Optimization of Culture Conditions for Partially Purified Cellulase Production by Oceanobacillus Species Isolated from Wood Industry Soil in Chennai, India

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Abstract— The enormous population of bacteria in the environment permits screening and production of more efficient cellulases to aid in the conversion of lignocellulosic residues, treatment of waste papers as well as other industrial applications. Selected colonies of *Oceanobacillus profundus* were screened for cellulase activity on agar media containing 1% CMC; for assaying the cellulose production, dinitrosalicylic acid method was employed by measuring the amount of glucose liberated in µmol/mL/min and the effect of pH, temperature, carbon source as well as nitrogen source on the enzyme was optimized followed by partial purification of enzyme using precipitation and dialysis procedure. Specific enzyme activity of the crude sample was found to be 1.504 IU/ml; optimum conditions for CMCase activity produced by the strains were; pH 7.2, 40º C, maltose as well as ammonium nitrate; the partially purified cellulase was observed to have a molecular size of 35kDa after characterization by SDS-PAGE. Application of cellulase in digestion of waste papers and wools, production of ethanol amongst others is now being adopted making demand for the enzyme increased.

Index Terms— Cellulase, CMCase activity, optimization, *Oceanobacillus profundus*, wood industry

1 Introduction

Cellulose has been classified as the most abundant biomass on earth found primarily in the cell wall of plants, it is a linear polymer of glucose units joined by ß-1,4 glycosidic bonds and having a crystalline nature (1-2). The synergistic action of three cellulase enzymes catalyzes the hydrolysis of cellulose (3) which include endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and ß-glucosidase (EC 3.2.1.21) (4). The ability to degrade and

utilize cellulose as a source of carbon is exhibited by a vast number of microorganisms with the catalytic action of cellulases produced during their development on cellulosic materials (5).

It is therefore very important to identify the microorganisms capable of the synthesis of these cellulosic enzymes as well as the degree of activity. Several microorganisms that are capable of degrading cellulose have been isolated and identified. It has been discovered that fungi are the major cellulase-producing microorganisms while a report on limited species of actinomycetes and bacteria possessing this attribute has also been made (6).

A list of few of the fungal species identified are *Trichoderma, Fusarium, Penicillium Chaetomium, Aspergillus* and *Myrothecium* (7). There is the existence of anaerobic and aerobic cellulolytic bacteria (8). Bacteria belonging to the genera *Clostridium, Cellulomonas, Cellulosimicrobium, Thermomonospora, Bacillus, Ruminococcus, Erwinia, Bacteriodes, Acetovibrio, Streptomyces, Microbispora, Fibrobacter* and *Paenibacillus* have been observed to produce different kinds of cellulase when incubated under anaerobic or aerobic conditions (9,10,11).

The yield of cellulase appear to depend upon a complex relationship involving a variety of factors like inoculum size, pH value, temperature, presence of inducers, medium additives, aeration and growth time (12). Studies have indicated that cellulases are utilized in industrial applications such as animal feed, pulp and paper, food, chemicals, fuel, brewery and wine, textile and laundry as well as agriculture (13).

This study was aimed at understanding best conditions favorable for the high yield of cellulase by Oceanobacillus profundus. In addition to this, the application of the enzyme in the degradation of used paper products was also analyzed.

2 Materials and Methods

2.1 Bacterial Strain and Culture Medium

The bacterial strain, *Oceanobacillus profundus* (GenBank accession no. JQ659602) was isolated from soil collected from a wood industry in Chennai, India. This was cultured in nutrient agar medium at 35ºC for 24 hours and the cultures were stored for further use.

2.2 Enzyme Assay (Screening for Carboxymethyl Cellulose Activity)

Selected colonies were screened for cellulase activity on agar media containing 1% CMC. The plates were incubated at 35°C for 48 hours to visualize the hydrolyzing zone, the incubated plates were flooded with aqueous solution of 0.3% Congo red for 15 minutes and counterstained with 1M NaCl (6). The microorganisms having clear zones which signifies cellulase activity were selected for cellulase production and identification.

