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Research Article

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Partial Purification of Cellulase Produced By A Bacterium Isolated from Wood Decompost

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ABSTRACT

Cellulases are a group of hydrolytic enzymes capable of degrading cellulose to the smaller glucose units and have a wide range of applications in bioethanol production, textile, drug, detergent, food-feed and pulp-paper industry. This study was designed to assess the cellulase production of a bacterium isolated from wood decompost. Cellulase producing bacteria (CPB) isolate obtained from wood decompost sample was tentatively characterized on the basis of their morphological and biochemical characteristics and identified to be Gram negative, cocci, cream pigmented, non-motile; catalase, methyl red, indole, nitrate, citrate, VP, glucose, lactose, fructose tests were shown to be positive. The CPB isolate exhibiting maximum zone size on the CMC media was selected. This isolate was then evaluated for enzyme production, and the crude sample (extracellular) showed 0.818 U/ml specific enzyme activity by DNS method. The protein content of the crude enzyme was estimated to be 0.62 mg/ml. Cellulase was partially purified using ammonium sulphate precipitation and the maximum 0.919 U/ml cellulase activity was recorded in the fraction precipitated upon 30-60% saturation. Comparative analysis of crude enzyme and partially purified enzyme on the CMC plate, showed that the zone of hydrolysis obtained was found to be 3.4mm and 4.6mm respectively.

Key words: *Ammonium sulphate, Assay, Cellulase, Morphology, Purification.*

INTRODUCTION

Cellulose is the most abundant biomass on earth[19].Cellulose accounts for 50% of the dry weight of plant biomass and approximately 50% of dry weight of secondary sources of biomass such as agricultural wastes[2]. Cellulolysis is the process of breaking down cellulose into smaller polysaccharides called cellodextrins or completely into glucose units by the participation of microbial cellulolytic enzymes[20].

Cellulases are produced by a number of microorganisms such as bacteria, yeast and fungi. There is increasing interest in cellulase production by bacteria because bacteria have high growth rate as compared to fungi and has good potential to be used in cellulase production[10]. Cellulolytic property of some bacterial genera such as *Cellulomonas* species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* species have been reported[17].

The major industrial application of cellulases are in textile industry for bio-polishing of fabric, producing stonewashed look of denims, in household laundry detergents for improving fabric softness and brightness besides, they are used in animal feeds for improving the nutritional quality and digestibility, in processing of fruit juice and in baking ,and de-inking of paper[15]. Other emerging applications are in bioadhesive and mucoadhesive drug delivery system, in pharmaceutical coating process, as compressibility enhancers, as gelling agents, thickening and stabilizing agent, as binder in granulation process, as taste making agent, as disintegrated agent, as fillers in solid dosage form. Cellulosic

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derivatives are in general strong, reproducible, recyclable and biocompatible, being used in biomedical applications such as blood purification[18].

The present study was attempted to extract the cellulase enzyme from an isolate obtained from wood decompost, and to determine its protein content. Moreover it was undertaken to purify enzyme and to compare its activity with that of the crude cellulase.

MATERIALS AND METHODS

Isolation and Primary Screening of Cellulase Producing Bacteria (CPB):

The cellulase producing bacteria was obtained from a wood decompost collected from the botanical garden, Chandigarh in sterilized containers. Serially diluted wood sample was spread on Nutrient agar plate and incubated at 37°C for 24 hours. After the incubation bacterial colonies were observed. Then the colonies were picked individually, and streaked on carboxy methyl cellulose (CMC) agar plates and further incubated at 37°C for 48 hours. After incubation, the plates were flooded with 1% congo red and 1M NaCl to check the cellulolytic activity of isolated strain.

Identification of CPB:

The isolate was identified by means of morphological characterization (gram staining, motility activity) and biochemical characterization (catalase, methyl red, indole, nitrate, citrate, voges-proskauer, gelatin hydrolysis, sugar fermentation and starch hydrolysis tests)[3].

