

# **Isolation of Cellulolytic Bacteria from Soil Sample and Production of Cellulase and Its Purification.**



**A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIAL  
FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF  
SCIENCE IN BIOTECHNOLOGY**

**Submitted by  
Mst. Shabrina Khanom  
Student ID: 16376002  
March, 2018**

**Biotechnology Program  
Department of Mathematics and Natural Sciences  
BRAC University  
Dhaka, Bangladesh**

## **Declaration**

I, hereby certify that the thesis work entitled “Isolation of Cellulolytic Bacteria from Soil Sample and Production of cellulase and Its Purification” submitted to the Department of Mathematics and Natural Science, BRAC University in partial fulfillment of the requirement for the degree of Master of Science in Biotechnology is a record of work carried out by me under supervision and guidance of my supervisor Dr. Mahboob Hossain, Professor of Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and the contents of this report in full or parts have not been submitted to any other university and institution for any degree or diploma.

**Candidate:**

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**( Mst. Shabrina Khanom )**

**Certified by:**

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**Dr. Mahboob Hossain**

**Supervisor**

Professor

Microbiology Program

Department of Mathematics and  
Natural Sciences

BRAC University

Dhaka, Bangladesh

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**Mst. Shabrina Khanom**

**March, 2018**

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## List of Abbreviations

<b>CBD</b>	Cellulose Binding Domain
<b>GHs</b>	Glycosyl Hydrolases
<b>CesA</b>	Cellulose Synthase
<b>CD</b>	Catalytic Domain
<b>CBM</b>	Cellulose Binding modules
<b>CAC</b>	Catalytically Active Complex
<b>NA</b>	Nutrient Agar
<b>CMCase</b>	Carboxymethyl cellulase
<b>DNS</b>	Dinitrosalicylic Acid
<b>CipA</b>	Cellulosome Integrating Protein
<b>VP</b>	Voges Proskauer
<b>MIU</b>	Motility Indole Urease



## **Abstract**

The cellulase producing bacteria were isolated from cow dung and characterized by morphological and biochemical analysis. Hence, studies pursue to unfold a novel cellulase that can overcome existing challenges in biorefinery, reduce biofuel production cost, as well as have tremendous applications in industrial processes. Further partial purification of cellulase was carried out by dialysis and ammonium sulphate precipitation. The partially purified enzyme was then purified by gel filtration chromatography using sephadex G-75 matrix and also to determine molecular weight by SDS-PAGE. Bacterial isolate were grown on Carboxy Methyl Cellulose (CMC) agar at various optimum conditions such as parameters like pH, temperature, incubation period prior to examine. In the present study, the result showed that cellulase producing bacteria can grow at optimized condition. The best isolate so far had an extracellular crude enzyme activity of 0.076 U/ml. The use of microorganisms for the production of enzymes offers a promising approach for its large scale production and as a possible food supplement or in the pharmaceutical industry.

# Chapter 1

# 1. Introduction

## 1.1 Background

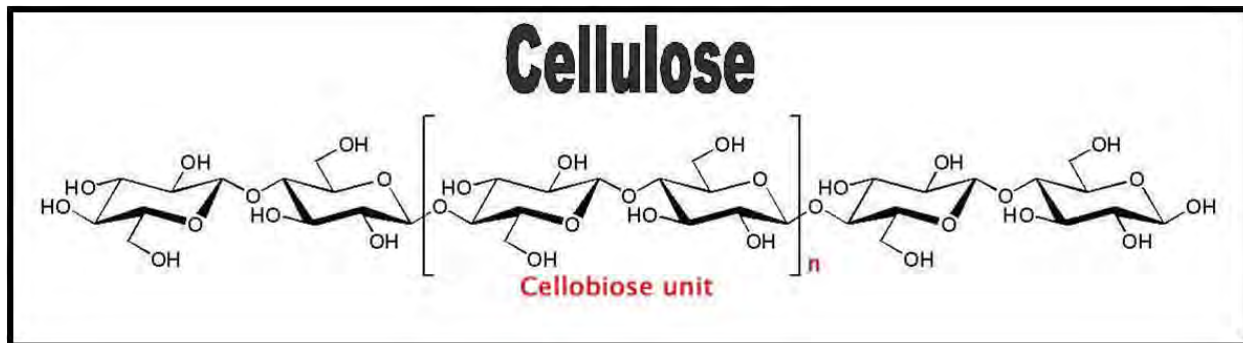
Cellulase is a nonexclusive name for the gathering of compounds which catalyze the hydrolysis of cellulose and related cellu-oligosaccharide subsidiaries. Cellulose comprises mostly of long polymers of  $\beta$  1-4, connected glucose units and structures a crystalline structure (Shallom & Shoham, 2003). The cellulase complex is contained three noteworthy parts: Carboxymethyl cellulase (CMCases) or Endo- $\beta$ -glucanase (EC 3.2.1.4), Exo- $\beta$ -glucanase (EC 3.2.1.91) and  $\beta$ -glucosidase (EC 3.2.1.21) (Kaur *et al.*, 2007). Cellulases from different sources have particular highlights as they display particular pH optima, dissolvability relying upon the amino corrosive organization. Warm steadiness and correct substrate specificity may likewise differ with the starting point (Parry *et al.*, 2001).



