

Production and Purification of Cellulase Enzyme by Endophytic *Bacillus* sp. Isolated from *Rhizophora Mucronata*

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Abstract

Mangroves occupy a relatively unexplored site in endophytic microorganism isolation so they can represent a new source in obtaining more enzymes with different potentialities. The present study was carried out to evaluate the ability of *Bacillus* sp isolated from mangrove species *Rhizophora mucronata* for the production of Cellulase enzyme. After production, Proteins from the culture filtrate were precipitated by Ammonium Sulfate (60%). The precipitate was reconstituted with assay buffer and dialyzed against same buffer. Then the dialyzed sample applied on DEAE Cellulose column and eluted with 1M NaCl in buffer. Purified enzyme was found to be 65KDa by SDS PAGE. The optimum conditions for the enzyme activity were found to be at a temperature 37°C and PH 7.0. The Km and Vmax values of the enzyme were 1.25mg/ml and 14.6 IU/ml, respectively.

Highlights

- Mangrove endophytic bacteria was isolated and screened for cellulase activity.
- Endophytic *Bacillus* sp. Isolated from *Rhizophora mucronata* used for the production of Cellulase enzyme.
- Cellulase enzyme was purified by Ammonium Sulfate precipitation, Dialysis and Column chromatography.
- The optimum conditions for the enzyme activity were found to be at a temperature 37°C and PH 7.0.
- The Km and Vmax values of the enzyme were 1.25mg/ml and 14.6 IU/ml, respectively.

Keywords: Endophytic bacteria, *Rhizophora mucronata*, cellulase enzyme

Endophytes are a suite of microorganisms that grow intra- and/or intercellularly in the tissues of higher plants without causing over symptoms on the plants in which they live, and have proven to be rich sources of bioactive natural products (Bacon and White, 2000). Mutual interaction between endophytes and host plants may result in fitness

benefits for both partners. Currently, endophytes are viewed as an outstanding source of bioactive natural products because there are so many of them occupying literally millions of unique biological niches (higher plants) growing in so many unusual environments. Thus, it appears that these biotypical factors can be important in plant selection,



since they may govern the novelty and biological activity of the products associated with endophytic microbes (Strobel and Daisy, 2003). Endophytes provide a broad variety of bioactive secondary metabolites with unique structure, including alkaloids, agrochemicals, antibiotics, immuno suppressants, antiparasitics, antioxidants, and anticancer agents.

Mangroves are salt tolerant marshy vegetation found mainly along the tropical and subtropical intertidal regions of the world comprising trees and shrubs, adapted to thrive in shallow, muddy, salt and brackish waters. The endophytic microorganisms of mangroves occupy a relatively unexplored site in microorganism isolation so they can represent a new source in obtaining more enzymes with different potentialities. Mangrove associated bacteria has not been explored for enzymes to a satisfactory level as much as fresh water microbes. Mangroves are therefore can be considered as a resource of endophytic bacteria serving an end number of enzymes that finds their use in industrial processes. Kumaresan and Suryanarayanan studied the extracellular enzyme production by the foliar endophytic fungi of *R.apiculata* and demonstrated their involvement in mangrove litter degradation.

Screening of mangrove endophytic bacterial resources for novel metabolites and enzymes and their application are major goal of current research to accomplish environment friendly technological development. Hence the present study was focused on the potential of endophytic bacteria isolated from *Rhizophora mucronata* in the production and purification of cellulase enzyme.

Materials and Methods

Isolation of endophytic bacteria

Healthy leaf, stem and root samples of mangrove species *Rhizophora mucronata* were collected from mangrove forest situated in Chettuva backwaters, Thrissur, in sterile bags and transported to the laboratory aseptically. Isolation of the endophytic bacteria was carried out by method previously described (Gayathri *et al.*, 2010).

Screening for Cellulase activity

All the isolated endophytic bacterial strains were streaked on basal agar media containing 1% CMC, and incubated at $37\pm 2^\circ\text{C}$ for 48 hours. After incubation, plates were washed with 1% Congo red solution and the plates were further treated by flooding with 1M NaCl for 15 minutes. The

formation of a clear zone of hydrolysis indicated cellulose degradation.

Production of Cellulase

Medium for Cellulase production contained (g/L) KH_2PO_4 1.0, K_2HPO_4 1.145, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 0.4, $(\text{NH}_4)_2\text{SO}_4$ 5.0, $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ 0.05 and $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$ 0.00125. 10 g/L of CMC was used as carbon source. The pH was adjusted to initial pH 7.0 by 1M NaOH. 10 ml inoculum was added to the medium and Fermentation was done at a temperature of 37°C for 7 days. After incubation, the culture filtrates were analysed for Cellulase activity and protein content.

