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Isolation, purification and characterization of carboxymethyl cellulase (CMCase) from endophytic *Fusarium oxysporum* producing podophyllotoxin

Refaz Ahmad Dar[1]*, Iram Saba[1], Mohd. Shahnawaz[2], Manisha Kashinath Sangale[2], Avinash Bapurao Ade[2], Shabir Ahmad Rather[1], Parvaiz Hassan Qazi[1]

[1]Microbial Biotechnology Division, Indian Institute of Integrative Medicine (CSIR), Sanatnagar, Srinagar, Jammu and Kashmir, India; *Corresponding Author: refazahmad@gmail.com
[2]Department of Botany, University of Pune, Pune, Maharashtra, India

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ABSTRACT

Endophytic fungus *Fusarium oxysporum* is a rich source of cellulases. In the present study, the highest activity was reported at 28˚C, pH 5.6 with 2% Carboxymethyl cellulose (CMC) as carbon source. CMC was purified using Sephadex G and DEAE cellulose chromatography to 15.9 folds and the molecular weight was determined to be 84 kDa by SDS-PAGE analysis and was subsequently characterized. The purified enzyme was stable over the pH range from 4.0 to 8.0 and at temperatures below 50˚C. The enzyme was highly active on CMC and reduced or no activity on Avicel, cellobiose and it was suggested to be CMCase/endoglucanase. The activity of endoglucanase was enhanced in the presence of $MgCl_2$, $CoCl_2$, $FeCl_3$, $CaCl_2$, $FeCl_2$ and intensive to $HgCl_2$. The purified enzyme showed its optimum activity at pH 5.0 - 6.0 and was quite stable at 50˚C for 30 min and retained 45% of original activity.

Keywords: Endophyte; *Fusarium oxysporum*; *Juniperus recurva*; Podophyllotoxin; CMCase

1. INTRODUCTION

The most important source of carbon on this planet is cellulose, and is being synthesized by both land plants and marine algae at the rate of 0.85×10^{11} tonnes per annum [1,2]. The combined and co-operative action of endocellulases, exocellulases (cellobiohydrolases and glucanohydrolases) and beta-glucosidases (β-D-glucoside glucohydrolase), leads to the degradation of cellulose into glucose [3,4]. The random hydrolysis of internal glycosidic linkages due to endocellulases leads to diminishing the length of the polymer followed of gradual increase of reducing sugar concentration. Whereas the removal of cellobiose from reducing or the non-reducing ends due to hydrolysis of cellulose by exocellulase results in rapid release of reducing sugars, the polymer length experiences a little change. The synergetic action of endocellolose and exocellulose on cellulose leads to the production of cellooligosaccharides and cellobiose. The beta-glucosidase cleaved both the cellooligosaccharides and cellobiose and results in the production of glucose [5,6]. Cellulases have been widely used in various industries such as agriculture, bioconversion, detergents, fermentation, food, pulp and paper and textile for biomass and genetic engineering [7-9]. Throughout the biospshere these cellulases are distributed and are being manifested in microbes such as bacteria, fungi and actinomycetes [10-12]. In literature there are various reports on degradation of cellulose by fungi [13,14]. The cellulolytic microbes utilize cellulosic substrates during the growth phase and produce a complex array of glycolsacyl hydrolases and all the components of this multienzyme system including its specification and mode of action are reported in *Trichoderma* sp. (filamentous fungi) [15]. At present, many research groups around the globe are screening new cellulases to identify the enzymes with high specific activity and stability which have significant biotechnological applications [16,17]. Various workers reported that the production costs of cellulases are highly associated with the microbial strains capable of its production [18,19]. Various factors such as pH, temperature, incubation period, cations, carbon, and nitrogen sources are responsible for the yield of enzyme production [20,21].

Podophyllotoxin, a well-known naturally occurring aryltetralin lignin occurs in few plant species and is used as a precursor for the chemical synthesis of various anti-cancer drugs like etoposide, teniposide and etopophose phosphate [22]. In the present study known, endophytic fungal strain producing podophylotoxin was used for production of cellulase. Since it was identified to be the *Fusarium oxysporum* producing podophyllotoxin and it was shown to possess cellulase enzyme complexes as well. In the present study an attempt has been made to determine some other factors that would be responsible for the optimal production of cellulase by the fungus.

