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Purification and Characterization of Carboxymethyl Cellulase (CMCase) from P*enicillium ochrochloron* isolated from forest soil of Neyyar Wild Life Sanctuary, India.

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Abstract:

A fungal strain TBG-71F isolated from the soil samples collected from Neyyar wild life sanctuary of Kerala is a rich source of cellulases and was identified as *Penicillium ochrochloron* based on morphological, micro-morphological and ITS1-5.8S-ITS2 sequence similarity. In the present study the highest Carboxymethyl Cellulase (CMCase) activity was reported at 27°C, pH 5.5 with 1% Carboxymethyl cellulose (CMC) as carbon source. CMC was purified using Sephadex G200 and to Sephadex G50 chromatography to 15.9 folds and the molecular weight was determined to be 55 kDa by SDS-PAGE analysis and was subsequently characterized. The purified enzyme has an optimum activity over the pH range from 4.0 to 5.0 and at temperature 60^{o}C. The enzyme was highly active on CMC and it was suggested to be CMCase/endoglucanase.

Key Words: Soil fungi; *Penicillium ochrochloron*; ITS1-5.8S-ITS2 rDNA, CMCase

Introduction:

Lignocellulosic complex is the most abundant biopolymer on the earth comprising about 50% of world biomass and is hence a prominent source of renewable non-fossil carbon on this planet, being synthesized at the rate of 0.85 x 10^{11} tons per annum [1, 2]. Lignocellulosic biomass mainly contains cellulose, a homopolymer of glucose, in bound form along with hemicelluloses and lignin and typically, lignocellulosic biomass contains about 40-60% cellulose, 20-40% hemicelluloses, and 10-25% lignin [3]. The combined and co-operative action of endocellulases (EC

3.1.1.4), exocellulases (cellobiohydrolases EC 3.2.1.91 and glucanohydrolases EC 3.2.1.74) and beta-glucosidases (β-D-glucoside glucohydrolase EC 3.2.1.21), leads to the degradation of cellulose into glucose [4]. Endocellulases hydrolyze internal glycosidic linkages in a random fashion, which results in a rapid decrease in polymer length and a gradual increase in the reducing sugar concentration. Exocellulases hydrolyze cellulose chains by removing mainly cellobiose either from the reducing or the non-reducing ends, which leads to a rapid release of reducing sugars but little change in polymer length. Endocellulases and exocellulases act synergistically on cellulose to produce cellooligosaccharides and cellobiose, which are then cleaved by β-glucosidase to glucose [5]. The monomeric sugars produced enzymatically from cell wall polysaccharides can be used for biorefining to produce a range of bio-materials. Fermentation, chemical catalysis, or other processes may be used to create novel products such as ethanol or other valuable chemical intermediates that can be used as chemical feed stocks in manufacturing processes [2].

There are various reports on fungi producing cellulases [6], but only a few have proved high activities for commercial success [7]. Unlike other genera, *Penicillium* synthesize more balanced cellulolytic complexes, which efficiently degrade cellulose and cellulose containing residues and were found to be more stable [8]. Recently, many research groups around the globe are screening new cellulases to identify the enzymes with high specific activity and stability which has significant biotechnological applications [5]. The present study deals with the endoglucanase activity of a fungal strain (TBG-71F) isolated from the forest soil of Neyyar wild life sanctuary in Kerala, which was identified as *Penicillium ochrochloron* based on morphological characters and ITS-28S sequence analysis. An attempt has been made to determine some of the factors that are responsible for the optimal production of CMCase by this fungus and also its partial purification and characterization.

Materials and Methods:

Organism and culture conditions:

The strain of the fungal strain TBG-71F was isolated from the forest soil samples collected from Neyyar wild life sanctuary of Kerala State, India. The strain was cultured at 28 ± 1^0C and was routinely maintained on potato dextrose agar (PDA) medium by periodic transfers.

Characterization of Fungal Isolate

The cultural characteristics of the colonies were observed on Czapek's agar and potato-dextrose agar (PDA) plates at 28°C for 7 days. Morphological characteristics of conidiogenous cells and conidia were observed by slide culture on the previously mentioned agar media at 28°C for 3 to 7 days. The isolates were identified based on their morphological and cultural characteristics according to the criterions laid down [9, 10].