Enzyme Assay

Cellulase activity was assayed using Dinitrosalicylic acid (DNS) method. 0.2 ml of culture filtrate was added to 0.8 ml of 1% CMC prepared in 0.2M sodium phosphate buffer (pH 7.0) in a test tube and incubated at 37°C for 60 minutes. The reaction was terminated by adding 3.0 ml of Dinitrosalicylic acid (DNS) reagent and by subsequently placing the reagent tubes in water bath at 100°C for 15 min. The absorbance was recorded at 570 nm against the blank (of 0.2M sodium phosphate buffer). One unit of Cellulase activity was expressed as one μmol of glucose liberated per ml enzyme per minute. Protein determination was carried out by Lowry's method. Specific activity was defined as units per mg of protein.

Purification of Cellulase enzyme

Seven days old broth culture, grown under optimized growth conditions was centrifuged at 9000 g for 10 min. The cell free supernatant was precipitated with ammonium sulfate (40-80% saturation). Precipitates were resuspended in 100mM sodium phosphate buffer, pH 7.0 (buffer A) and dialyzed against same buffer. After dialysis the concentrated sample was applied onto a DEAE Cellulose column equilibrated with buffer A and eluted with the assay buffer containing 1M NaCl (buffer B). The active fractions with enzyme activity were pooled and used for characterization. The entire process was carried out in $0-4^\circ\text{C}$.

Molecular weight determination by SDS PAGE

Molecular weight of purified enzyme was determined by SDS PAGE. SDS-PAGE was performed on a 10% polyacrylamide gel by the method of Laemmli (1970). After electrophoresis, the proteins were stained with Coomassie



brilliant blue R-250. The molecular weight standards used were from a high molecular-weight calibration kit (Fermentas).

Effect of temperature and pH on activity of Enzymes

In order to find out the optimum temperature and pH for the enzyme activity, enzyme assay was carried out at different temperatures (25°C, 30°C, 37°C, 45°C and 55°C) and at different pH (5.0, 6.0, 7.0, 8.0 and 9.0).

Determination of Km value of purified Cellulase

Cellulase activity was assayed with variable amounts of CMC as substrate (1.0, 2.0, 3.0, 4.0 and 5.0mg). The data was plotted as linear graph and the values of V_{max} and K_m were calculated from the graph.

Results and Discussion

Isolation and screening of Cellulase producing endophytic bacteria

Nutrient agar plates inoculated with mangrove plant samples showed morphologically different bacterial colonies. In total 26 colonies were recovered. All the bacterial isolates were screened for Cellulase by Congo red staining method. Out of 26 isolates, 12 (46.15%) exhibited Cellulase activity. Bacterial isolate BS7 was selected as potent Cellulose hydrolyzer which was identified biochemically as *Bacillus* sp. Gayathri *et al.*, (2010) have reported that 104 colonies were recovered from 5 different mangrove species.

Production and purification of Cellulase

Submerged fermentation was carried out for production. After 7 days, fermented medium was centrifuged at 9000g for 10 minutes. Proteins from the culture filtrate were precipitated by Ammonium Sulfate (60%). It was found that of 40-60% ammonium sulphate saturation is common for purification of bacterial Cellulase (Sudan and Bajaj, 2007). The precipitate was reconstituted with assay buffer and dialyzed against same buffer. The dialyzed sample was then subjected to ion exchange chromatography on DEAE

Cellulose column and eluted with 1M NaCl in assay buffer. All the fractions are assayed for Cellulase activity and the fractions with enzyme activity were pooled which was found to have a specific activity of 68.3 IU/mg. Specific activity of the enzyme was sequentially increased at each step and final increment was more than 4 folds. In a study by Bajaj *et al.*, (2009) Cellulase was purified 9.06 folds from *Bacillus* strain M-9 with DEAE-cellulose chromatography. A brief summary of purification steps was given in table 1.

Molecular weight determination by SDS PAGE

Pooled Cellulase fractions were subjected to SDS PAGE to estimate the molecular weight and it was 65KDa. The molecular weight of Cellulase varies with different bacteria. Lee *et al.*, (2008) and Bajaj *et al.*, (2009) purified Cellulase from *Bacillus* strains which had molecular weight of 54 kDa. In *Pseudomonas fluorescens* it is 36 kDa (Bakare *et al.*, 2005)

Effect of temperature and pH on activity of Enzyme

Purified enzyme preparation was recorded to show activity over a broad range of temperature (25°-55°C) with the optimal activity at 37°C and declined thereafter (Fig.1).

