Submerged fermentation and characterization of carboxymethyl cellulase from a rhizospheric isolate of *Trichoderma viride* associated with *Azadirachta indica*

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Analysis of process parameters influencing the submerged fermentation of carboxymethyl cellulase by *Trichoderma viride* isolated from *Azadirachta indica* rhizosphere showed that the highest enzyme yield was obtained with carboxymethyl cellulose and ammonium sulphate as nutritional supplements at an optimum pH, temperature, incubation period and inoculum volume of 5.0, 30°C, 72 h and 1% (v/v), respectively. Sodium dodecyl sulphate polyacrylamide gel electrophoresis revealed the apparent molecular weight of the purified enzyme to be 55 kDa. The enzyme exhibited a \( V_{\text{max}} \) value of 83.7 µg/min and \( K_m \) value of 0.53 mg/ml. Optimal activity of the enzyme at 50°C and pH 5.0 emphasizes its potential to be utilized in textile industry operations.

**Keywords:** *Trichoderma viride*, carboxymethyl cellulase, submerged fermentation, media optimization.

**Introduction**

Cellulases are a group of fibrolytic enzymes which disrupt the cellulosic component of plant cell wall into simple and easily metabolizable sugars. Three types of cellulase enzymes synergistically involved in cellulose hydrolysis process include cellobiohydrolase, endoglucanase or carboxymethyl cellulase (CMCase) and β-glucosidase. With recent developments in biotechnology, there has been an increased interest to use cellulose digesting microorganisms to convert cellulosic biomass to simpler sugars that can be used in animal feed, waste water treatment and in brewing industry.

Although a large number of microorganisms contribute to cellulose degradation, a few produce significant levels of the enzyme. Few bacteria and actinomycetes have been reported to yield cellulase activity. However, under natural conditions fungi are the main cellulase producers. In general, bacterial cellulases are constitutively produced, whereas fungal cellulases are produced only in the presence of cellulose.

Among fungi, *Trichoderma* sp. has been utilized industrially under both submerged fermentation (SmF) and solid-state fermentation (SSF). To date, the production of cellulolytic enzymes has been intensively studied under SmF with different microorganisms in comparison to SSF. The present investigation aims at isolation, identification of cellulolytic fungi from the rhizosphere, assessment of cultural conditions that determine the optimal production of cellulase under submerged fermentation and characterization of the enzyme.

**Experimental Section**

**Isolation and Screening of Fungi Producing Cellulase**

Fungal forms were isolated from *Azadirachta indica* (neem tree) rhizosphere soil by serial dilution and agar plate technique on potato dextrose agar (PDA) and screened for cellulase production using a minimal agar medium containing (g/l): yeast extract, 1; K2HPO4, 4; NaCl, 2; MgSO4.7H2O, 1; MnSO4, 0.05; FeSO4.7H2O, 0.05; CaCl2, 2H2O, 2; NH4Cl, 2; agar, 20 and distilled water at pH 6.0, supplemented with 1% (w/v) carboxymethyl cellulose (CMC). The medium was point inoculated with the spores of individual fungal isolate obtained from the PDA plates and incubated at 27°C for 5 days.

Following incubation, the plates were flooded with 3 ml aqueous solution of Congo Red (0.1% w/v) for 30 min. The plates were further washed with 1 ml of NaCl solution (1 M) and left for 15 min. Formation of clear zones around the colonies indicated cellulolytic activity by the isolates. The fungal isolate
demonstrating highest zone of cellulose break down was identified as *Trichoderma viride* on the basis of colony morphology and microscopy. This culture was selected for further studies.

**Inoculum Preparation**

Spore suspension was prepared by mixing a loop full of fungal spores in 10 ml of sterile distilled water. A uniform spore suspension was obtained by mixing vigorously, which was measured for absorbance under white light.

**Optimization of Cultural Conditions**

1 ml of fungal spore suspension (10⁷ spores/ml) was used as inoculum. The inoculated flasks were incubated under shaking condition (130 rpm). The effect of different cellulosic substrates such as 1% (w/v) (dried leaf, saw dust, cellulose powder, CMC and filter paper) and 1% (w/v) nitrogen sources (peptone, yeast extract, beef extract, casein, ammonium chloride, ammonium sulphate and sodium nitrate) were tested.

Various physical parameters such as pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0), temperature (25, 30, 35, 40, 50 and 55°C) and incubation time (24, 48, 72, 96, 120 and 144 h) were optimized by conventional methods for maximal enzyme production. Different inoculum sizes such as 0.1, 0.3, 0.5, 1.0, 3.0, 5.0, 7.0 and 10.0% (v/v) were tested for their ability to induce cellulase production in the production medium.

**Assay of Cellulase**

The fermented broth was filtered and centrifuged at 5000 rpm for 30 min at 4°C. The clear cell free supernatant served as the crude enzyme. Cellulase assay was carried out by 3, 5- Dinitrosalicylic acid (DNS) method using glucose as standard. One unit of cellulase activity was defined as the amount of enzyme that released one μg of reducing sugar per minute under standard assay conditions.