The isolated fungal colonies were cultured on PDA plates and incubated at 28±1°C for 5 days for complete sporulation. Morphological characteristics such as colony diameter, conidial colour, exudates, reverse colony colour etc were observed and

recorded. Microscopic characteristics were observed by preparing wet mounts of the fungal mycelia with lactophenol cotton blue stain [11]. The slides were observed under a binocular light microscope (NIKON, Japan) and photographed.
The genomic DNA was prepared from the TBG-71F strain using modified CTAB Method [12]. The ITS1-5.8S-ITS2 region was amplified and sequenced using fungal-specific primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS4 (5′-TCCTCCGCTTATTGAT ATGC-3′) as previously described [13]. The sequences were searched against those already known at NCBI GenBank (http://www.ncbi.nih.gov/index.html) using BLAST search option. The sequences of ITS region were aligned with the sequences of similar fungi retrieved from NCBI database using CLUSTAL W, and a phylogenetic tree was constructed using the neighbor-joining algorithm [14]. Evolutionary analyses were conducted in MEGA version 6.0 [15] with the bootstrap analysis of 1000 replicates [16].

Preparation of crude enzyme
The spore suspension of *P. ochrochloron* TBG-71F was transferred to 500ml Erlenmeyer flasks containing 100 ml of sterilized medium composed of following composition: Carboxy Methyl Cellulose 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 room temperature for 5 days. Culture filtrates were collected by filtration (Whatman no. 1) and centrifuged at 10,000 rpm for 15 minutes at 4°C temperature in the centrifuge (Hermle Z323K, Germany) to remove the spores of the organism. The crude enzyme thus obtained was subjected to enzyme assay.

Determination of Mycelia Dry Weight
For this purpose, the fermented broth was filtered, using pre-weighed Whatman no.1 filter paper. It was washed with distilled water thrice and then dried at 100 ^{0}C over night in a hot air oven and weighed.

Cellulase Enzyme assay
Filter paper activity (FPA) for total cellulase activity in the culture filtrate was determined according to the method reported by Hankin and Anagnostakis [17]. Aliquots of appropriately diluted cultured filtrate as enzyme source was added to whatman no. 1 filter paper strip (1x6 cm; 50 mg) immersed in one milliliter of 0.05 M Sodium citrate buffer of pH 5.0. After incubation at 50^{0}C for 1 hrs, the reducing sugar released was estimated by Dinitrosalicylic acid (DNS) method of Nelson and Somogyi [18]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1µ mole of reducing sugar from filter paper per ml per min. Carboxy methyl cellulase (CMCase) activity was determined by measuring the amount of reducing sugars released from CMC using the method of Nelson and Somogyi [18]. The standard assay mixture contained 1.5% w⁄ v CMC in a final volume of 0.1-ml of 50-mmol l^{-1} acetate buffer at pH 4.5. The mixture was incubated at 40^{0}C for 15 min. Colour development was measured at 520 nm. One unit of enzymatic activity was defined as the amount of enzyme that released 1µ mol of

reducing sugar equivalent per minute under the assay conditions described. The β-glucosidase activity was assayed according to the method by Pointing [19]. The total protein content was determined by Bradford method [20].

Optimization of production conditions:
To optimize the CMCase production, effects of fermentation conditions such as the different carbon source, incubation period, temperature, and pH were studied. Effect of various carbon compounds viz., crystalline cellulose, Carboxy methyl cellulose (CMC) and glucose were used for studying. The basal broth was distributed into different flasks and 1.0 (%w/v) of each carbon sources were added to the basal medium and after culture inoculation, the flasks were incubated for at room temperature for 6 days and the crude CMCase activity was measured under standard assay conditions. To determine the effective incubation period and temperature for cellulase production, fermentation was carried out at different temperature (25°C, 27°C, 29°C, 31°C and 33°C) for 6 days. Enzyme assay was carried out at interval of 24 hours by DNS method. Effect of pH on enzyme production was determined by producing the cellulase at different pH ranging 4-8, was adjusted by using 1N HCl or 1N NaOH. All the experiments were performed in triplicates.

