chromatography and or gel filtration. For many industrial applications, partially purified enzyme preparations are good enough whereas for
analytical and medical applications enzymes must be highly purified and must be free from undesirable contaminants (Ghosh and Ghosh, 2003). Purification of microbial enzymes faces the problems of contamination substances present in the starting material and also the separation of the enzyme from other cellulytic enzymes produced by the microorganisms. Normally tissue homogenate contain different proteins and the purification become difficult. However in practice only four different steps are needed for the purification of proteins. In some cases a single chromatographic step is enough based on the purpose of purification. For structural and functional studies, 100% purification is not necessary. Enzymes can be purified several fold but the yield of the enzymes at the end may be very poor. Industrial enzymes are purified as little as possible i.e. for the removal of interfering materials. Since additional stages are costly in terms of equipment, manpower and loss of enzyme activity, unnecessary purification is to be avoided. As a result, some commercial enzyme preparations consist especially of concentrated fermentation broth, plus additives to stabilize the enzyme’s activity (Wilson and Walker, 2000). It is important to retain maximum activity of the enzyme during its preparation. The factors, which cause inactivation, are heat, proteolysis, suboptimal pH, oxidation, denaturation, irreversible inhibitors and loss of co-factors and co-enzymes.

The main objective of the first stage for the recovery of an extracellular product is the removal of large solid particles and microbial cells usually by centrifugation or filtration. In the next stage the broth is fractioned or extracted into major fractions using ultrafiltration, reverse osmosis, adsorption or ion exchange or gel filtration or affinity chromatography, liquid-liquid extraction, two phase aqueous extraction or precipitation. Afterwards, the product containing fraction is purified by fractional precipitation. Further more precise chromatographic techniques and crystallization are done to obtain a product, which is highly concentrated and essentially free from impurities. Other products are isolated by using modification of this flow system (Stanbury et al., 1997).

The enzyme obtained from the above steps is pure. Electrophoretic method is used for checking the purity and also for determining the molecular weight of the purified enzyme sample. The molecular weight can be detected by comparing with the molecular weight of marker proteins. By using SDS-PAGE the subunits of the
enzyme if present can be separated. It is important to determine the kinetic properties i.e. $K_m$, $V_{max}$, thermal stability and pH stability of the enzyme.

The present study involves the purification and characterization of carboxymethyl cellulase (Endoglucanase, CMCase) produced on pretreated sugarcane bagasse by Cladosporium sp. NCIM 901.

5.1 Review of literature

Purification of carboxymethyl cellulase (CMCase) from various sources including bacteria, fungi is being conducted for many years. Considering their wide range of applications in various industries, the purification and characterization studies of carboxymethyl cellulase (CMCase) from new sources draws much attention. Progress on the mode of action, active site architecture, and three-dimensional structures of bacterial and fungal cellulases has led to prediction of their role in cellulose hydrolysis and to realization of their wide range of potential application. Cellulases from various sources have shown their distinctive features as they carry their specific pH optima, solubility and amino acid composition. Thermal stability and exact substrate specificity may also vary with the origin. The optimum pH generally lies between 4 and 5 and temperature is 40-50°C (Bhat, 2000; Parry et al., 2002).

Purification and characterization of β-glucosidase and CMCase from Humicola insolens were carried by Subrahmanyeswara et al., (1988). The enzymes were homogeneous; the main differences being in amino acid profile and substrate specificity. The purified β-glucosidase hydrolyzed cellulose mainly to glucose along with small quantity of cellobiose from non-reducing end. Carboxymethyl cellulase was produced by Cryptococcus sp. S-2 on cellobiose and purified by ultrafiltration, DEAE-5PW anion exchange column and TSK-Gel G3000SW gel filtration. The purified enzyme had a molecular mass of 34 kDa. The optimum temperature and pH of the enzyme were at 40–50°C and 3.5, respectively. The enzyme was stable over pH range of 5.5–7.5 and retained about 50% of its maximum activity after heating at 90°C for 1 hr (Thongekkaew et al., 2008).

A complete cellulase system (CMCase, cellobiohydrolase and β-glucosidase) was produced by Mucor circinelloides (NRRL 26519) on lactose, cellobiose and
sibmacell 50. The purified CMCase (specific activity 43.33 U/mg protein) exhibited a molecular weight of 27 kDa. The optimum temperature and pH for the enzyme were 55°C and 4.0–6.0, respectively. The enzyme was stable at pH 4.0–7.0 up to 60°C. It hydrolyzed CMC and insoluble cellulose substrates (Avicel, Solka-floc and SigmaCell 50) to soluble cellodextrins. The addition of 5mM MgCl₂ and 0.5mM CoCl₂ to the reaction mixture caused an increase of 27±5 and 44±14% in enzyme activity, respectively (Saha, 2004). Working on the same lines, Yaoi and Mitsuishi (2004) isolated xyloglucan-specific endo-β-1,4-glucanase (XEG), xyloglucanase, having molecular weight 80 kDa and a pl 4.8 from the fungus Geotrichum sp. M128. The enzyme was not active toward carboxymethyl cellulose, avicel or barley glucan.