**Estimation of Protein Content**

The soluble protein content of the enzyme sample was determined by Lowry’s method using crystalline bovine serum albumin as standard.

**Purification of CMCase**

The crude enzyme extract obtained after fermentation was subjected to 80% (w/v) ammonium sulphate precipitation. Ion-exchange chromatography was performed using DEAE-sepharose column. Fractions were collected and tested for CMCase activity. Active fractions were pooled and dialyzed against 10 mM Tris-HCl buffer (pH 5.5).

**Characterization of CMCase**

The effect of pH on CMCase activity was investigated by incubating the reaction mixture with different buffers: 0.1 M citrate buffer (pH 3.5), 0.1 M phosphate buffer (pH 5.5, 6.8 and 7.5) and 0.1 M Tris-HCl buffer (pH 9.5). Optimum temperature was determined by incubating the enzyme substrate reaction mixture at different temperatures (20, 30, 40, 50, 60, 70, 80, 90 and 100°C) for 30 min, followed by enzyme assay. Substrate concentration was evaluated using CMC solutions of various concentrations (0.1 to 2.0 mg/ml). The Michaelis constant (Km) was determined from the value of maximal velocity (Vmax).

**Determination of Molecular Weight**

The apparent molecular weight of purified CMCase was determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using Bio-Rad Mini-Protein Tetra Cell vertical electrophoresis unit and broad range pre-stained protein marker (New England Biolabs, UK).

**Statistical Analysis**

Effect of each parameter was studied in triplicate and the data have been graphically presented as mean ± standard deviation (n=3). ANOVA was performed using Microsoft Excel 2007. P values < 0.05 were considered significant with a confidence limit of 95%.

**Results and Discussion**

An impressive increase in the application of cellulases in various fields over the last few decades demands extensive research in improving its quality and large scale production. Among the various cellulases available, fungal cellulases gain a cutting edge advantage over other cellulases due to greater yield, efficiency, facilitated crystallization and higher degree of purity. Production of cellulase in large quantities requires study of process parameters that affect the growth and enzyme production ability of any microbial form.

**Effect of Different Cellulosic Substrates**

In case of inducible enzymes like fungal cellulases, the availability and nature of the substrate can be determinative factors for secretion of the enzyme. Of all the substrates examined for their suitability for maximum production of cellulase, CMC supported the highest yield of the enzyme (Fig. 1). This may be attributed to the chemical nature of CMC. The structure of CMC has been engineered to decrystallize cellulose, which creates amorphous sites for CMCase.
to act. The carboxymethyl groups (-CH$_2$-COOH) bound to some of the hydroxyl groups of glucopyranose backbone of CMC molecule render its soluble and chemically reactive nature, thus making it a highly specific substrate for endoacting cellulases. Due to more soluble nature of CMC, in comparison to filter paper and cellulose powder used in the study, it facilitated greater substrate availability to the fungal culture and thus better secretion of the hydrolytic enzyme.

Our result is in accordance with those of earlier work conducted on cellulase production from $T.$ harzianum using different carbon sources, where CMC was reported to be the best substrate for substantial amount of enzyme production$^{15}$.

**Effect of Nitrogen Supplementation**

In the present study, (NH$_4$)$_2$SO$_4$ acted as the best nitrogen source when compared to the organic nitrogen and rest of the inorganic nitrogen sources (Fig. 2). This could probably be due to the presence of both ammonium as well as sulphate ions that are essential for the conidial cell growth and enzyme production$^{16}$.

Incorporation of different inorganic nitrogen sources in the production media for cellulase production by $T.$ viride GCBT-11 revealed that among all the nitrogen sources tested, (NH$_4$)$_2$SO$_4$ supported maximum production of cellulases (CMCase 1.68, FPase 0.926 U/ml/min)$^{17}$.

**Effect of Initial pH of the Medium**

CMCase yield by $T.$ viride appeared to depend on pH value. The effect of pH on cellulase production was determined at pH range of 4.0 to 9.0. pH 5.0 favoured maximum enzyme production (55.88 µg/min) during the submerged fermentation. Earlier, maximum CMCase (1.46 U/mL) from $T.$ reesei was produced at optimum conditions of 28°C, pH 5.0 and cellulose as the carbon source.$^{18}$ Cellulase from $T.$ viride was maximally produced at pH 5.5, yeilding exoglucanase (2.16 U/ml), endoglucanase (1.94 U/ml) and β-glucosidase (1.71 U/ml)$^{19}$.