Purification of cellulase
All the purification steps were performed at 4^0C unless otherwise stated. CMCase was purified by ammonium sulfate precipitation followed by gel filtration chromatography on Sephadex G200 and Sephadex G50 column. For the purification of extracellular cellulases the crude culture supernatant prepared 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. The dialyzed samples were added on to Sephadex G200 column (30 cm) pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0. The cellulases were eluted at a linear flow rate of 30 cm/h. Different fractions of the enzymes were pooled, concentrated by dialysis and applied to Sephadex G50 column. Elution was performed at a flow rate of 30 cm/h. The concentrated fractions were subjected to enzyme activity. Protein concentration was determined by Bradford method [20] using bovine serum albumin as standard.

Determination of molecular weight:
The apparent molecular weight of the purified cellulase from fungal isolate was determined on 8% sodium dodecyl sulfate-polyacryamide gel electrophoresis (SDS-PAGE) essentially as described by Laemmli [21]. The gels were stained with coomassie brilliant Blue R-250 and de-stained in acetic acid: methanol: water (1:4:5 v/v) and visualized.

Effect of Temperature and pH on the activity of purified enzyme:
To evaluate the optimum temperature of the enzyme, the activity was determined by

carrying out the assay at several temperatures between 30 and 90°C. 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).

Results and Discussion

Taxonomic Identification of the fungal strain:

The fungal isolate was identified by morphological traits and fungal ITS1-5.8S-ITS2 region sequence analysis. The colonies on Czapek's agar and Potato Dextrose agar plates showed rapid growth, attaining a diameter of 9.0-14.0 cm. in 7 days at 27°C. Colonies appeared as closely interwoven fine vegetative hyphae with cottony surface and buff (on Czapek's agar) to green (on PDA) in colour, while reverse side is mostly yellow-orange in color. The penicilli were abundant in number and more in marginal than central colony areas. On observation of morphological and microscopical characters, fungus represented the properties of *Penicillium* genus as it displayed monovertecillate. Conidiophores were erect, septate, and branched. Conidia appeared in globose to ovate in shape and born as 2-3 in chains, which typically forms brush like head (Figure 1). Genomic DNA was extracted from the isolate TBG-71F and then the complete ITS and 5.8S rDNA were amplified using primers ITS1 and ITS4. The PCR fragment length was 580 bp, which coincided with the expected size and was sequenced. A homology search was performed with the resulted 540 bp sequence using BLAST algorithm (NCBI). It showed 99% homology to *Pencillium ochrochloron*. The CLUSTAL W programme was used for multiple alignment of ITS1-5.8S-ITS2 region of TBG-71F with similar sequences retrieved from GenBank and a phylogenetic analysis was done using MEGA6 software. The topology of the resultant tree was evaluated by bootstrap analysis of the Neighbour-joining method based on 1000 resamples (Figure 2). Based on the morphological, micro-morphological and ITS1-5.8S-ITS2 sequence similarities, the isolate TBG-71F was identified as *Penicillium ochrochloron.* Different species of *Penicillium* including *P. echinulatum* 9A02S1 [22], *P. decumbens* [23], *P. brasilianum* IBT20888 [24] have been reported to have promising cellulase activities.