**Fig 1.1: Cellulase**

As of late, the compound, cellulase finds wide application to an assortment of fields, for example, material, paper and mash, nourishment and creature encourage, fuel and concoction industry. Furthermore, they can be utilized as a part of waste administration, the pharmaceutical industry, protoplast creation, hereditary building and contamination treatment.

Cellulose is the most copious natural compound in our earth. It's a direct glucopolymer of anhydrous glucose chain, hydrolysis by corrosive and chemical "cellulase" (Sami *et al.*, 1989).



**Fig 1.2 : Cellulose structure**

The capability of cellulose as an option vitality source has empowered research into bioconversion forms which hydrolyse cellulose to solvent sugars for feedstock in alcoholic maturations and other mechanical procedures (Coughlan *et al.*, 1990).

Cellulose has colossal potential as reasonable and sustainable source of energy, as it offers lessened abuse of non-renewable energy sources and natural contamination (Coral *et al.*, 1990). For as far back as a couple of years, there has been huge advances in distinguishing new cellulase assets that can productively and financially change over cellulose into fermentable sugars.

Cellulases are inducible chemicals which are orchestrated by vast number of microorganisms either cell-bound or extracellular amid their development on cellulosic materials (Lee & Koo, 2001).

Cellulose is ordinarily debased by a catalyst called cellulase. Cellulase alludes to a class of chemicals created mainly by growths, microscopic organisms, and protozoans that catalyze the cellulolysis (or hydrolysis) of cellulose (Immanuel *et al.*, 1990). This protein is delivered by a few microorganisms, generally by microscopic organisms and parasites. Cost of cellulase in enzymatic hydrolysis is viewed as a central point (Kanokphorn *et al.*, 2011).

Microscopic organisms have high development rate when contrasted with growths can possibly be utilized as a part of cellulose creation. Cellulolytic property of some bacterial genera, for example, *Cellulomonas* species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* species were accounted for (Nakamura *et al.*, 1982). Chemical generation is firmly controlled by microorganisms and for enhancing its profitability these controls can be improved. Cellulase yields seem to rely upon a perplexing relationship including an assortment of components like inoculum estimate, pH, temperature, nearness of inducers, medium added substances, air circulation, and development time (Robson *et al.*, 1989).

## **1.2 Literature review**

### **1.2.1 Enzyme and historical resume**

With a specific end goal to assign those impetuses, Friedrich Wilhelm Kuhne instituted the name "compound" in 1878. They were additionally titled as "indications of nature's eagerness" as they stimulate a large portion of the synthetic responses happening inside the body. In, 1837 Jones Jacob Berzelius found the reactant idea of these natural diastases. In 1857, Pasteur showed the alcoholic aging which clarifies that the synergist activity could be actuated by living cell, for example, sorted out matures (microbes and yeast) or by non living substances, for example, disorderly ages (diastases). Kuhne proposed protein to be disorderly matures while Eduard and Hans Buchner did the alcoholic maturation with yeast remove asserting cell free aging in 1897. In 1926, James B. Sumner confined and filtered urease and affirmed the proteinaceous idea of the catalysts. From that point forward around 250 chemicals have been acquired in unadulterated crystalline frame, for example, trypsin, catalase, Rnase and so forth. In 1983, the cellulosome idea was found in *Clostridium thermocellum*, in which the cellulases were composed into a high atomic weight cellulolytic complex. In the up and coming years, different cellulase qualities from this bacterium were cloned and sequenced and their secluded structure was recognized (Cavaco *et al.*, 1998).