2. MATERIALS AND METHODS

2.1. Organism and Culture Conditions

The strain of the endophytic fungus *Fusarium oxysporum* producing podophyllotoxin was procured from the fungal repository of Indian Institute of Integrative Medicine (CSIR), Srinagar, Jammu and Kashmir State, India, which had earlier been isolated from *Juniperus recurva*a (a medicinal plant). The strain was cultured at 28˚C and was routinely maintained on potato dextrose agar (PDA) medium by periodic transfers. The spore suspension of *Fusarium oxysporum* was transferred to 500ml Erlenmeyer flasks containing 100 ml of sterilized medium composed of following composition: glucose 1.0%; yeast extract 1.0%; KH_2PO_4 0.6%; K_2HPO_4 0.04%; $MgSO_4 \cdot 7H_2O$, 0.05%; urea 0.05% and the pH was adjusted to 5.6. The flasks were incubated in a rotator shaker (180 rpm) at 28˚C [23].

2.2. Isolation of Enzymes

Extra cellular enzymes were isolated by filtering the culture through Whatman No. 1 filter paper. The Carboxymethyl cellulase (CMCase), cellobiase (1,4-*β*-glucosidase) and filter paper activity (Fpase) were measured using the methods described by Ghose (1987). One unit of enzyme activity (IU) was defined as the amount of enzyme that released 1 µmol of glucose per ml per minute. The protein content was determined by Bradford method [24].

2.3. Optimization of Growth Conditions

To determine the optimum temperature and pH of enzymes, the crude CMCase activity was measured under standard assay conditions, the temperature was varied from 25˚C to 50˚C and the pH was adjusted within the range of 4.0 to 8.0. All the experiments were performed in triplicates.

2.4. Purification of the Enzyme

All extraction steps were performed at 4˚C. Cellulase was purified from the fungal filtrate after concentration by freeze drying and storing at 4˚C. The enzyme protein was bulk precipitated by $(NH_4)_2SO_4$ (70%) and dissolved in a minimum volume (100 ml) of 0.1 M citrate phosphate buffer (pH 5.0). The enzyme was dialyzed against the same buffer for 24 h at 4˚C. 50 ml of enzyme protein was loaded onto a column chromatogram of 100 Sephadex G (18 × 2 cm) pre-equilibrated with 50 ml buffer. The column was eluted with the same buffer at 20 $ml \cdot h^{-1}$ and 5 ml fraction was collected. Fraction was analyzed for protein and activity of CMCase. The most active fractions were pooled, concentrated by freeze-drying and dialyzed as before. The pooled fraction was loaded (25 ml) onto column chromatograms of DEAE-cellulose (Diethyl-amino-ethyl-cellulose). The column was eluted with gradient of 0 - 0.8 M NaCl at a flow rate of 10 ml/h^{-1} and 5 ml fraction was collected and dialyzed once again to remove Na^+ and Cl^-.

2.5. Determination of Molecular Weight

The apparent molecular weight of the purified cellulase from fugal isolate was determined by sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn [25] with protein molecular weight ladder. The gels were stained with coomassie brilliant Blue R-250 and destained in acetic acid: methanol: water (1:4:5 v/v).

2.6. Effect of Cations on Endoglucanase Activity

The effect of different metal ions on production of the enzyme was determined by the addition of the corresponding ions at a concentration of 10 mM to the medium. The enzyme production was studied in the presence of ($MgCl_2$, $CoCl_2$, $FeCl_3$, $CaCl_2$, $FeCl_2$ and $HgCl_2$). The activity is expressed as a percentage of the activity in the absence of metal ion.

2.7. Stability of the Purified Enzyme

The thermal and pH stability of the purified enzyme was monitored by incubating the enzyme at various temperatures ranging from 25˚C - 50˚C for 30 min and pH ranging from 4 - 8. Then the treated enzyme was assayed for activity.

3. RESULTS AND DISCUSSION

3.1. Selection of Cellulase Producing Fungal Strain

The cellulase degrading activity of the endophytic fungal strain was assessed through agar diffusion assay **Figure 1**. The strain *Fusarium oxysporum* JRE1 pro-

duced the clear hydrolytic zone and therefore was maintained on PDA slants for further use.

3.2. Effect of Different Carbon Sources for Enzyme Production

Different carbon sources used for the cellulase production were wheat straw, wheat bran, crystalline cellulose and carboxymethyl cellulose (CMC). The best cellulase activity was found in the CMC, but extracellular enzymes were higher in shaking cultures than in static cultures. Different concentrations of the carbon sources were used. Among them the best CMCase activity was recorded with 2% CMC (16.8 U/ml) followed by 2% wheat bran (11.5 $U \cdot mg^{-1}$) where as wheat straw and crystalline cellulose does not showed any marked activity (**Figure 2**).