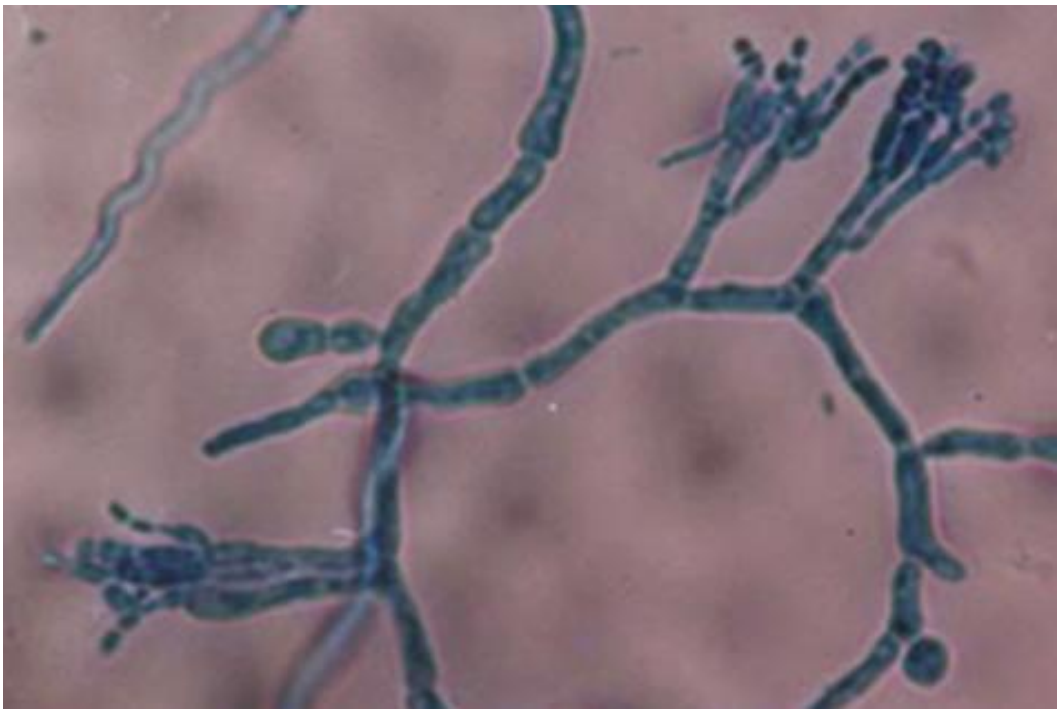

Figure 1: Microphotograph of Fungal Isolate TBG-71F showing Conidiophores with Conidia.

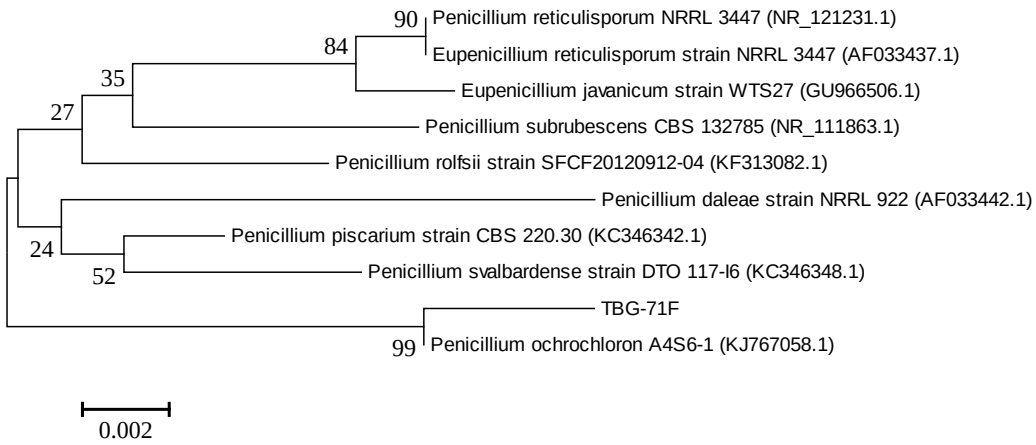

Figure 2: Neighbour-joining Phylogenetic tree showing Evolutionary relationships of TBG-71F:

The evolutionary history was inferred using the Neighbor-Joining method [14]. The optimal tree with the sum of branch length = 0.06014739 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [16]. The tree is drawn to scale, with

branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Jukes-Cantor method [25] and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 517 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 [15].

Effect of different carbon sources on enzyme production

Effect of supplementation of different carbon sources to basal medium on cellulase production by *P. ochrochloron* was examined. The carbon sources used were crystalline cellulose (CC), carboxymethyl cellulose (CMC) and glucose (G). Among these, CMC served as the best source for cellulase production followed by cellulose (table-1). Enzyme activities of even other individual components of cellulase, such as CMCase and β-glucosidase were also highest in culture filtrate of *P. ochrochloron* grown in the presence of CMC. The use of glucose as carbon source supported maximum growth of the fungal strain but resulted in minimum production of cellulases.

Table 1: Effect of Carbon Sources on Cellulase Production by *Pencillium ochrochloron* TBG-71F

Carbon Sources	**Mycelial dry weight (gL^{-1})**	**Cellulases**		
		FPase (UmL^{-1})	**CMCase (UmL^{-1})**	**β-glucosidase (UmL^{-1})**
Crystalline cellulose (CC)	2.12 ± 0.06	1.73±0.058	4.85±0.062	2.01±0.004
Carboxymethyl cellulose (CMC)	4.36 ± 0.30	1.92±0.008	7.59±0.075	2.84±0.037
Glucose (G)	5.42 ± 0.11	1.00±0.011	1.49±0.025	1.81±0.026

Note: Values are average from 3 replicates ± SD (Standard Deviation).