Using an anaerobic sulphidogenic bioreactor, Oyekola et al., (2007) isolated, purified and characterized two CMCases and a β-glucosidase. The maximum activity was observed at pH 6 and 6.5, respectively and temperature 50°C. The CMCase remained stable with no effect on activity after 60 min whereas, glucosidase retained only 30% activity. Carboxymethyl cellulases were purified 13 and 25-fold after sonication, PEG concentration and DEAE chromatography. Divalent ions like Cu, Ni and Zn were inhibitory while Fe, Mg and Ca showed stimulatory effects on enzyme activity at concentrations greater than 400 mg/L.

In another research work, Kaur et al., (2007) purified and characterized two endoglucanases (EG I and EG II) from a thermophilic fungus, Melanocarpus sp. MTCC 3922. The molecular weight of EG I and EG II with SDS-PAGE and isoelectric points were 40 and 50 kDa and 4.0 and 3.6 kDa, respectively. EG I and EG II showed maximum activity at 50 and 70°C and pH 6.0 and 5.0, respectively. EG I remained active over a pH range of 5.0–7.0 whereas, EG II showed loss of activity from 50 to 80°C. The presence of mercaptoethanol and SDS inhibited the EG I activity but showed no such effect on EG II.

In order to evaluate the role and mechanism of action of cellulase components during the solubilization of cellulose, Karnchanatat et al., (2008) purified a thermostable CMCase from culture supernatants of Daldinia eschscholzii (Ehrenb.Fr.) Rehm grown on 1.0% (w/v) CMC using ammonium sulfate precipitation, ion-exchange, hydrophobic interaction and gel filtration.
The molecular weight of the enzyme was 46.4 kDa. The isoelectric point of the enzyme was pH 4.9 while the optimum temperature was 70°C. The enzyme retained 85% of its maximum activity after 150 min at 50°C, but was rapidly inactivated at 70°C. The enzyme was inhibited by Hg²⁺, Cu²⁺ and Fe³⁺ and stimulated by Ca²⁺, Co²⁺, Mg²⁺, Mn²⁺, glycerol, DMSO, DTT and EDTA.

In order to compare thermal stabilities and pH optima, Qin et al. (2008) produced two cellulase components (C1 and C2) after expressing Cel5A (endoglucanase II) of Trichoderma reesei in Saccharomyces cerevisiae. Purified C1 had a larger molecular mass (57 kDa) than that of the native Cel5A (48 kDa) due to the different extents of asparagines-linked glycosylation. There was no significant difference in enzymatic activity between C1 and the native Cel5A from T. reesei. The cellulase component C1 treated with endoglycosidase H1 had molecular mass of 54 kDa and retained about 88% of its original activity. Crude C2 exhibited molecular mass greater than 85 kDa up to 200 kDa.

To explicate the production physiology of cellulases, Aniger F-119 was grown on different substrates to produce endoglucanase, exoglucanase and β-glucosidase. The fungus expressed high enzymes production at moisture level 70% (w/w), initial pH 4.5 and inoculum size 10% (v/w) at 32°C after 72h yielded 31.5, 46.0 and 215.2 IU/g original substrate. Supplementation of the culture medium with ammonium sulphate enhanced enzyme activities. Similarly, wheat bran at 20% was most suitable carbon source for obtaining high enzyme yields. The protein fraction obtained at, 20-40% ammonium sulphate saturation was most suitable for enzymes recovery. The enzymes i.e. endoglucanase, exoglucanase and β-glucosidase were found to be active and stable at temperatures 55, 65 and 60°C, pH 4.5, 5.5 and 4.5, respectively (Fadel, 2000). CMCase activities alter with varying pH and temperature and characterization of produced enzyme require knowledge about optimum pH, temperature stability and substrate specificity. Therefore, the enzyme is characterized to find out the best level of performance for enhanced efficiency of the process (Bhat, 2000; Parry et al., 2002).

It is obvious that carboxymethyl cellulase (Endoglucanase; CMCase) differ in molecular sizes, pH optima, and other characteristics independent of their source. This difference is also related to fermentation type. Since applications of CMCase
enzymes in various fields are wide spreading, it is important to understand nature and properties of these enzymes for efficient and effective usage. In this study, Carboxymethyl cellulase from Cladosporium sp. NCIM 901 produced by SSF on bagasse was purified and characterized.

5.2 Materials and Methods

Carboxymethyl cellulase (Endogluconase; CMCase) from Cladosporium sp. NCIM 901 was purified following the enzyme purification methodologies available in the literature. The strategy adopted included fractionation by ammonium sulphate followed by dialysis and gel filtration chromatography. Purity was checked using polyacrylamide gel electrophoresis.