2.3 Effect of Different Physical Parameters on Enzyme Production

2.3.1 Effect of pH on enzyme production

To determine optimal pH, the bacteria was inoculated in nutrient broth and incubated for 7days and the supernatant was filtered, centrifuged and treated with different pH ranges from 7.2 to 7.8. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The supernatant was subjected to quantitative assay.

2.3.2 Effect of temperature on enzyme production

To determine optimal temperature, the bacteria was inoculated in nutrient broth and incubated for 7days and the supernatant was filtered, centrifuged and treated with varying temperatures 37°C (room temperature), -14°C (refrigerator), 40°C (incubator) and 45°C (hot air oven). The supernatant was subjected to further assay.

2.3.3 Effect of carbon sources on enzyme production

In order to determine the optimized carbon source, various carbon sources such as dextrose, fructose, sucrose, and maltose are to be added individually at different concentrations (ranging from 0.5 -3.0% w/v) in to the broth medium. The organism was allowed to grow until its log phase at 37°C. Then, the culture filtrate was collected and subjected to quantitative assay for enzyme production.

2.3.4 Effect of nitrogen sources on enzyme production

Various nitrogen sources both organic and inorganic such as (ammonium nitrate, ammonium chloride, urea and yeast extract) were added individually at different concentrations (ranging from 0.5 -3.0% w/v) in to the broth medium. The organism was allowed to grow until its log phase at 37°C. Then, the culture filtrate was collected and subjected to quantitative assay for enzyme production.

2.4 Extraction of crude enzyme

After incubation, the cultures were filtered, centrifuged at 5000 rpm for 15 minutes and supernatant were used as source of crude enzyme. The crude enzyme solution was utilized for determination of enzyme activity. (14)

2.5 Quantitative assay – DNS Method

Cellulase activity was measured by the DNS (3, 5-dinitrosalicylic acid) method (15), through the determination of the amount of reducing sugars liberated from Carboxy Methyl Cellulose (CMC) solubilized in 100 mM Sodium Citrate buffer, pH 5.0. This mixture was incubated for 15 min at 55 °C and the reaction was terminated by the addition of DNS solution. The treated samples were boiled for 10 min, then 1ml sodium potassium tartarate and 2ml distilled water were added for color stabilization, and the optical density was measured at 540 nm. The cellulase activity was determined by using a calibration curve for glucose. One unit of enzyme activity is defined as the amount of enzyme that released 1 µmol of glucose per minute.

2.6 Bulk production for the optimized parameter

The optimized media was used in bulk production. The incubation period was 7days after which the sample was centrifuged at 5,000 rpm for 15 minutes, filtered, followed by isolating the protein by Salt precipitation (Ammonium sulphate).

2.7 Partial purification using ammonium sulphate

Using solid ammonium sulphate, the crude enzyme sample was brought to 80% saturation.

After the total amount of ammonium sulphate was dissolved, the mixture was placed in refrigerator overnight at 4°C. The suspension was then centrifuged at 8000 rpm for 15 minutes. The supernatant was discarded and the pellet was collected and dissolved in 10mM sodium phosphate buffer.

2.8 Dialysis

The dialysis membrane was first pre-treated to remove some undesirable impurities.

The dialysis tube was filled with the sample solution up to 2/3 of the tube volume using a micropipette. The sample was dialyzed against 10mM sodium phosphate buffer and incubated overnight on a magnetic stirrer in cool condition. The dialyzed sample was carefully drained using sterile syringe and stored in microfuge tubes.

2.9 Estimation of protein and molecular weight determination

The protein concentration in crude sample was determined by using Bradford method with Coomassie Brilliant Blue (G-250) as standard at 595nm (16), molecular weight of the sample was determined by SDS-PAGE (Sodium Dodecyl Sulfate –Polyacrylamide Gel Electrophoresis). Loaded right next to the purified protein were the standard protein markers, followed by the crude and dialyzed sample.

2.10 Filter paper assay

Whatman no.1 filter paper was used to examine the degradation rate by the enzyme with application on used blotted paper.