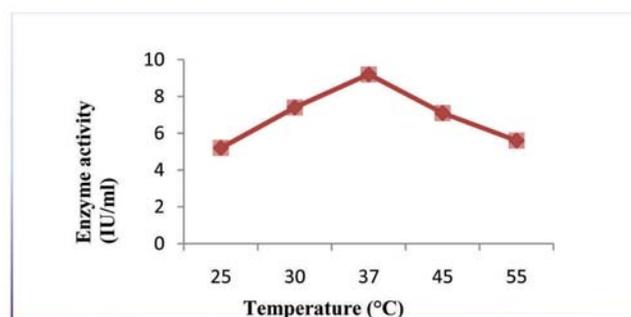


Fig. 1: Effect of temperature on activity of Enzyme

Table 1: Summary of Purification of Cellulase

Sl.No	Purification step	Enzyme activity (IU)	Protein (mg/ml)	Specific Activity (IU/mg)	Fold purification
1	Centrifugation	10	0.60	16.66	-
2	Ammonium sulfate precipitation	10.6	0.44	24.09	1.44
3	Dialysis	11.55	0.28	41.25	2.47
4	Ion exchange chromatography	13.66	0.20	68.3	4.09

The enzyme hydrolyzed CMC in the pH range of 5.0-9.0, and exhibited highest activity at pH 7.0 (Fig.2). Similar optimum enzyme activity was also found at pH 7.0 in *Pseudomonas fluorescens* (Bakare et al., 2005) and *Bacillus* strains (Sadhu et al., 2013). But lower optimum pH (5.0) for enzyme activity with broad ranges of pH stability was found in *Bacillus* strain M-9 (Bajaj et al., 2009).

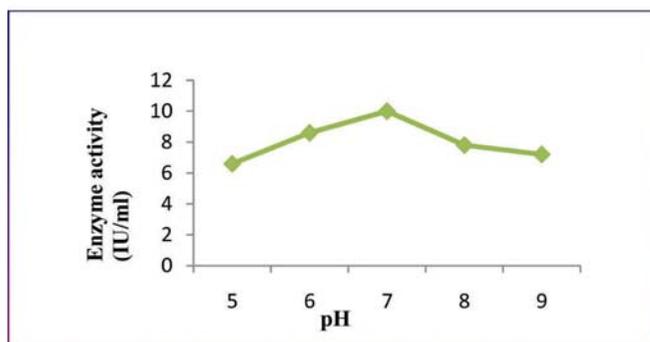


Fig. 2: Effect of pH on activity of Enzyme

Determination of K_m value of purified Cellulase

The K_m value is the amount of substrate needed to achieve half the maximal initial reaction velocity (Tong et al., 1980). It is a measure of enzyme affinity to substrate.

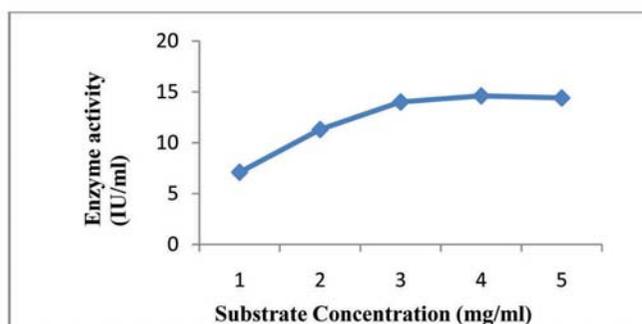


Fig. 3: Cellulase activity at different substrate concentrations

K_m value of purified cellulase was determined by linear Graph at varying substrate (CMC) concentration and found as 1.25 mg/ml and the V_{max} was found to be 14.6 IU/ml (Fig. 3). The K_m value of Cellulase was found 3.6 mg/ml

in *Pseudomonas fluorescens* (Bakare et al., 2005). Sadhu et al., (2013) purified a cellulase from *Bacillus* strain which was found to have a K_m value of 0.25mg/ml and V_{max} was 20 $\mu\text{mol/ml/min}$.

From the present study it may be concluded that the endophytic *Bacillus* sp isolated from mangrove plant is a potential Cellulase producer. The purified enzyme with broad range of temperature and pH stability could have potential applications in wide range of industries.

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