5.2.1 Enzyme production: Solid state fermentation was carried out in 250 mL Erlenmeyer flasks containing 5.0 g of PAA pretreated sugarcane bagasse and 15 mL Reese’s mineral medium (Reese and Mandel, 1963). The flasks were sterilized at 15 psi for 30 min for two consecutive days and inoculated with 2 mL of fungal spores. Following incubation for 5 d, 100 mL of citrate buffer (0.05 M, pH 4.8) was added to the flasks and kept under mild stirring (120 rpm) for 1 h. The slurry was filtered through muslin cloth, followed by whatman filter paper No.1. The crude extract was used for estimation of endoglucanase enzyme activity.

5.2.2 Enzyme purification: All operations were done at 0-4°C. After solid state fermentation, the crude filtrate was centrifuged at 12000 rpm for 15 min to remove any suspended material and supernatant was collected and used for sequential enzyme purification.

5.2.2.1 Ammonium sulphate precipitation: The method of De-Moraes et al., (1999) was followed for purification of carboxymethyl cellulase (CMCase). Different levels of ammonium sulphate (30, 60 and 80%) in citrate buffer (pH 4.8) were used for the enzyme precipitation. The respective amounts were added to 5 mL of crude enzyme solution and placed at 4°C for one to two hours with continuous stirring. These were centrifuged at 15300 x g for 15 minutes in refrigerated centrifuge (Microfuge, Remi, India). The pellets were collected carefully and the supernatant was discarded. The pellets were then dissolved in distilled water at a rate of 0.1 g/mL. The dissolved pellets were assayed for CMCase activity. The activity
of CMCase reflected the best level of (NH₄)₂SO₄ concentration (gram) for purification.

5.2.2.2 Dialysis: After ammonium sulphate precipitation the enzyme salt solution was dialyzed against the citrate buffer for 24 hours at 4°C to remove ammonium salt (Peshin and Mathur, 1999).

5.2.2.3 Gel filtration: The dialyzed fraction (5.0 mL) was subjected to the gel filtration on Sephadex G-75 column (1.6 cm × 60 cm), pre-equilibrated with 0.05 mol/L citrate buffer (pH 4.8) at a flow rate of 1.0 mL/min. Fractions of 5.0 mL were collected; examined at 280 nm and assayed for CMCase activity. The active fractions containing CMCase activities from the column were pooled and dialyzed against the citrate buffer for further analysis (Peshin and Mathur, 1999).

5.3 Determination of molecular weight of purified enzyme: The molecular weight of the enzyme was determined by SDS-PAGE following the method of Laemmli (1970), using a separating gel of 10% (w/v) acrylamide. The molecular mass of enzyme was determined by loading the standard markers along with the prepared enzyme.

Electrophoresis: The purified enzyme was subjected to electrophoresis studies to confirm purity. The molecular weight of CMCase was determined by using 12% SDS-PAGE (Laemmli, 1970).

Materials

1. Stock acrylamide solution
   30% acrylamide and 0.8% bisacrylamide

2. Polymerising agents
   a) Ammonium persulphate (10%). This was prepared freshly before use.
   b) TEMED (N, N, N', N'-tetramethyl ethylene diamine: was used as supplied.

3. Resolving gel buffer: 3.0 m Tris-HCl buffer, pH 8.8.

4. Reservoir buffer: Tris-Glycine, pH 8.3.
5. Native-PAGE:

**Stacking gel composition**

- Acrylamide-bisacrylamide: 2.50 mL
- Stacking gel buffer: 5.0 mL
- Ammonium persulphate (10%): 0.10 mL
- Distilled water: 11.50 mL
- TEMED: 0.02 mL

**Resolving gel composition (12.5%)**

- Acrylamide-bisacrylamide: 12.50 mL
- Resolving gel buffer: 3.75 mL
- Ammonium persulphate (10%): 0.15 mL
- Distilled water: 14.25 mL
- TEMED: 0.02 mL

**Sample preparation**

- Distilled water: 3.0 mL
- Stacking gel buffer: 1.0 mL
- Bromophenol blue (0.5%): 0.4 mL
- Glycerol: 1.6 mL

6. SDS – PAGE: 3.03 g of Tris, 14.4 g glycine and 1 g SDS was dissolved in 1 L distilled water and pH was 8.3.

**Stacking gel composition**

- Acrylamide-bisacrylamide: 2.5 mL
- Stacking gel buffer: 5.0 mL

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**Resolving gel composition (12.5%)**

- Acrylamide-bisacrylamide: 12.50 mL
- Resolving gel buffer: 3.75 mL
- SDS (10%): 0.30 mL
- Ammonium persulphate (10%): 0.15 mL
- Distilled water: 12.0 mL
- TEMED: 0.02 mL

**Sample preparation**

- Stacking gel buffer: 1.0 mL
- Bromophenol blue (0.5%): 0.4 mL
- SDS (10.0%): 1.6 mL
- Glycerol: 1.6 mL
- β-mercaptoethanol: 0.4 mL
- Distilled water: 3.0 mL

Prepared a mixture of sample and sample buffer in a concentration of 1:1 and heated in a boiling water bath for 3 min in the case of SDS-PAGE. For molecular weight determination of the subunits marker proteins were purchased from Fermentas (Wide range markers). 20 mg of purified CMCase was co-chromatographed with standard SDS proteins markers. Electrophoresis was performed by using a constant voltage of 50V at the time of pre running. When the sample was applied, it was done at a constant voltage of 100 V.
5.3.1 Protein staining

SDS-PAGE: Gel was stained using silver staining method described by Celis et al., (2006). Ultra pure water (> 18 megohm/cm resistance) for preparation of all buffers as well as during the washing steps is recommended.