Cellulose is a noteworthy polysaccharide constituent of plant cell dividers and a standout amongst the most inexhaustible natural mixes in the biosphere (Murai *et al.*, 1998). The cellulolytic catalyst arrangement of unmistakable microorganisms is regularly unique. A few

microorganisms discharge a lot of cellulolytic catalysts in culture media, while others albeit developing on cellulose, discharge practically no chemicals into the medium. In this way, it isn't certain that the cellulolytic compounds are really extracellular or not. By and large, high cellulase exercises are found in culture filtrates just in the stationary period of development and it can be contended that the compounds are discharged via autolysis (Hofsten *et al.*, 1972).

Cellulases are inducible catalysts integrated by microorganisms amid their development on cellulosic material. These catalysts can either be free, especially in high-impact microorganisms, or gathered in a multicomponent compound complex called "cellulosomes" in anaerobic cellulolytic bacteria (Sukumaran *et al.*, 2005). Microorganisms are currently being broadly investigated for cellulase generation in light of their quick development, articulation of multienzyme buildings, solidness at extremes of temperature and pH, lesser criticism hindrance, ability to colonize a wide assortment of ecological specialties, and capacity to withstand assortments of natural anxiety. They deliver cellulases that are steady under outrageous conditions, amid bioconversion forms, which quicken enzymatic hydrolysis, maturation, and item recuperation. These traits incited a look for quickly developing microbes ready to integrate dynamic cellulases under brutal conditions (Lynd *et al.*, 2002). There are a few reports on generation of cellulases from microorganisms, for example, *Bacillus*, *Clostridium*, *Cellulomonas*, *Ruminococcus*, and *Streptomyces spp.* *Bacillus spp.* are most looked for after as they create differing scope of cellulases that are steady under outrageous conditions (Rawat *et al.*, 2012).

### **1.2.2 Types and action**

Five general types of cellulases based on the type of reaction catalyzed:

- Endocellulases (EC 3.2.1.4): randomly cleave internal bonds at amorphous sites that create new chain ends.
- Exocellulases or cellobiohydrolases (EC 3.2.1.91): cleave two to four units from the ends of the exposed chains produced by endocellulase, resulting in tetrasaccharides (Zverlov *et al.*, 2012). or disaccharides, such as cellobiose. Exocellulases are further classified into type I, that work processively from the reducing end of the cellulose chain, and type II, that work processively from the nonreducing end.
- Cellobiases (EC 3.2.1.21) or beta-glucosidases: hydrolyse the exocellulase product into individual monosaccharides.

- Oxidative cellulases: depolymerize cellulose by radical reactions, as for instance cellobiose dehydrogenase (acceptor).
- Cellulose phosphorylases: depolymerize cellulose using phosphates instead of water.

Within the above types there are also progressive (also known as processive) and nonprogressive types. Progressive cellulase will continue to interact with a single polysaccharide strand, nonprogressive cellulase will interact once then disengage and engage another polysaccharide strand.

### 1.2.3 microorganism used for cellulase production

Table 1. Selected bacterial and fungal strains for glycosyl hydrase production.		
Name	Enzymes types	
<b>Bacteria (aerobic)</b>		
<i>Acidothermus cellulolyticus</i>	NC/HC	T
<i>Bacillus sp.</i>	NC/HC	M/AT
<i>Bacillus pumilus</i>	NC/HC	M/AT
<i>Bacillus substilis</i>	NC/HC	M/T
<i>Bacillus agaradhaerens</i> JAM-KU023	NC/HC	T/A
<i>Brevibacillus sp.</i> strain JXL	NC/HC	T
<i>Cellulomonas flavigena</i>	NC/HC	T/AT
<i>Cellulomonas fimi</i>	NC/HC	M
<i>Geobacillus thermoleovorans</i>	NC/HC	T/AT
<i>Paenibacillus campinasensis</i> BL11	NC/HC	T
<i>Paenibacillus strain</i> B39	NC	T
<i>Streptomyces sp.</i>	NC/HC	M/T
<i>Thermoactinomyces sp.</i>	NC/HC	T
<i>Thermomonospora curvata</i>	NC/HC	T
<i>Thermomonospora fusca</i>	NC/HC	T
<b>Bacteria (anaerobic)</b>		
<i>Acetivibrio cellulolyticus</i>	Cellulosome/NC	M
<i>Bacteroides cellulosolvens</i>	Cellulosome	M
<i>Clostridium acetobutylicum</i>	Cellulosome	M
<i>Clostridium cellulolyticum</i>	Cellulosome/NC	M
<i>Clostridium cellulovorans</i>	Cellulosome/NC	M
<i>Clostridium josui</i>	Cellulosome	M
<i>Clostridium papyrosolvens</i>	Cellulosome	M
<i>Clostridium thermocellum</i>	Cellulosome/NC	T
<i>Ruminococcus albus</i>	Cellulosome	M
<i>Ruminococcus flavefaciens</i>	Cellulosome	M