Enzyme Production and Extraction:

Cellulose production medium (CMC broth) for CPB was prepared in 250 Erlenmeyer flask. Then the production medium was autoclaved at 15 psi pressure for 20 minutes; cooled it and inoculated with 1 ml of the isolated culture. The medium was incubated at 37°C for 72 hours with an agitation speed of 150 rpm. After incubation, the broth was taken out in centrifugation tubes and centrifuged in cooling micro centrifuge (REMI, ISO certified) at 16,000 rpm for 20 minutes at 4°C. The pellet was discarded and supernatant was taken as the source of crude enzyme.

Cellulase Assay:

The activity of cellulase was assayed using DNS (Dinitro-salicylic acid) method by estimation of reducing sugars released from CMC solubilised in 0.05M citrate buffer[11]. Crude enzyme was added to 1% CMC in 0.05 M citrate buffer and incubated at 45°C for 30 minutes. The reaction was stopped by addition of 3 ml of DNS reagent in the reaction mixture. Then the mixture was incubated at 100°C for 10 minutes in the water bath. Sugars liberated were determined by measuring absorbance at 540 nm. One international unit (IU) of cellulase activity refers to the amount of enzyme that released 1 µM of glucose per ml of the sample per minute under assay conditions[5] as mentioned above.

Protein Estimation:

Concentration of protein in crude extracellular extract containing cellulase was determined by Lowry's method[8]. Different concentrations of BSA (Bovine Serum Albumin) as standard protein in a range of 0.02-0.2mg/ml were reacted with Lowry's reagent C and D and the absorbance at 620nm was read. A standard graph was plotted between concentration of protein on X axis and absorbance at 620nm on Y axis. 100µl of extract was reacted with Lowry's reagent C and D and absorbance at 620nm was read. This absorbance was compared with the standard graph in order to get the concentration of protein in crude extracellular extract.

Partial Purification of Cellulase Enzyme and Determination of its Activity:

The crude enzyme was partially purified by ammonium sulphate fractionation in which the culture filtrate was subjected to protein fractionation by differential ammonium sulphate precipitation[1]. Fractionation of protein was done by addition of small increments of solid ammonium sulphate at 4ºC with constant stirring to obtain three fractions i.e. 0-30%, 30-60% and 60-90%. When all the ammonium sulphate was dissolved at the end of each fractionation range, the mixture was allowed to stand for 30 minutes to 1 h. The mixture was centrifuged at 10,000 x g for 30 minutes at 4ºC. The pellet was collected and supernatant was used as the starting material for next fractionation. The collected precipitate of each fractionation

range was resuspended in small volume of 0.05M citrate buffer pH 7.0 and checked for the enzyme activity by spectrophotometric technique using CMC as substrate as mentioned above under “cellulase assay” heading.

Comparison of the Activity of the Crude Enzyme and Partially Purified Enzyme:

To compare the enzymatic activity of crude and partially purified sample, Congo red on CMC plate was used for evaluation as discussed above (under the heading “Isolation and primary screening of CPB”). The zones of hydrolysis were observed and compared around the two wells containing the crude and partially purified enzyme samples.

RESULTS AND DISCUSSION

Isolation and Primary Screening of CPB:

The bacterial isolates from wood sample were recovered on nutrient agar medium, after serial dilutions. After incubation for 24 hours at 37°C, growth of bacterial colonies was observed and screened for their cellulolytic ability on CMC agar plates (Fig. 1). CMC inoculated plates were flooded with congo red dye and NaCl solution. The appearance of clear zone around the colonies indicated their cellulolytic property (Fig. 2). The isolate which showed clear zone around them was identified as CPB and characterized morphologically and biochemically[16].