Procedure: To achieve the best results, that are high sensitivity and low background, it is very important to follow closely the incubation time of all steps as given in the protocol.

Solutions

1. **Fixation solution**: 50% ethanol (or methanol), 12% acetic acid, 0.05% formalin. To make 1 liter, add 120 mL of glacial acetic acid to 500 mL of 96% ethanol and 500 µL of 35% formaldehyde (note: commercial formalin is 35% formaldehyde). Complete to final volume with deionized water.

2. **Washing**: 20% ethanol (or methanol). To make 1 liter, add 200 mL 96% ethanol to 800 mL of deionized water.

3. **Sensitizing solution**: 0.02% (w/v) sodium thiosulfate (Na₂S₂O₃). To make 1 liter add 200 mg of sodium thiosulfate anhydride to small volume of deionized water, mix well and bring to the final volume of 1 liter.

4. **Staining**: 0.2% (w/v) silver nitrate (AgNO₃) and 0.07% formalin is prepared fresh. To make 1 liter add 2 g of AgNO₃ to a small amount of deionized water and 760 µL of 35% formaldehyde. The final volume was made with deionized water. The solution was cooled at 4°C before using.

5. **Developing solution**: 6% (w/v) sodium carbonate (Na₂CO₃), 0.0004% (w/v) sodium thiosulphate (Na₂S₂O₃), 0.05% formalin. To make 1 liter add 60 g Na₂CO₃ to a small amount of deionized water and dissolve. Add 4 mg of sodium thiosulphate anhydride to a small volume of deionized water and dissolve. Mix both solutions, add 500 µL of 35% formaldehyde and bring to the final volume with the water.

6. **Terminating solution**: 12% acetic acid. To make 1 liter, add 120 mL of glacial acetic acid to 500 mL of deionized water. Mix well and bring to the final volume with water.
7. **Drying solution**: 20% ethanol. To make 1 liter, add 200 mL of ethanol to 800 mL of deionized water. Mix well.

**Steps**

1. After electrophoresis, remove the gel from the cassette and place into a tray containing appropriate volume of fixing solution. Soak the gel in this solution approximately 2 h. Fixation will restrict protein movement from the gel matrix and will remove interfering ions and detergent from the gel. Fixation can also be done overnight. It may improve the sensitivity of the staining and decrease the background.

2. Discard the fixative solution and wash the gel in 20% ethanol for 20 min. Change the solution three times to remove the remaining detergent ions as well as fixation acid from the gel.

3. Discard the ethanol solution and add enough volume of the sensitizing solution. Incubate for 2 min with gentle rotation. It will increase the sensitivity and the contrast of the staining.

4. Discard the sensitizing solution and wash the gel twice, 1 min each time, in deionized water. Discard the water.

5. **Add the cold silver staining solution and shake for 20 min to allow the silver ions to bind to proteins.**

6. After staining is complete, pour off the staining solution and rinse the gel with a large volume of deionized water to 20-60 sec to remove the excess of unbound silver ions. Repeat the washing once more.

7. Rinse the gel shortly with the developing solution. Discard the solution.

8. Add new portion of the developing solution and develop the protein image by incubation the gel in 300 mL of developing solution for 2-5 min. The reaction can be stopped as soon as the desired intensity of the bands is reached.

9. **Stop the reduction reaction by adding 50 mL of terminating solution directly to the gel that is still immersed into developing solution. Gently agitate the gel during 10 min. As soon as “bubbling” of the solution is over, the development is stopped.**
5.4.5 Effect of different reagents on activity: To examine effects of different additives on the purified enzyme, the enzyme activity was performed by the presence of metal ions. CMCase enzyme was incubated in 5 mM (final concentration) of MnCl₂, CaCl₂, MgCl₂, EDTA, HgCl₂, EDTA and DTT for 30 min, in sodium citrate buffer pH 5.5 at room temperature and the activities were determined thereafter using CMC as substrate. The EDTA and DTT used at 10 mM concentration at pH 5.5. The activity assayed in the absence of metal ions or reagents were recorded as 100%.

5.4.6 Substrate concentration determination: The purified enzyme was incubated with substrate (CMC) and the reducing sugar produced was measured colorimetrically at 575 nm with DNSA (Dinitrosalicylic acid) reagent. Kinetics parameters were measured with Graph Pad Prism version 4.00 for windows (Graph Pad Software, San Diego, CA, U.S.A.; www.graphpad.com). For Km determination, the data were plotted to fit the Michaelis Menten equation by non-linear regression.