Filamentous fungi (aerobic)		
<i>Acremonium cellulolyticus</i>	NC/HC	M
<i>Acrophialophora nainiana</i>	HC/HC	M
<i>Aspergillus acculeatus</i>	NC/HC	M
<i>Aspergillus fumigatus</i>	NC/HC	M/T
<i>Aspergillus niger</i>	NC/HC	M
<i>Aspergillus oryzae</i>	NC/HC	M
<i>Fusarium solani</i>	NC/HC	M
<i>Humicola grisea</i> var. <i>thermoidea</i>	NC/HC	T
<i>Irpex lacteus</i>	NC/HC/LN	M
<i>Penicillium funmiculosum</i>	NC/HC	M
<i>Penicillium atrovenetum</i>	NC/HC	T
<i>Penicillium citrinum</i>	NC/HC	M
<i>Phanerochaete chrysosporium</i>	NC/HC/LN	M
<i>Schizophyllum commune</i>	NC/HC	M
<i>Sclerotium rolfsii</i>	NC/HC	M
<i>Sporotrichum cellulophilum</i>	NC/HC	T
<i>Talaromyces emersonii</i>	NC/HC	T
<i>Thielavia terrestris</i>	NC/HC	T
<i>Trichoderma koningii</i>	NC/HC	M
<i>Trichoderma reesei</i>	NC/HC	M
<i>Trichoderma viride</i>	NC/HC	M
Anaerobic fungi		
<i>Anaeromyces elegans</i>	NC/HC	M
<i>Anaeromyces mucronatus</i>	NC/HC	M
<i>Caecomyces</i> CR4	NC/HC	M
<i>Neocallimastic frontalis</i>	Cellulosome	M
<i>Neocallimastic hurleyensis</i>	Cellulosome	M
<i>Neocallimastic patriciarum</i>	Cellulosome	M
<i>Orpinomyces joyonii</i>	Cellulosome	M
<i>Orpinomyces</i> PC-2	Cellulosome	M
<i>Piromyces communis</i>	Cellulosome	M
<i>Piromyces equi</i>	Cellulosome	M
<i>Piromyces</i> E2	Cellulosome	M
AT: Alkali tolerant; HC: Hemicellulase; LN: Ligninase; M: Mesophilic; NC: Noncomplexed cellulase; T: Thermophilic.		

### 1.2.4 Mode of action of cellulase

Cellulases [1,4-(1,3; 1,4)-  $\beta$ -D-glucan 4-glucanohydrolase, EC 3.2.1.4] are fundamental in the carbon cycle in nature. In most cellulolytic microorganisms, a few cellulase segments delivered in the culture filtrate together constitute a "cellulase framework," and insoluble cellulosic substrates are changed over to dissolvable sugar by their synergistic activity.