The cost and availability of substrate is crucial in the successful production of cellulases in an economic way. Here we have compared five different substrates for the production of cellulase enzymes by *P. ochrochloron.* The choice of an appropriate substrate is of great importance as cost effective production can be achieved by the use of cheap easily available substrates. The substrate not only serves as a carbon source but also produces the necessary inducing compounds for the organism [26]. It has been reported that endoglucanase activity was repressed by glucose and induced by CMC [27]. Lucas *et al*. [28] demonstrated that CMC was preferred substrate for endoglucanase production. We also observed similar results with comparatively low cellulase activity in the presence of glucose, while CMC proved to be a strong inducer of cellulase enzymes.

Effect of Incubation period, Temperature and pH CMCase production

Optimization of incubation period and temperature were done by inoculating *P. ochrochloron* strain TBG-71F in CMC medium and incubating at different

temperatures for 6 days (Figure 3). The fungal strain displayed significant cellulase activity within a temperature range of 25-33^{0}C with maximum activity at 27^{0}C on 96 hrs of incubation (7.59 UmL^{-1}). The CMCase production is much higher than that reported for *P. pinophilum* grown in cellulose-supplemented mineral media (1.9 U ml^{-1}), but lower than the production by *P. purpurogenum* (24 U ml^{-1}) [29, 30]. The CMCase yield by *P. ochrochloron* appears to depend on pH of the medium (Figure 4). Results show that CMCase production, gradually increased as the pH value increased from 5-6 and reached its optimum (7.7 UmL^{-1}) at pH of 5.5. The optimal pH for fungal cellulases varies from species to species, though in most cases the optimum pH ranges from 3.0 to 6.0 [31].

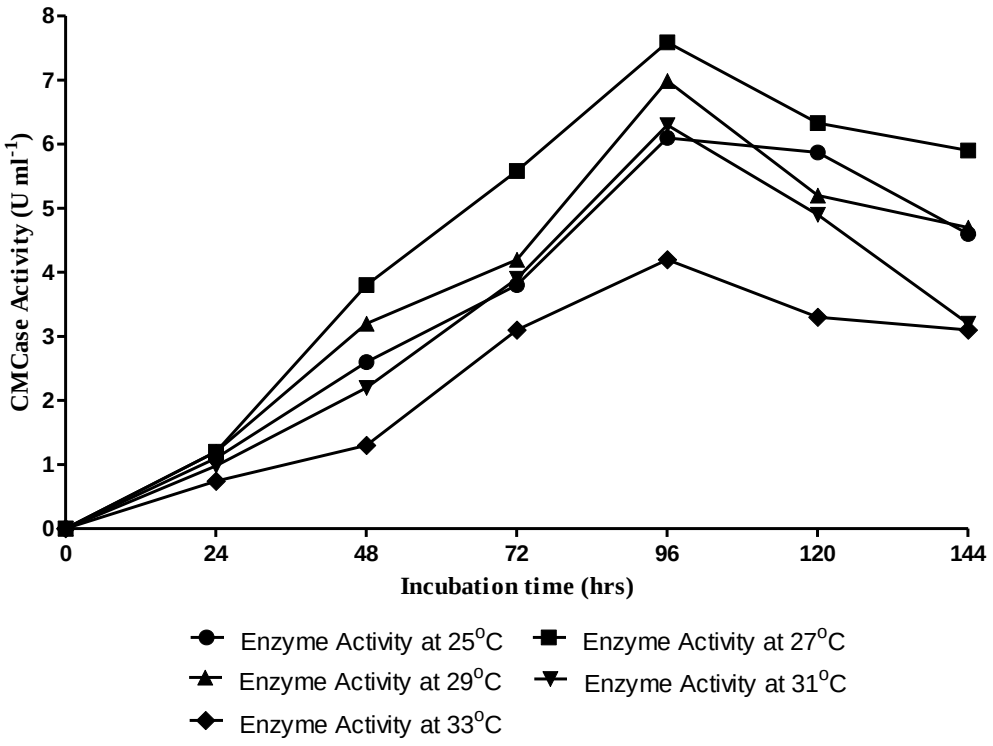

Figure 3: Effect of Incubation Time and Temperature on CMCase activity of *P. ochrochloron* TBG-71F