3.3. Purification and Electrophoresis of Enzyme

The Supernatant from 7-day-old submerged culture of the *Fusarium oxysporum* grown on 2% CMC, 1% soluble starch, and 0.4% yeast extract was used for endocellulase purification. Fractionation of concentrated, dialysed culture filtrate using ion-exchange chromatogramphy on DEAE-Sepharose separated three peaks of endocellulase activity. Fractions of the first and second peak had major amounts of the enzyme activity (over 75%). The major endocellulase component was further purified on a Phenyl-Sepharose column (**Table 1**). The major endocellulase component was purified 15.9 fold with a yield of 27.7% to a specific activity of 2.9 $U \cdot mg^{-1}$ of protein. A single protein band was observed by SDS-PAGE (**Figure 3**), indicating that the major endocellulase had been purified to homogeneity.

Figure 1. Agar plate showing clear zones of hydrolysis formed by *F. oxysporum.*

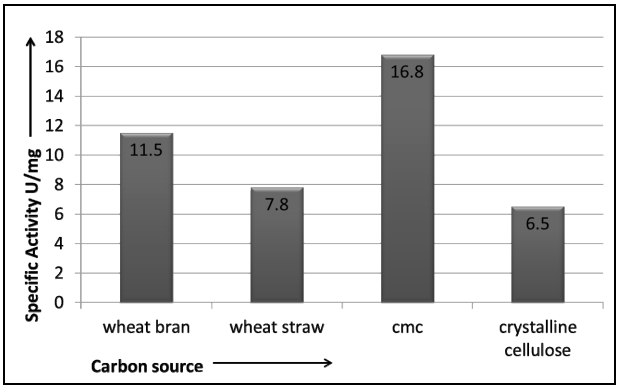

Figure 2. Production of cellulase production by *F. oxysporum* on different carbon source.

3.4. Effect of Metal Ions

Among different metals examined the endoglucanase production was enhanced in the presence of metal cations like $MgCl_2$, $CoCl_2$, $MnCl_2$ and $CaCl_2$ and to some extent by $FeCl_2$. The result also shows that endoglucanase was insensitive to $HgCl_2$ (**Table 2**).

3.5. Effect of pH

The enzyme was active over a broad range of pH (4.0 - 8.0), showing maximum activity at pH 5.6. The effect

Table 1. Details of the purification of the enzyme CMCase.

Step	Total protein (mg)	Total activity (U)	Specific activity ($U \cdot mg^{-1}$)	Recovery (%)	Purification (fold)
Crude extract	188.7	203.4	0.9	100	1
70% $(NH_4)_2SO_4$	41.6	290.8	8.3	90.6	4.9
DEAE-Sepharose	14.2	170.6	16.8	34.1	10.5
Phenyl-Sepharose	2.9	98.2	27.7	16.3	15.9

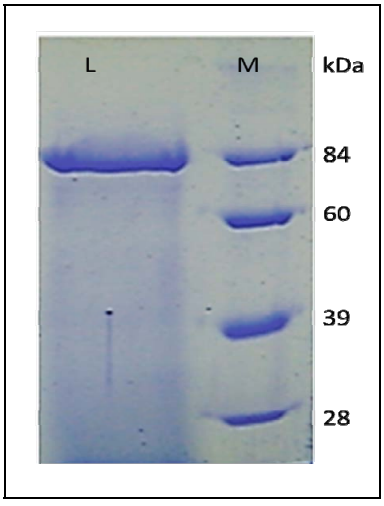

Figure 3. SDS-PAGE (8%) analysis of cellulase b purified from *F. oxysporum*. M: Protein ladder; L: Purified bands of CMCase.

of pH on stability of the enzyme was studied by using CMC as a substrate under the standard assay condition. The pH-stability profile of the CMCase was determined by the residual activity measurement showed 75% of its original activity was retained between pH 4.0 - 7.0 (**Figure 4**).

3.6. Effect of Temperature

The enzyme displayed significant activity within a temperature range of 25˚C - 37˚C with maximum activity at 28˚C and at pH 5.6. In order to examine the temperature stability of the CMCase, the purified cellulase was maintained after 30 min incubation at temperatures ranging from 25˚C to 50˚C and pH 5.6. The enzyme retained more than 70% of its maximum activity after 30 min exposure to temperatures of 25˚C - 37˚C and 45% after 30 min exposure at a temperature of 50˚C (**Figure 5**).