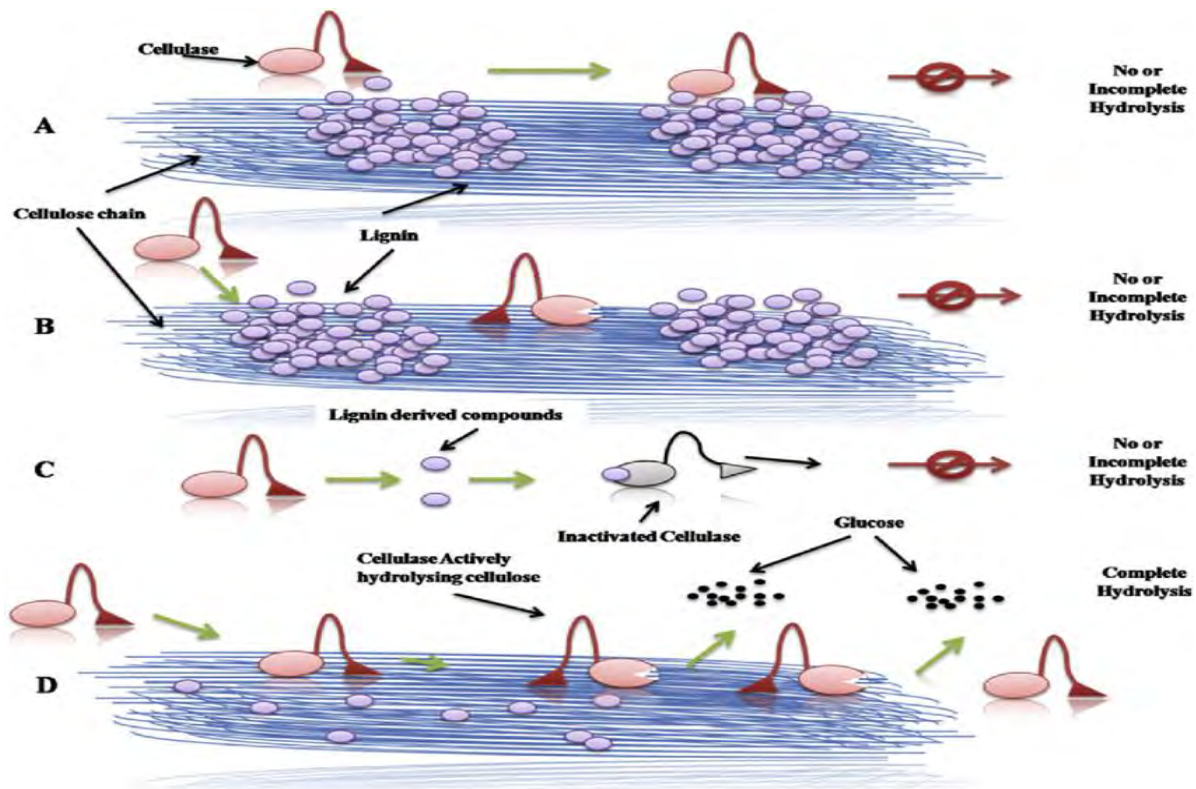


Fig 1.3: Inhibition of cellulase by lignin. a) Non-productive adsorption of cellulase onto lignin, b) Physical blockage of cellulase progress on lignocellulose chain, c) enzyme inhibition due to soluble lignin-derived compounds, and d) normal functioning of cellulase on cellulose chain to release glucose in presence of no or very low amount of lignin.