1ml of 10mM sodium phosphate buffer (pH 7.0) was added to a test tube with different volume of enzyme diluted in sodium phosphate buffer. At least two dilutions were made of the enzyme sample investigated. The temperature was adjusted to 50°C and a filter paper strip was added and the suspension mixed. The content in the tube was incubated at 50°C for one hour. After one hour, 3.0ml of DNS was added and mixed. The tube was transferred to a rack on the table. The tubes were then boiled for exactly 10 minutes and transferred to a cold water bath with addition of distilled water. The tubes were mixed, the pulp allowed to settle for 20 mins, color formation was then measured against the spectro zero at 540nm. This experiment was replicated for the application in used blotted paper.

3 Results

3.1 Screening for CMCase Activity

The isolate appeared white colonies on CMC agar. After staining with 0.3% congo red and 1N sodium chloride, halo zone was detected on CMC plate (Fig. 1).

3.2 Optimization of Culture Conditions

The optimum conditions of Oceanobacillus profundus were observed in the substrate and tabulated (Table 1 – 4). The maximum enzyme activity was recorded at a pH-7.2 with ammonium nitrate as nitrogen source and maltose as carbon source at 40°C (Fig. 2-5). The optimized culture condition was used for the bulk production process.

3.3 Quantitative assay – DNS Method

The crude enzyme solution was utilized for determination of enzyme activity. Cellulase activity was measured by the DNS method, from carboxy methyl cellulose (CMC). The specific enzyme activity of the crude sample was found to be 1.504 IU/ml.

3.4 Ammonium sulphate precipitation

Subsequent to addition of the salt to the supernatant in a beaker, it was left for stirring on the magnetic stirrer for one hour; the supernatant was kept in the refrigerated condition overnight. The protein content was seen to have precipitated out due to the ammonium sulfate added.
The supernatant reached 80% saturation ant the precipitated samples were cooled and used for dialysis process.

3.5 Dialysis

After the dialysis bag has been left in cool condition overnight on a magnetic stirrer, Pellets were seen in the bag indicating that dialysis has taken place and the salt content associated with the protein in the dialysis bag has dialyzed to the buffer solution outside the tube due to osmosis.

3.6 Estimation of protein and molecular weight determination (SDS-PAGE)

The protein concentrations in crude sample were determined with Coomassie Brilliant Blue (G-250) as standard. The SDS-PAGE was performed in order to determine the molecular size of the obtained protein. The protein samples were loaded in their respective wells in increasing concentration (450 - 550 µg/ml). It was observed from Fig. 6 below that the isolated enzyme has molecular weight of 35 kDa. The bands were compared with the standard marker run alongside.