A CMCase enzyme was purified from this strain and biochemically characterized. The podophylotoxin

Table 2. Effect of different metal ions on CMCase production by *F. oxysporum*.

Metal ion	Final conc. (mM)	Relative activity (%)
None	0	100
$CaCl_2$	10	100
$CoCl_2$	10	109
$HgCl_2$	10	43
$MgCl_2$	10	116
$MnCl_2$	10	158
$FeCl_2$	10	95

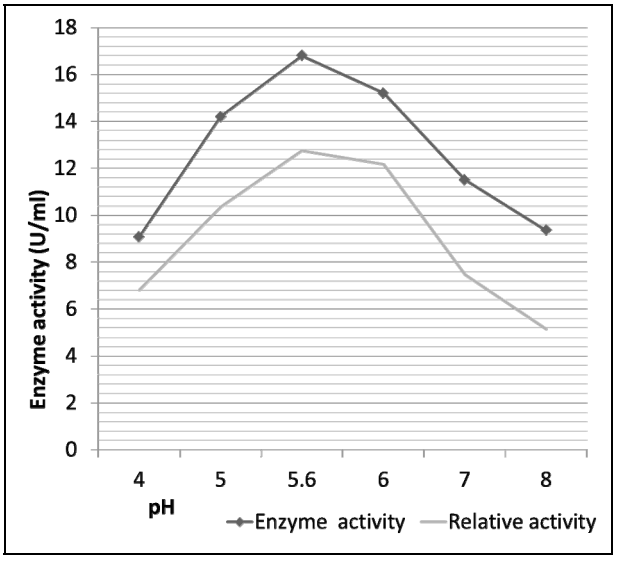

Figure 4. Cellulase activity at different pH (4 - 8).

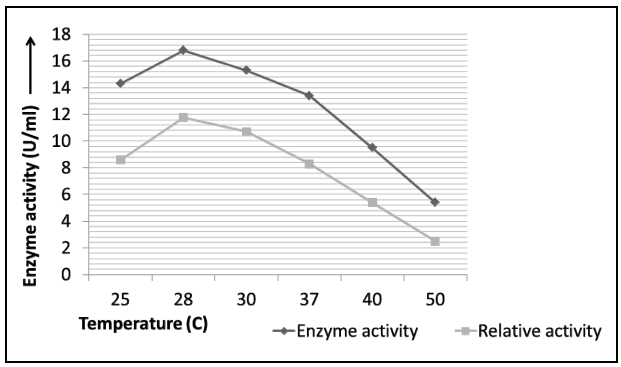

Figure 5. Cellulase activity at different temperature (25˚C - 50˚C).

producing endophytic fungus already isolated and identified as *Fusarium oxysporum* was subjected to submerged fermentation using different residues as raw material. The enzyme was highly active on carboxymethyl cellulose (CMC) but much reduced or no activity was observed on Avicel, cellobiose and it was suggested to be a CMCase/endoglucanase. Independent researchers have also reported the CMCase from the same strain of the fungus, but here we are reporting for the first time the endophytic fungal strain producing podophylotoxin that is a well known anticancer drug. Kumar *et al.* [26] also reported highest CMCase activity with CMC as a carbon source in *Paenibacillus polymyxa*. A similar trend has also been reported in *Bacillus* sp. [27]. The enzyme was stable over a broad pH range and was in agreement with the previous reports [27-30]. The temperature also plays an important role in activity and the stability of the enzymes. Over a broad range of temperature, the enzyme was also found stable and was also supported by many workers around the globe [27-30]. Finally, the microorganism is promising for industrial application since it grows quickly in submerged condition in simple and of low cost substrates and secretes the enzymes extracellularly and shares features which are frequently required for industrial application.

To the best of our knowledge no one has yet reported CMCase from the endophytic fungi (*F. oxysporum*) from the Sonamarg Area of Kashmir valley, India. This is the first report of CMCase production from the *F. oxysporum* from this region.

4. CONCLUSION

Based on the study carried out, it can be concluded that the enzyme was highly active on carboxymethyl cellulose (CMC) but much reduced or no activity was observed on Avicel, cellobiose and it was suggested to be a CMCase/endoglucanase. The enzyme was stable over a broad pH range and temperature and can be produced commercially at the cheapest cost. Thus we conclude that

a single endophytic fungal strain which is a source of anticancer molecule (podophyllotoxin) will be a very good source for bioethanol production from low cost substrates.

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