Fig. 1: Isolation of cellulolytic bacteria

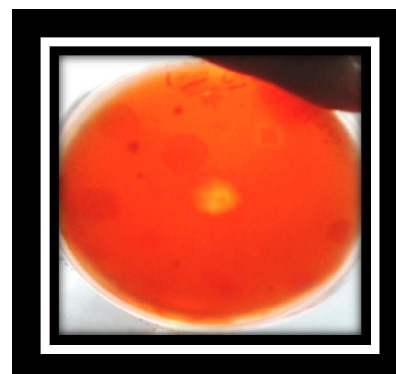


Fig. 2: Screening of cellulolytic bacteria (using congo red & NaCl)

Identification of CPB:

The morphological and biochemical data of CPB are tabulated in Table 1 & 2.

Table 1: Morphological characteristics shown by CPB isolate

Morphological Characteristics	CPB
Colony colour	Creamy
Cell margin	Regular
Cell shape	Cocci
Gram reaction	Negative
Motility test	Non-motile

Table 2: Biochemical characteristics of CPB isolate

Biochemical tests	CPB
Catalase	Positive
Methyl red	Positive
Indole	Positive
Nitrate reduction	Positive
Citrate	Positive

Hydrolysis of gelatin	Negative
Starch hydrolysis	Positive
Voges- Prosakauer	Positive
Sugar fermentation	
Glucose	Positive
Sucrose	Negative
Lactose	Positive
Mannitol	Negative
Fructose	Positive

Our biochemical results were comparable with an earlier report of Shaikh[16], who isolated a CPB belonging to genus *Bacillus* and *Pseudomonas*.

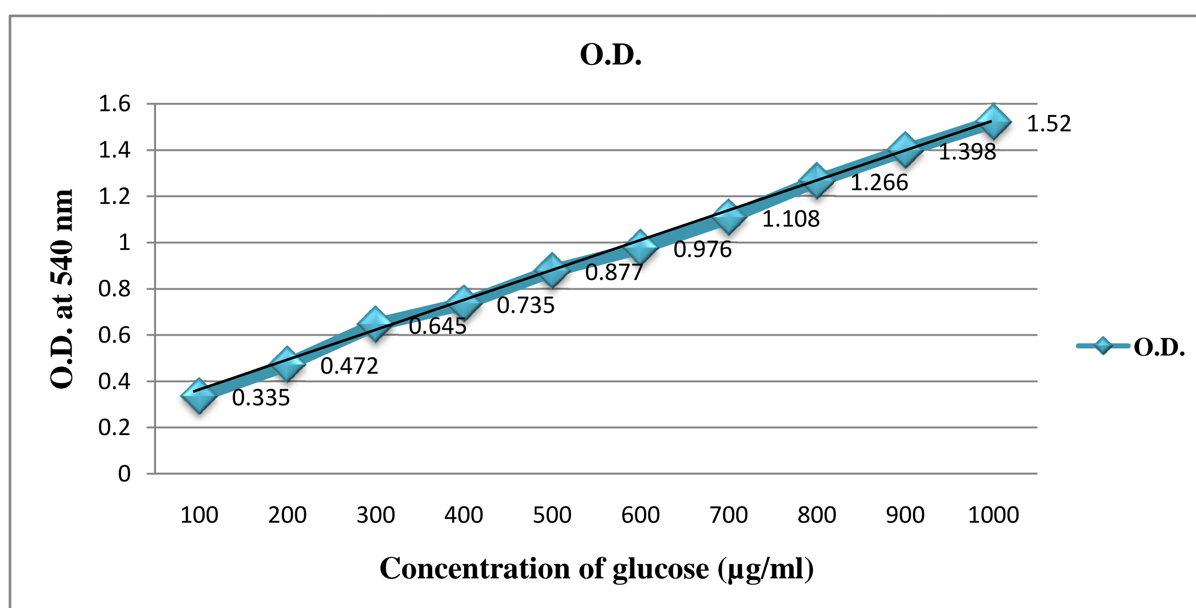
DNS Assay of Crude Cellulase Enzyme:
Upon performing DNS Assay of the crude enzyme sample in triplicates, O.D. of reaction tube was 0.775, 0.787 and 0.796 at 540 nm (Table 3).