5.5 Enzyme and other assays: Endoglucanase activity of cell free culture filtrates was estimated by the method described by Ghose et al., (1983) using carboxymethyl cellulose 2% (w/v) dissolved in citrate buffer (0.05 M) of pH 4.8 as a substrate. One IU of activity on the substrate was defined as μ mole of glucose released per min at 50°C after 30 min using a glucose standard curve. Total soluble protein and reducing sugars were estimated by the method described by Lowry et al., (1951) and Miller (1959), respectively.

5.6 Results and Discussion

Cellulose break down involves a synergistic activity of cellulolytic enzymes. Among these carboxymethyl cellulase (CMCase, endoglucanase) constitutes a key enzyme involved in the cellulose conversion. The substrate sugarcane bagasse used for the crude enzyme production had an organic carbon 48%, total nitrogen 0.5%, cellulose 41.3%, hemicellulose 21.4% and 12.1% lignin. Sugarcane bagasse, being a recalcitrant plant material, is considered as a waste; however it has been shown to be a promising substrate for production of different hydrolytic enzymes by solid state fermentation and by submerged fermentation.
Moist gels can be kept in 12% acetic acid at 4°C in sealed plastic bags or placed in the drying solution for 2 h prior to vacuum drying.

Native PAGE: After electrophoresis, gels were washed briefly in water and incubated for 1.5 hour at 25°C in 100 mM malic acid without shaking. Gels were rinsed in water for 5 min and stained overnight in ruthenium red (0.03% w/v in distilled water). Stained gels were washed in water to remove excess ruthenium red. Carboxymethyl cellulase was visualized as white bands.

5.4 Characterization of purified CMCase

5.4.1 Optimum temperature: For determination of the optimum temperature of the enzyme, the activity was determined by carrying out the assay at several temperatures between 30 and 90°C.

5.4.2 Optimum pH: The pH profile of the enzymes was evaluated by incubating the enzymes for 10 min at 50°C in appropriate buffers: 50 mM sodium acetate (pH 3–4.5), 50 mM sodium citrate (pH 5–5.5) and 50 mM sodium phosphate buffer (pH 6–9).

5.4.3 Heat stability: The thermostability of CMCase was studied by heating it at different temperatures (40-90°C) for 15 min. Percentage of the original activity retained after heat treatment at 90°C for 15 min was calculated.

5.4.4 Substrate specificity: The substrate specificity of the purified enzyme was determined by performing the assay with CMC (carboxy methyl cellulose), Filter paper, microcrystalline cellulose, Phosphoric acid swollen cellulose, cotton, chitin, starch and cellulose. All substrates were taken at the concentration of 10 mg/mL. Cellobiose and Para nitro phenyl β-D-gluco pyranoside (PNPG) were taken as 10mM concentration in sodium citrate buffer (pH 5.5) at 50°C for 30 min. After the reaction completed the enzyme activity was assayed.

The activity for hydrolyzing filter paper carried out by mixing 0.5 mL of enzyme solution in 0.5 mL of 10mM sodium citrate buffer (pH 5.5) and one strip of whatman filter paper No.1 (size 1 cm x 6 cm). The mixture was incubated at 50°C for 60 min. The amount of reducing sugar produced by the reaction was measured by DNS method (Miller, 1959).
5.6.1 Purification of Carboxymethyl cellulose: Carboxymethyl cellulase produced from the isolate *Cladosporium* sp. NCIM 901 under solid state fermentation was purified by ammonium sulphate precipitation and gel filtration chromatography on sephadex G-75. The total crude enzyme solution was 250 mL. Ammonium sulphate precipitation has been widely used for the concentration of the protein from dilute samples. Different concentrations (30, 60 and 80%) of the ammonium sulfate were used for the purpose. After conducting some preliminary trials, 80% concentration was selected for the enzyme precipitation. After standing overnight, the precipitate formed was collected by centrifugation. Later, the enzyme was loaded on the column packed with sephadex G-75 and fractions of 5 mL were collected and analyzed for enzyme activity.

Table 5.1. Characteristics of the Carboxymethyl cellulase enzyme from *Cladosporium* sp. NCIM 901 strain at different steps of purification.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (mL)</th>
<th>Enzyme activity (IU)</th>
<th>Total protein (mg)</th>
<th>Specific activity (IU/mg)</th>
<th>Purification fold</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell free filtrate</td>
<td>250</td>
<td>120.0</td>
<td>150.4</td>
<td>0.79</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Ammonium sulphate precipitation</td>
<td>15</td>
<td>95.0</td>
<td>64.3</td>
<td>1.47</td>
<td>1.77</td>
<td>79.1</td>
</tr>
<tr>
<td>Sephadex G-75</td>
<td>5</td>
<td>23.0</td>
<td>1.40</td>
<td>16.42</td>
<td>20.78</td>
<td>19.16</td>
</tr>
</tbody>
</table>