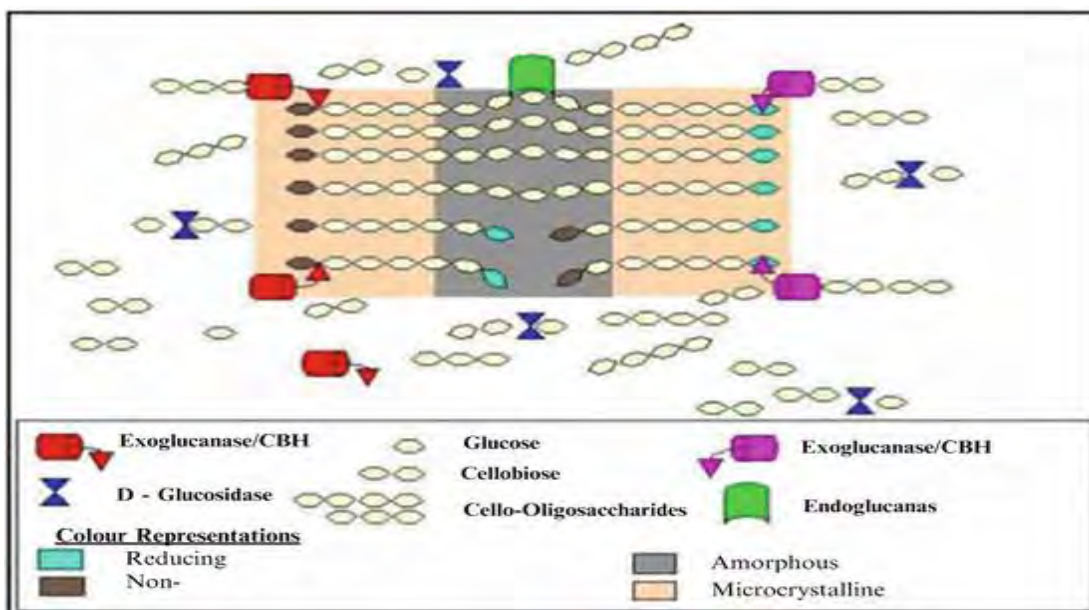


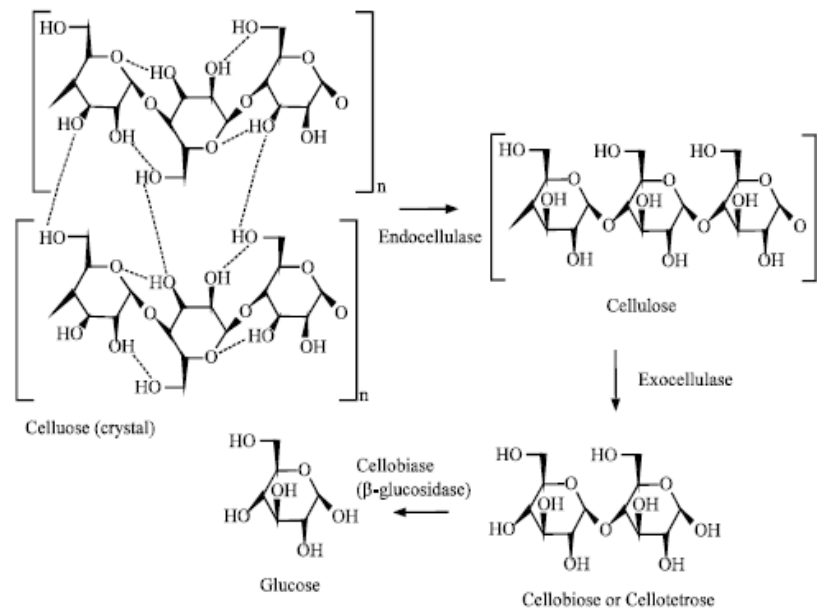
Fig 1.4 : Mode of action of cellulase

### 1.2.5 Cellulase complexes

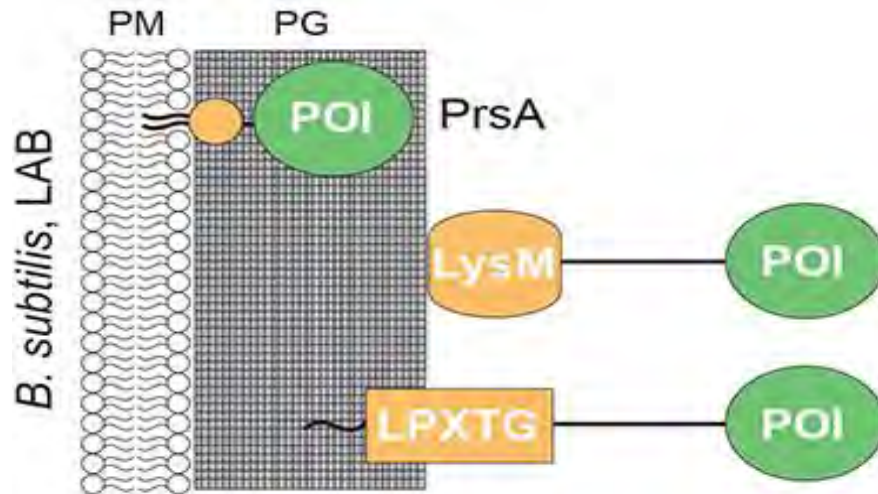
In numerous microscopic organisms, cellulases in-vivo are mind boggling catalyst structures sorted out in supramolecular edifices, the cellulosomes. They contain approximately five diverse enzymatic subunits speaking to in particular endocellulases, exocellulases, cellobiases, oxidative cellulases and cellulose phosphorylases wherein just endocellulases and cellobiases take part in the real hydrolysis of the  $\beta$  (1 $\rightarrow$ 4) linkage.

The cellulase complex from *Trichoderma reesei*, for instance, contains a part marked C1 (57,000 daltons) that isolates the chains of crystalline cellulose, an endoglucanase (around 52,000 daltons), an exoglucanase (around 61,000 daltons), and a beta-glucosidase (76,000 daltons).