Table 3: O.D. of Crude Cellulase enzyme

S. NO.	Crude Enzyme (ml)	Water (ml)	DNSA Reagent (ml)	Heat in boiling water bath for 30 minutes	O.D. (540nm)
1.	0.1	0.9	1		0.775
2.	0.1	0.9	1		0.787
3.	0.1	0.9	1		0.796

The concentration of liberated concentration of glucose was calculated to be 436µg/ml, 442µg/ml and 448µg/ml resp. according to the standard graph (Fig. 3). Average of the three concordant readings of our crude sample of enzyme was taken to check the amount of glucose produced i.e., 442µg/ml when 0.1 ml of the crude cellulase enzyme was used.

Fig. 3: Standard curve of DNS assay



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Enzyme Activity of Crude Sample:

Enzyme activity as calculated from the formula below was found to be 0.818 U/ml

$$\text{Enzyme Activity, I.U} = \frac{\text{Net Amount of Sugar Produced} * \text{Dilution Factor}}{180 * \text{Incubation Time}}$$

Where 180 = Molecular weight of glucose

30 minutes = Incubation time

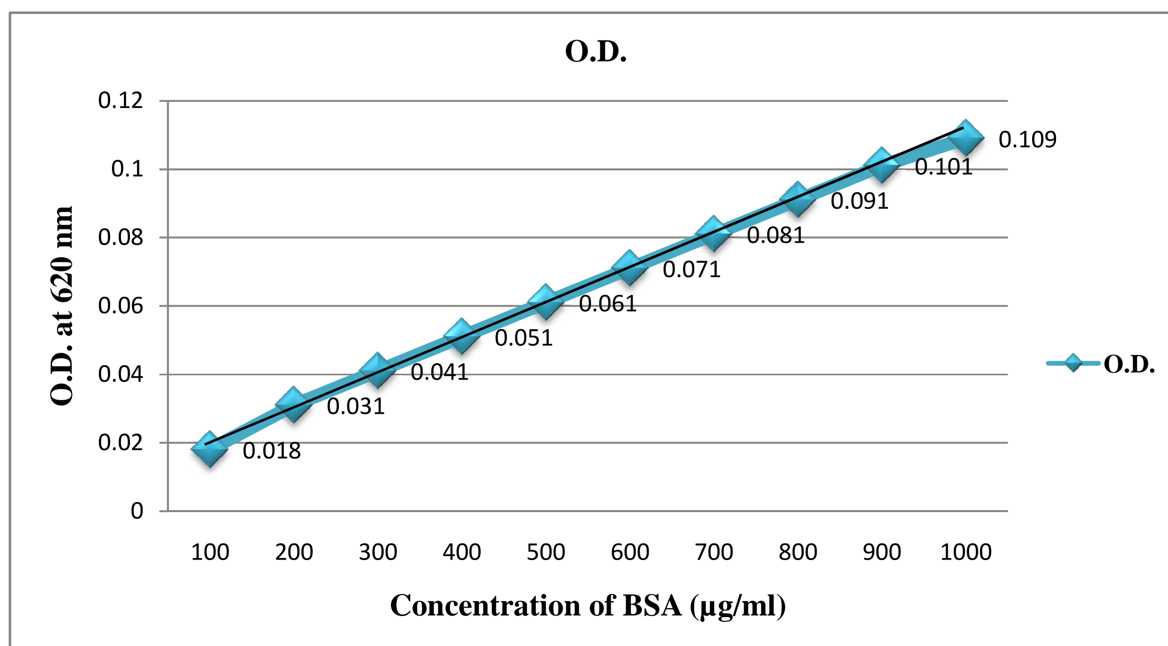
$$\text{Enzyme activity} = \frac{442 * 10}{180 * 30} = 0.818 \text{ IU/ml.}$$

The activity of cellulase enzyme isolated in our study is comparable with an earlier report in which cellulase activity of 0.460 IU/ ml was recorded by Pothiraj[12]. In another study the isolate *P. chrysogenum* showed an enzyme activity of 0.552 IU/ml and *T. reesei* showed enzyme activity of 0.363 IU/ml[6]. In another study on three cellulolytic bacterial strains, belonging to the genera *Bacillus*, the enzyme activity of *Bacillus cereus* was 0.440 IU/ml and 0.410 IU/ml, that of *Bacillus subtilis* was 0.357 IU/ml and *Bacillus thuringiensis* was 0.334 IU/ml[2].