**Effect of Incubation Temperature**

The maximum CMCase yield (67.05 µg/min) from the rhizospheric $T.$ viride was recorded at 30°C. Being a mesophilic fungus, the membrane of $T.$ viride isolate was stable when the incubation temperature was maintained at mesophilic range, resulting in significant conversion of the substrate to reducing sugars. Decrease in the enzyme yield at lower or elevated temperatures probably resulted from reduced metabolic activity and impaired action of cell membrane of the fungus.

Other studies investigating the effect of incubation temperature on cellulase biosynthesis by $T.$ viride reported that the CMCase production was maximum at 30ºC and as temperature was further increased, there was a gradual reduction in the enzyme production$^{17}$. This is in absolute agreement with the present findings.

**Effect of Incubation Time**

The incubation time for achieving the maximum enzyme yield is governed by the characteristics of the culture and is based on growth rate and enzyme production. The results demonstrated that CMCase activity increased steadily and reached maximum (81.66 µg/min) at 72 h of incubation. Further increase in the incubation period led to reduction in CMCase production. This might be due to impaired fungal metabolism resulting from the depletion of macro and micronutrients in the fermentation medium upon prolonged incubation. Such an event leads to the inactivation of enzymes secretion$^{20}$. 

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Fig. 1—Effect of different substrates on enzyme production, Data represent mean ± S.D. (n=3); $P < 0.05$

Fig. 2—Effect of nitrogen supplements on CMCase production, Data represent mean ± S.D. (n=3); $P < 0.05$
Earlier, optimization of time course for fermentation of *T. viride* was investigated from 0-168 h, where maximum CMCase production was observed at 72-96 h. Further increase in incubation time reduced the enzyme production.

**Effect of Inoculum Size**

Lower inoculum size subjects any microbial culture to multiply at a slower rate and hence insufficient utilization of the substrate to produce metabolites. Presence of abundant spores in inoculum facilitates rapid proliferation and biomass synthesis. Beyond a certain inoculum load, enzyme production may decrease due to the depletion of nutrients and developed oxygen tension in the medium resulting from enhanced biomass. These factors may individually or collectively result in decrease of metabolic activity.

It is evident from our study that inoculum load has a significant role to play in determining the amount of enzyme secreted in the fermentation medium. Inoculum load as less as 1% (v/v) resulted in the highest enzyme titre (85.83 µg/min). Beyond this level there was a gradual decrease in enzyme production. Effect of inoculum size on cellulase production by some fungi cultured on pineapple waste was previously studied. Decrease in the amount of glucose production resulted at inoculum sizes above 6% and 8% for fermentations using *A. niger* and above 4% and 6% for fermentations using *T. longibrachiatum*.

**Effect of pH on CMCase Activity**

The purified CMCase from *T. viride* showed a specific activity of 168.27 µg/mg/min. The effect of pH on enzyme activity indicated that CMCase was active at pH range of 4-8, with the highest activity (65.88 µg/min) recorded at pH 5 (Fig. 3). Our result is in agreement with that of Irfan *et al.* who reported that *T. viride* FBL1 showed maximum CMCase activity at pH 5.5 in citrate phosphate buffer.

**Effect of Temperature on CMCase Activity**

Maximum CMCase activity (89.94 µg/min) was recorded at 50°C. When CMCase activity was determined above or below 50°C, a gradual decline in the activity was observed (Fig. 4). Similarly, CMCase secreted by *T. viride* exhibited highest activity at pH 5.0 and 50°C.

**Effect of Substrate Concentration**

Apart from the physical conditions, rate of enzymatic reaction is also dependent on substrate concentration. CMCase activity showed a progressive increase at substrate concentrations from 0.1 to 2.0 mg/ml. CMC concentration of 0.8 mg/ml resulted in maximum CMCase activity (83.7 µg/min), beyond which a slight reduction in the activity was noted. *Km* and *Vmax* values were determined as 0.53 mg/ml and 83.7 µg/min, respectively. These results are significant as the small *Km* value indicates a high affinity of the enzyme towards the substrate. This result is in close proximity to the *Km* of a CMCase (0.53 mg/ml) obtained from *Aspergillus flavus*.

**Determination of Molecular Weight**

Following SDS-PAGE, the purified CMCase was observed as a single band of homogenous monomeric protein. When compared to the standard molecular weight markers, the apparent molecular weight of the CMCase was found to be 55 kDa. Our result is in close proximity to that of an earlier study where the purified cellulase from fungal species like *T. viride*, contained only one subunit (molecular weight 58 kDa) on SDS-PAGE.

**Conclusion**

Cultural parameters have a profound effect on the production of CMCase by *T. viride*. Incorporation
of CMC and ammonium sulphate, an acidic pH and temperature around 30°C facilitated highest yield of the enzyme. Taking into account the thermostable nature of the CMCase, there is no doubt that this rhizospheric isolate of *T. viride* may be utilized for industrial production of cellulases. However, in order to enhance the productivity, it is necessary to screen different mutant strains of *T. viride*, develop recombinant strains and evaluate their performances at greater scale bioprocess.

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References