4 Figures

Figure 1 CMCase Activity

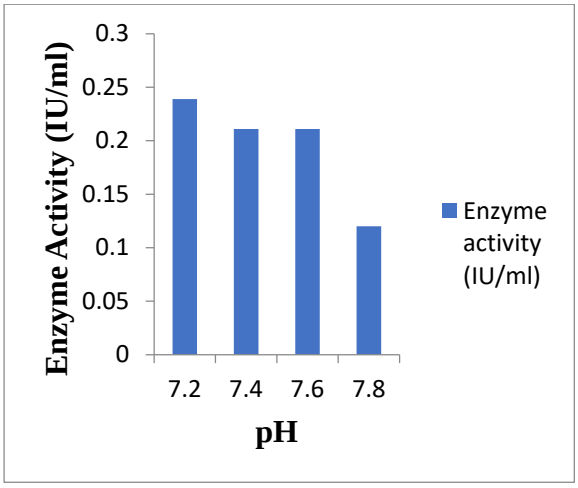

Figure 2 Effect of pH on enzyme activity

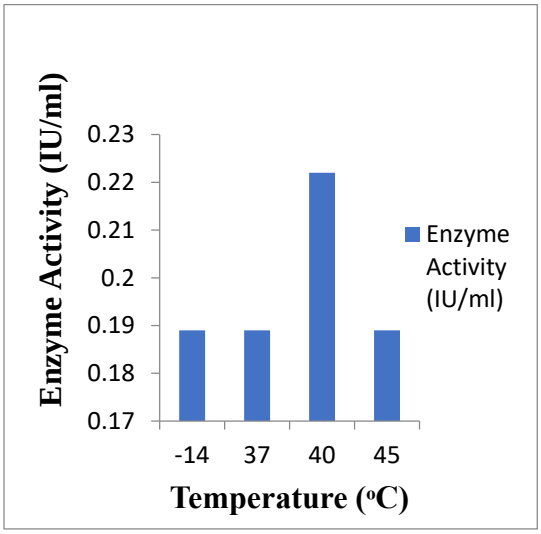

Figure 3 Effect of Temperature on enzyme activity

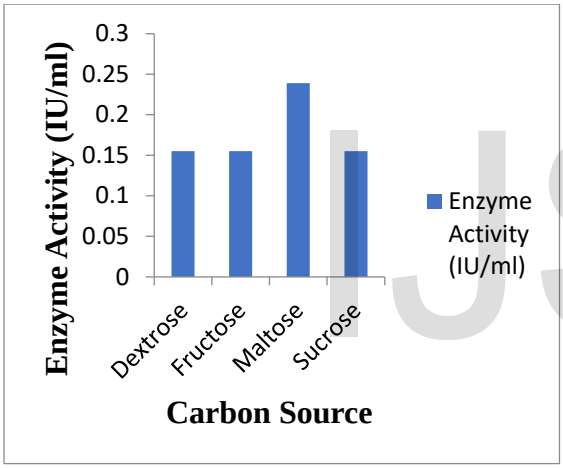

Figure 4 Effect of Carbon source

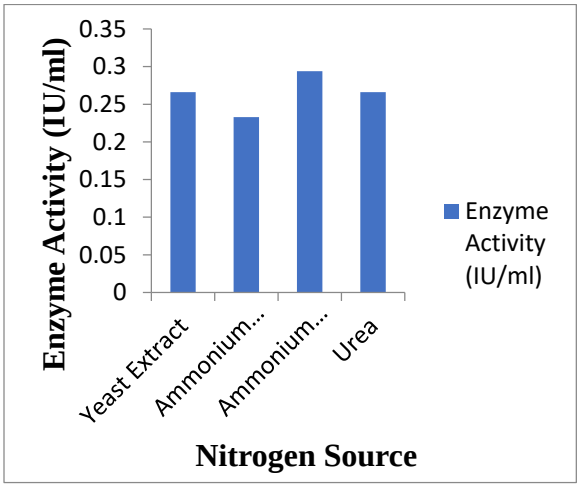

Figure 5 Effect of Nitrogen source

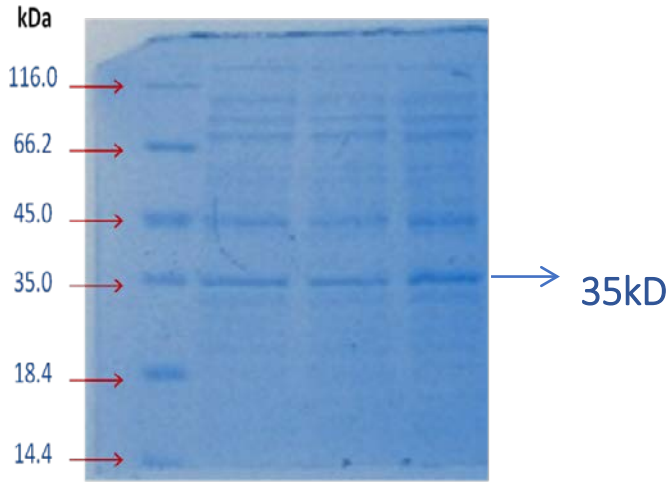

Figure 6 Visualization of bands by SDS-PAGE

Figure 7 Filter paper before degradation

Figure 8 Filter paper after degradation

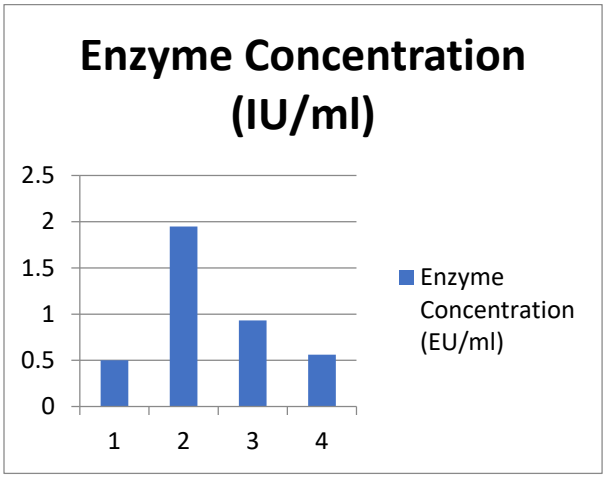

Figure 9 Filter paper assay

Figure 10 Used blotted paper before degradation

Figure 11 Used blotted paper after degradation

5 TABLES

Table 1: Optimization of pH

Parameter	Different values	Optical density (540nm)	IU/ml
	7.2	0.043	0.239
pH	7.4	0.038	0.211
	7.6	0.038	0.211
	7.8	0.036	0.200

Table 2: Optimization of temperature

Parameter	Different temperatures	Optical Density (540nm)	IU/ml
	-14ºC	0.034	0.189
Temperature	37 ºC	0.034	0.189
	40 ºC	0.040	0.222
	45 ºC	0.034	0.189

Table 3: Optimization of carbon source

Parameter	Different Sources	Optical Density (540nm)	IU/ml
	Dextrose	0.028	0.155
Carbon Source	Fructose	0.028	0.155
	Maltose	0.043	0.239
	Sucrose	0.028	0.155

Table 4: Optimization of Nitrogen Source

Parameter	Different Sources	Optical Density (540nm)	IU/ml
	Yeast Extract	0.271	1.504
Nitrogen Source	Ammonium Chloride	0.250	0.250
	Ammonium Nitrate	0.256	1.421
	Urea	0.224	1.243

6 Application of Enzyme

The cellulase enzyme was used in the partial degradation of used blotted paper. The concentrations of the buffer were 0.5ml, 1ml, 2ml, and each containing 1ml enzyme; with the control having 1.5ml buffer only. The pictures showing the degradation are shown below.

7 Discussion