The Table 5.1 gives the summary of results of different steps involved in CMCase purification process. Initially the crude culture filtrate had total enzyme activity of 120 IU, total protein 95.0 mg and the specific activity of 0.79 IU/mg protein. It was subjected to ammonium sulphate precipitation as it contained carboxymethyl cellulase. Each of the purification step resulted in enhanced specific activity. The ammonium sulfate precipitation contained 95.0 IU of the enzyme with 64.3 mg protein. The maximum specific activity (16.42 IU/mg protein) was obtained after gel chromatography through sephadex G-75 followed by 1.47 IU/mg protein by that obtained after ammonium sulphate precipitation, while a minimum of 0.79 IU/mg protein was observed in case of culture filtrate.
Correspondingly, there was decrease in the total protein content from 150.4 mg (crude extract) to 1.40 mg (after gel chromatography through sephadex G-75). As observed the total protein content was decreased from starting to final proceeding steps. This decrease in protein content indicates the separation of protein impurities from the desired enzyme fraction (CMCase). The results of the study are in line with the findings of Han and Srinivasan (1969) wherein they noted enhanced enzyme activity and decreased total protein contents when the crude extract (enzyme filtrate) from Alcaligenes faecalis was treated with (NH₄)₂SO₄ (40-60%).

Each step of the process increased the purification fold: 1.77 fold purification was calculated after ammonium sulphate precipitation whilst the maximum purification was observed after gel filtration (20.78 fold). Concomitantly, there was a decrease in the yield of enzyme after every step carried out during the process. The enzyme yield reduced to 79.1% after ammonium sulphate precipitation that reached to its minimum level i.e. 19.16%, after gel chromatography through Sephadex G-75. The results indicated that every step in the enzyme purification resulted in the removal of undesirable fractions of protein therefore; there was decline in enzyme yield after the process of purification. The data indicated that the purified carboxymethyl cellulase showed a 20.78 fold purification higher specific activity and with 19.16% overall yields compared to culture filtrate.

The precipitate obtained through saturated ammonium sulphate treatment was dissolved in 15 mL of 100 mM sodium citrate buffer at pH 5 and finally concentrated to 2.0 ml by polyethylene glycol. Concentrated solution of 1mL of the above was subjected to gel filtration chromatography in a column 30×1.8 cm. The elution was 10 mL/h. From the elution profile single enzyme activity peak and single protein activity peak were obtained. The enzyme activity peak was somewhat coincided with the protein peak obtained. The elution profile is shown in Figure 5.1. The active fractions obtained were pooled and subjected to electrophoresis and also used for the characterization studies of carboxymethyl cellulase.

The purified fraction, when subjected to Native PAGE and SDS-PAGE a single band was observed. The electrophoresis studies revealed the molecular weight of 33 kDa by using the program total lab. Figure 5.2 and 5.3 show the Native-PAGE electrophoresis and molecular weight of purified enzyme protein after SDS-PAGE.
A. Protein content in the fractions of gel chromatography.

B. Carboxy methyl cellulase activity in the fractions of gel chromatography.

Fig. 5.1 Elution profile of carboxymethyl cellulase enzyme in gel chromatography.
Lane 1: Purified enzyme of SSF; Lane 2: Molecular weight marker proteins

Fig. 5.2. Characterization of carboxymethyl cellulase by native-PAGE.

Lane 1: Purified enzyme of SSF; Lane 2: Ammonium sulphate precipitate of SSF; Lane 3: Crude enzyme of SSF; Lane 4: Molecular weight marker proteins

Fig. 5.3. Characterization of carboxymethyl cellulase by SDS-PAGE.
Jatinder et al., (2006) have detected two isoforms of endoglucanase in the zymogram of Melanocarpus sp. produced by solid state fermentation of rice straw and wheat straw. Ammonium sulphate precipitation has been widely used for the concentration of the protein from dilute samples. Peshin and Mathur (1999) recovered 40% β-glucosidase activity at 60-90% saturation from crude extract using ammonium sulphate precipitation method. Similarly Usama and Hala (2008) recovered 79% of endoglucanase activity using ammonium sulphate precipitation (70% saturation) from crude filtrate of Aspergillus niger grown on water hyacinth under solid state fermentation. In our study, we recovered 79.1% carboxymethyl cellulase yield with 1.77 fold purification.

Kamchanatat et al., (2008) observed 45.31 IU/mg protein after ammonium sulphate precipitation during the purification of endoglucanase from crude culture of wood-decaying fungus Daldinia cschscholzii (Ehrenb.:Fr.) Rehm. The difference in the results as compared with present study may be due to the difference in the concentration of the ammonium sulphate used for precipitation of enzymic protein. The results are also supported by those of Kaur et al., (2007); who observed a specific activity of 24.61 mol/min/mg protein, 4.51 fold purification and 13.46% enzyme recovery during the process of CMCase purification.

Likewise, Li et al., (2003), during five step purification of carboxymethyl cellulase from Chaetomium thermophilum CT2 have observed a specific activity of 38.7 IU/mg, 8.8% enzyme recovery and 20.4 fold purification. The higher value of specific activity was observed in the above than the present study which could be due to the extent of purification i.e. more steps were involved in the purification process resulting in the increased purification of enzymic protein. The results of the present study were also comparable with those Oyekolu et al., (2007) wherein they found 6.5 IU/mg specific activity, 2.4% enzyme recovery and 11 fold enzyme purification during their research work.