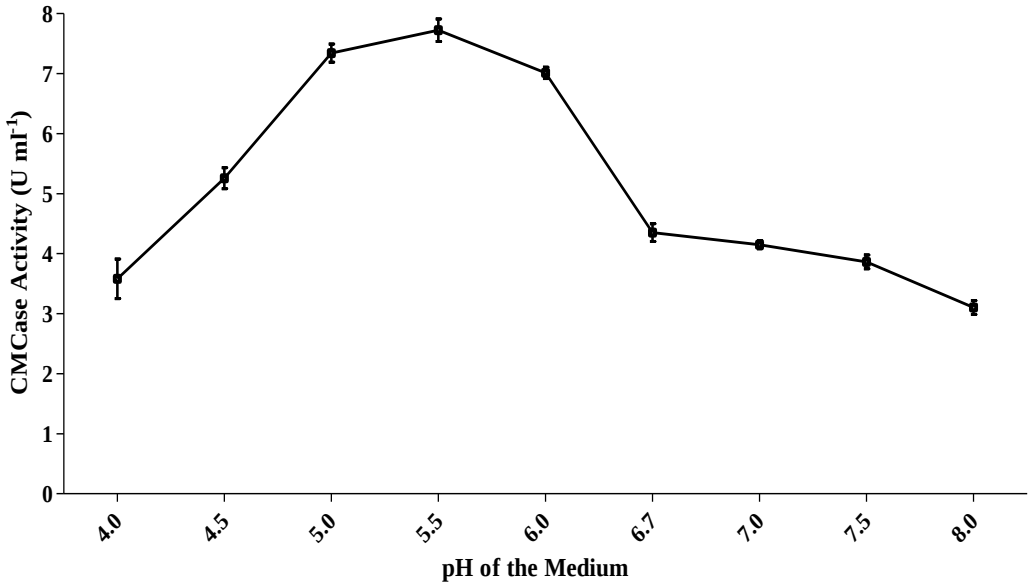

Figure 4: Effect of Initial pH on CMCase activity of *P. ochrochloron* TBG-71F

Purification and electrophoresis of enzyme:
Supernatant from 6-day-old submerged culture of the *P. ochracloron* TBG-71F grown on basal media with 1% CMC was used for endoglucanase purification. Fractionation of concentrated, dialyzed culture filtrate using a column packed with sephadex G-200 produced three peaks of endoglucanase activity. Fractions of the first and second peak had major amounts of the enzyme activity (over 75%). The major endoglucanase component was further purified on a Sephadex G-50 column (Table 2). The major endoglucanase component was purified 19.2 fold with a yield of 29.01% to a specific activity of 50.21 Umg−1 of protein. A single protein band with a molecular size of 58 kDa was observed by SDS-PAGE (Figure 5), indicating that the major endoglucanase had been purified to homogeneity. The endoglucanases purified from *P. notatum* NCIM NO-923 also had a mass of ~55 kDa [32] while endoglucanases from *P. simplicissimum* H-11 and *P. pinophilum* MS20 were found to have molecular masses of 33.2 kDa [33] and 42 kDa [34] respectively. It was reported that molecular weight of endoglucanase enzyme isolated from *Trichoderma sp*. is around 55 kDa [35].