According to recent research, a large population of microorganisms have been discovered to have the potentials of producing cellulases that function in degradation and bioconversion of cellulosic materials. In this study the Oceanobacillus profundus, a gram positive cocci were seen to produce significant quantity of enzyme at optimized conditions. Other studies have shown the production of cellulases by species such as Microcerotermes diversus (17), Bacillus subtilis (18), Cellulomonas fimi (19), with cellulase hydrolysis capacity as follows 1.220 IU/ml, 0.865UI/ml and 0.984 IU/ml respectively. In the present study, the filter paper assay revealed that the cellulase enzyme was capable of partially degrading the Whatman No.1 according to (20) after one hour and overnight incubation respectively. Different dilution factors with Phosphate buffer were used. The dilution including 0.5 ml of buffer gave the highest activity in both of the incubations.

8 Conclusion

In the bid to exploit cellulases for their applications in industrial, bio-medical engineering and other biotechnological approaches, bacteria possess an attractive potential; this is as a result of their rapid growth rate, extreme habitat variability and the complexity of enzymes they synthesize. Isolation and purification of cellulases from more bacterial species will go a long way in contributing to the population of microorganisms having the ability to produce this enzyme as well as understanding the distinct feature enabling them of this synthesis. Furthermore, improvement of these cellulolytic bacteria will provide more yield of cellulases that can widen the area of application to other relevant fields. In the future, these enzymes can be used in the production of sustainable energy such as biofuel which is now a form of clean energy in order to reduce the use of fossil fuels known to pollute the ozone layer of the earth.

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