The results are also in close conformity to those reported by Murashima et al., (2002) who observed 46.5 IU/mg specific activity, 18.6% enzyme recovery and 12.6 fold purification during purification and characterization of CMCase from
Rhizopus oryzae. However, the purification fold and enzyme recovery in the present study were high. In another study, there was much higher specific activity (213.1 IU/mg) as reported by Qin et al., (2008) which may be due to the involvement of different techniques as adopted in their study i.e. the use of ultrafiltration and filtration through CM-Sepharose FF column. Likewise, Saha et al., (2004) reported a much higher value for purification fold i.e. 408 after a four step purification of CMCase from Mucor circinelloides NRRL 26519. Such great differences could be due to the differences in methods adopted for the purpose. During the present study, maximum purification i.e. 3.05 fold was attained after gel filtration. The purification procedure is very important it results in a significant increase in the specific activity of CMCase.

5.6.2 Characterization studies: After purification, the enzyme carboxy methyl cellulase was characterized to find the conditions at which it showed best performance. This information would be helpful in future application to obtain appreciable efficiency. Effect of substrate concentration on enzyme activity was studied. The produced CMCase was characterized for pH, temperature, heat stability and molecular mass. The details of the effects of various parameters are summarized below.

5.6.2.1 Substrate concentration: The degradation of carboxymethyl cellulose by CMCase was assayed by measuring the reducing groups released using the DNS method respectively. In this study kinetic parameters were determined using CMCase as a substrate in a range of 1-15 mg/mL. Michaelis Menten equation was found to fit the reaction of carboxymethyl cellulase from the Cladosporium sp. (Figure 5.4). V\text{max} and K\text{m} were calculated using Graphpad prism software. V\text{max} found to be 18.92/min and K\text{m} was found to be 7.2 mM for CMCase. Above 12 mg of substrate the enzyme activity seemed to decrease.
5.6.2.2 Optimum pH of the purified enzyme: The effect of various pH values on the CMCase activity is presented in Figure 5.5. The data illustrate that the enzyme activity was studied at different pH values and the highest enzyme activity was found at 5.5 pH. When the pH of the enzyme medium was increased there was a declining trend in the enzyme activity. However with further increase in pH to 7.5 the enzyme activity was decreased gradually as the pH increased. Likewise, when the CMCase activity was measured at pH values lower than 4.5, it showed a decreasing trend with decrease in pH. Generally, the enzyme showed maximum activity at pH 5.5 followed by 7.5. The results were compared with the results obtained by the work conducted by Saha (2004) who reported optimum pH for CMCase in the range 4.0-6.0. Also, Kaur et al., (2007) characterized EG II and found that it showed maximum activity at pH 5.0 which is close to the present results.
5.6.2.3 Optimum Temperature of the purified enzyme: The effect of different temperatures on the relative activity of purified CMCase is presented in Figure 5.6. It was found that the enzyme showed maximum activity at 50°C. However, when the temperature increased above 50°C the activity of the enzyme was affected negatively and gradually reduced. When the enzyme activity was determined below 50°C, a gradual decline in the CMCase activity was observed. As the enzymes are proteins in nature. The denaturation of enzymic protein occurs at elevated temperatures therefore, after certain level of temperature increase (above 50°C), the enzyme activity decreased rapidly. The present results are compared with the results obtained by Oyekola et al., (2007) and Kaur et al., (2007). They reported the same temperature (50°C) as optimum for CMCase activity. The results are in close agreement with the findings of Thongekkaew et al., (2008) who reported 40–50°C as an optimum temperature during the characterization of CMCase produced from Cryptococcus sp. S-2. The results are also supported by Fadel (2000) who found that the 55°C as a best temperature at which the enzyme was most active and stable. Saha (2004) also reported the same temperature i.e. 55°C as optimum for CMCase activity.
Fig. 5.6. Effect of different temperature on the CMCase activity.

These values are in agreement with those detected for the majority of the purified extracellular endocellulases from thermophilic fungi. In general, the endocellulases of thermophilic fungi are thermostable, with optimal activity between 55 and 80°C at pH 4.0–6.0 (Maheshwari et al., 2000).

5.6.2.4 Heat stability of the purified CMCase enzyme: Finding the heat stability of enzymes has considerable importance from the application viewpoint. The relationship of CMCase activity with the change in temperature is represented in Figure 5.7. As shown in the data presented in graph there is an inverse correlation between temperature increase and enzyme stability. The results revealed that the maximum enzyme stability was found at 50°C. However, by increasing in temperature beyond this the stability of the enzyme decreased rapidly and it exhibited only 30% stability at 90°C. Elevated temperature may cause the denaturation of enzymes thus resulting in decreased enzyme activity. In general, temperature significantly affected the enzyme stability. Previously, Kaur et al., (2007) also reported a significant loss in CMCase activity from 50 to 80°C that confirms present results. The results are also in line with those reported by Thongekkaew et al., (2008) that CMCase retained about 50% of its maximum activity after heating at 90°C. Likewise, the results are supported by recent findings of Karnehanatat et al., (2008) who observed a rapid decrease in enzyme activity at 70°C.
5.6.2.5 Substrate specificity of purified CMCase: The substrate specificity of the purified CMCase was tested by assaying the hydrolyzing activity on various cellulosic substrates like CMC (carboxy methyl cellulose), Filter paper, Microcrystalline cellulose, Phosphoric acid swollen cellulose, cotton, chitin, starch, cellulbiose, cellulose, and Para nitro phenyl glucopyranoside (PNPG) and the data are presented in Table 5.2.