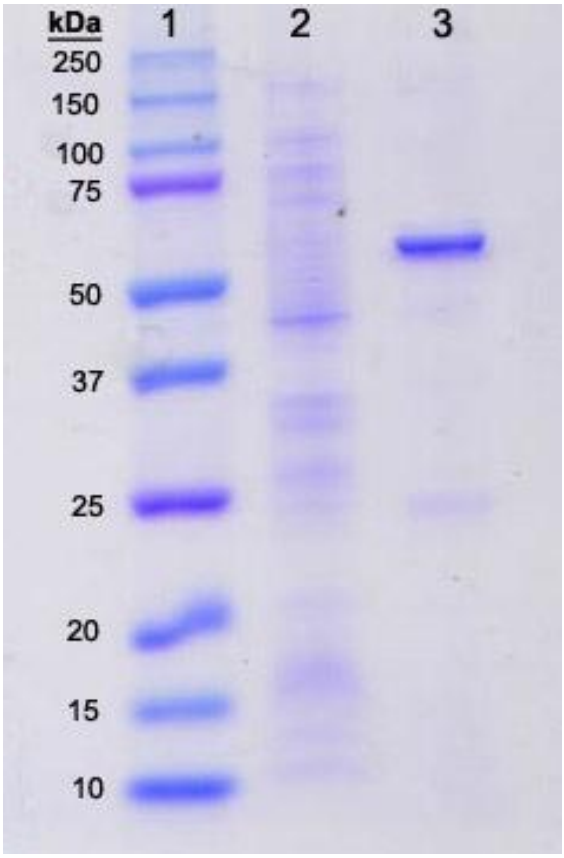

Figure 5: SDS PAGE (12%) Showing the Purified CMCase: Lane 1 represents the broad range protein weight marker, Lane 2 represents crude protein and Lane 3 represents purified protein to homogeneity.

Table 2: Profile of step wise endoglucanse (CMCase) purification from *P. ochrochloron*TBG71F under submerged Fermentation

Purification step	Specific Activity (U mg^{-1})	Yield (%)	Purification (fold)
Culture Filtrate	2.68	100	1
$(NH_4)_2SO_4$ precipitation	4.03	77.59	1.53
Gel Filtration on Sephadex G-200	21.89	45.42	8.35
Gel Filtration on Sephadex G-50	50.21	29.01	19.20

Effect of Temperature and pH on the activity of purified enzyme

It was found that the enzyme exhibited maximum activity at 60°C. The enzymatic activity was negatively affected by temperature above 60 °C and gradually reduced (figure 6A). The results show that the endoglucanase (CMCase) purified from *P. ochracloron* TBG-71F has higher optimum temperature than other characterized cellulases from the genus. Cellulases from *P. notatum* NCIM NO-923 and *P. occitanis* showed maximum activity at 50 ^{o}C and 55 ^{o}C respectively [32, 36]. When

the enzyme activity was analysed at different pH values, the highest enzyme activity was found at 4.5pH and showed a declining trend in the enzyme activity at higher and lower pH (figure 6B).

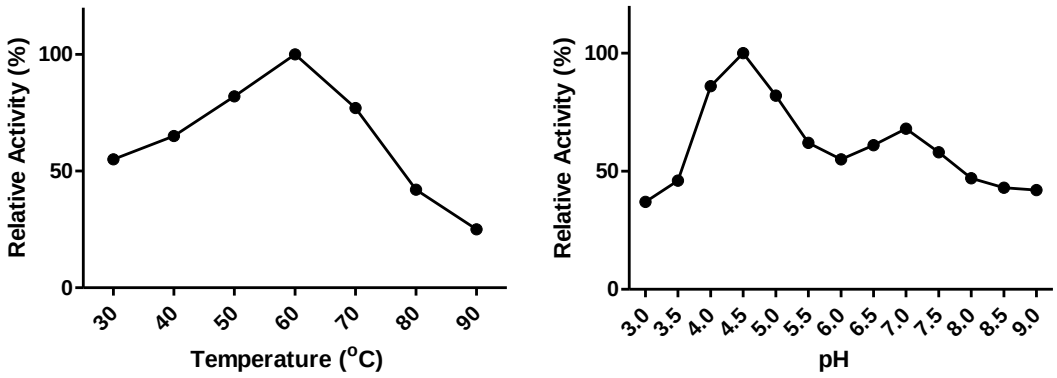

Figure 6A & B: Effect of pH and temperature on carboxy methyl cellulase (CMCase) activity of *Penicillium ochracloron* TBG-71F.

Conclusion

The results of the present investigation demonstrate the identification and characterization of a fungal strain TBG-71F exhibiting potential to secrete endoglucanase (CMCase) maximally when CMC was used as carbon source in the culture medium, at 96 hrs of incubation, at 27 ^{o}C and at an initial pH of 5.5. The fungal strain have been characterized and identified as *Penicillium ochracloron* based on morphological characters and ITS1-5.8S-ITS2 sequence homology analysis. The enzyme was purified to homogeneity and was found to have a molecular size of 58 kDa. The optimum enzymatic activity is at a temperature of 60 ^{o}C and pH of 4.5. The strain secretes high levels of CMCase, and that makes it a good candidate for industrial applications.

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