Protein Estimation in Crude Enzyme by Lowry's method:

Concentration of protein in the extracellular crude extract containing crude cellulase was determined by comparing the absorbance readings of the test sample with the standard graph using BSA in Lowry's method (Fig. 4).

Fig. 4: Standard curve of BSA stock solution for protein estimation



Since the absorbance of unknown sample (at 660nm) was recorded to be 0.076, hence from the standard graph, the final concentration of the cellulase sample was calculated to be 680µg/ml or 0.68mg/ml.

The protein concentration of cellulase enzyme as isolated in our study is comparable with an earlier report in which the concentration of protein in crude cellulase enzyme was found to be 0.268mg/ml from a CPB isolated from cow dung[7]. In another report on cellulase from a fungal isolate PISS-3, an amount of 0.1mg/ml protein content was found[14]. Protein content of crude sample of cellulase enzyme produced by *Aspergillus* sp. isolated from forest litter soil was found to be 1.65 mg/ml[9].

Partial Purification of Cellulase Enzyme and Determination of Its Activity:

Partially purified cellulase obtained upon saturation using 30-60% ammonium sulphate was assayed using DNS method in a similar way as for the crude sample. When performed in triplicates, the absorbance (at 540 nm) of the partially purified cellulase sample was recorded to be 0.859, 0.869 and 0.882 (Table 4). This corresponds to 490, 496, 503 µg/ml glucose concentration from standard graph and the the average of the 3 readings was calculated to be 496.3 µg/ml.

Table 4: O.D. of Partially Purified enzyme

S. No.	Partially purified enzyme (ml)	Water (ml)	DNSA Reagent (ml)		O.D. (540nm)
1.	0.1	0.9	1	**Heat in boiling water bath for 30 minutes.**	0.859
2.	0.1	0.9	1		0.869
3.	0.1	0.9	1		0.882

Enzyme activity as calculated from the formula (as done for crude enzyme) for partially purified cellulose was found to be 0.919 IU/ml.

The activity of partially purified enzyme isolated in our study is comparable with an earlier finding reported by Das[4], who found the enzyme activity of partially purified sample to be 1.165U/ml, 0.995U/ml, 0.83U/ml, 0.536U/ml and 0.515U/ml for strains NRRLB 16746, colony 194, NRRLB 12377, colony 51 and colony 157 respectively isolated from soil sample.

Comparisons of Activity of the Crude and Partially Purified Enzyme:

A comparative analysis of the activity of the crude and partially purified cellulase was done on CMC plates using congo red and NaCl, which indicated a larger zone of hydrolysis (4.6 mm) by partially purified enzyme than the crude enzyme (3.4 mm) (Table 5, Fig.5).

An observed diameter nearly similar to that in our study was earlier reported by Rathore[13], who observed the size of zone of hydrolysis of cellulase enzyme to be 2mm, 5mm and 3mm in diameter for culture strains PSS-1, PSS-2 and BSS-1 respectively.

Table 5: Zone of hydrolysis of crude and partially purified enzyme

S. No.	Zone of hydrolysis	Diameter of clear zone (mm)
1.	Crude enzyme	3.4
2.	Partially purified enzyme	4.6

Fig. 5: Zone of hydrolysis of crude (C) and partially purified (P) enzyme

CONCLUSION

Based on the present study it can be concluded that wood decompost can be very good source for isolation of CPB which produced active cellulase. Isolates exhibiting high cellulase activity can prove to be of immense help to humans in production of pharmaceutical and many more products. Further genetic, biochemical and microbial engineering techniques are required to make use of full potential of these bacterial isolates for cellulase production.

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