The purified enzyme had significantly higher activity towards CMC, a soluble cellulosic substrate with β-1, 4-linkage, than any other substrates. The enzyme showed lower activity towards phosphoric acid swollen cellulose and filter paper. The purified enzyme could not hydrolyze crystalline cellulosic material such as cellulose powder which is probably due to the low affinity of the purified enzyme for crystalline cellulose. The purified enzyme did not exhibit detectable β-glucosidase or amylolytic activity. Furthermore, PNPG as the substrate for β-glucosidase was not degraded by the purified enzyme like other endoglucanases (Mackenzie et al., 1998). From these results, it seems more appropriate to refer to this enzyme as an endo type of cellulase. The present results were compared with other results. The endocellulase is similar to those isolated from other thermophilic fungi in its ability to degrade soluble forms of polysaccharides more readily than the crystalline cellulososes (Ganju et al., 1990; Roy et al., 1990).
Table 5.2: Substrate specificity of the purified CM cellulase of *Cladosporium sp*. NCIM 901.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Linkage</th>
<th>Enzyme activity (IU/mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMC</td>
<td>β-1,4</td>
<td>14.6</td>
</tr>
<tr>
<td>Filter paper</td>
<td>β-1,4</td>
<td>8.2</td>
</tr>
<tr>
<td>Microcrystalline cellulose</td>
<td>β-1,4</td>
<td>4.3</td>
</tr>
<tr>
<td>Phosphoric acid swollen cellulose</td>
<td>β-1,4</td>
<td>9.2</td>
</tr>
<tr>
<td>Cotton</td>
<td>β-1,4</td>
<td>0.8</td>
</tr>
<tr>
<td>Chitin</td>
<td>β-1,4</td>
<td>0.0</td>
</tr>
<tr>
<td>Starch</td>
<td>α-1,4</td>
<td>0.0</td>
</tr>
<tr>
<td>Cellobiose</td>
<td>β-1,4</td>
<td>0.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>β-1,4</td>
<td>0.0</td>
</tr>
<tr>
<td>PNPG</td>
<td>β-1,4</td>
<td>0.0</td>
</tr>
</tbody>
</table>

*The data presented are averages and standard errors of two independent experiments.

5.6.2.6 Effect of different reagents on activity of CMCase: The effect of different additives on CMCase activity of the purified CMCase was examined in the presence of various additives at 5mM concentration. Most of the additives used in this study except for CoCl₂ and HgCl₂ enhanced the CMCase activity as presented in Table 5.3. The Hg²⁺ strongly inhibited CMCase activity of the purified enzyme. It has been suggested earlier that the inhibition by Hg²⁺ ions is not just related to binding the thiol groups but may be the result of interactions with tryptophan residue or the carboxyl group of amino acids in the enzyme (Lamed et al., 1994). However the enzyme activity was enhanced by CaCl₂ and MgCl₂. It was not affected by ethylenediaminetetraacetic acid (EDTA), Dithiothreitol (DTT) assayed at pH 5.5.
Table 5.3: Effect of different reagents on the activity of purified CMCase.

<table>
<thead>
<tr>
<th>Effector</th>
<th>Concentration (mM)</th>
<th>Relative enzyme activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>100 ±0</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>5</td>
<td>110 ± 8</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>5</td>
<td>120 ± 3</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>5</td>
<td>82 ± 12</td>
</tr>
<tr>
<td>CoCl₂</td>
<td>5</td>
<td>64 ± 4</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>5</td>
<td>62 ± 3</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>102 ± 4</td>
</tr>
<tr>
<td>DTT</td>
<td>10</td>
<td>102 ± 12</td>
</tr>
</tbody>
</table>

*The data presented are averages and standard errors of two independent experiments.

CMC have versatile applications in various fields and low cost agricultural wastes used as substrate act as carbon and nitrogen sources. The CMCase of *Cladosporium* sp. was purified by ammonium sulphate precipitation and by using gel filtration chromatography on Sephadex G-75. The purified fraction, when subjected to native-PAGE and SDS-PAGE showed a single band. The purified enzyme showed maximum activity at pH 5.5 and was stable at pH 4.4 to 6.0. The enzyme showed maximum activity at the temperature of 50°C. The result from this study reveals that *Cladosporium* sp. showed higher CMCase activity and the purified enzyme was used in various applications.
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