Various "mark" groupings known as dockerins and cohesins have been recognized in the genomes of microbes that create cellulosomes. Contingent upon their amino corrosive arrangement and tertiary structures, cellulases are isolated into class and families (*Bayer et al.*, 1998).



**Fig 1.5 : Mode of action of cellulase enzyme**



**Fig 1.6 : Methods used to display cellulases and their complexes. Proteins can be anchored to the plasma membrane through fusion to PrsA, but lysozyme treatment is necessary to expose this cellulase to the external environment. More conventionally, proteins are cell surface displayed either through noncovalent interactions with cell wall peptidoglycan by protein fusion to the LysM domain, or through covalent attachment to cell wall peptidoglycan.**

### **1.2.6 Applications of cellulase in different industries**

Cellulases have pulled in much intrigue in view of the assorted variety of their application. The major mechanical uses of cellulases are in material industry for 'bio-cleaning' of textures and creating stonewashed look of denims, and also in family unit clothing cleansers for enhancing texture non-abrasiveness and splendor. Utilization of catalysts in material, nourishment, cleanser, calfskin and paper businesses requests distinguishing proof of exceptionally stable proteins dynamic at outrageous pH and temperature.

**Table 1.2: Applications of cellulases** (Kuhad *et al.*, 2011).

<b>Industry</b>	<b>Applications</b>
Agriculture	Plant pathogen and disease control; generation of plant and fungal protoplasts; enhanced seed germination and improved root system; enhanced plant growth and flowering; improved soil quality; reduced dependence on mineral fertilizers.
Bioconversion	Conversion of cellulosic materials to ethanol, other solvents, organic acids and single cell protein, and lipids; production of energy-rich animal feed; improved nutritional quality of animal feed; improved ruminant performance; improved feed digestion and absorption; preservation of high quality fodder.
Detergents	Cellulase-based detergents; superior cleaning action without damaging fibers; improved color brightness and dirt removal; remove of rough protuberances in cotton fabrics; anti-redeposition of ink particles.
Fermentation	Improved malting and mashing; improved pressing and color extraction of grapes; improved aroma of wines; improved primary fermentation and quality of beer; improved viscosity and filterability of wort ; improved must clarification in wine production; improved filtration rate and wine stability.
Food	Release of the antioxidants from fruit and vegetable pomace; improvement of yields in starch and protein extraction; improved maceration, pressing, and color extraction of fruits and vegetables; clarification of fruit juices; improved texture and quality of bakery products; improved viscosity fruit purees; improved texture, flavor, aroma, and volatile properties of fruits and vegetables; controlled bitterness of citrus fruits.

Pulp and Paper	Co-additive in pulp bleaching; biomechanical pulping; improved draining; enzymatic deinking; reduced energy requirement; reduced chlorine requirement; improved fiber brightness, strength properties, and pulp freeness and cleanliness; improved drainage in paper mills; production of biodegradable cardboard, paper towels, and sanitary paper.
Textile	Bio-stoning of jeans; bio-polishing of textile fibers; improved fabrics quality; improved absorbance property of fibers; softening of garments; improved stability of cellulosic fabrics; removal of excess dye from fabrics; restoration of color brightness.
Others	Improved carotenoids extraction; improved oxidation and cooler stability of carotenoids; improved olive oil extraction; improved malaxation of olive paste; improved quality of olive oil; reduced risk of biomass waste; production of hybrid molecules; production of designer cellulosomes.

The organic parts of handling of cellulosic biomass turn into the core of future research including cellulases and cellulolytic microorganisms. Cellulases are as a rule monetarily delivered by a few enterprises all inclusive and are broadly being utilized as a part of sustenance, creature encourage, maturation, agribusiness, mash and paper, and material applications. With present day biotechnology apparatuses, particularly in the zone of microbial hereditary qualities, novel chemicals and new compound applications will wind up plainly accessible for the different businesses. Changes in cellulase exercises or granting of wanted highlights to catalysts by protein designing are most likely different territories where cellulase look into needs to progress.

### **1.2.7 Aim of the study**

The present study was attempted with the following objectives:

- To isolate and screen of cellulolytic bacteria from cow dung
- Biochemical characterization of the isolate
- Production of cellulose
- Optimization of production conditions(time, temperature, pH, chemical concentration etc)
- Partial purification of cellulase and
- Performing gel filtration chromatography using sephadex G-75 for final purification of cellulase.

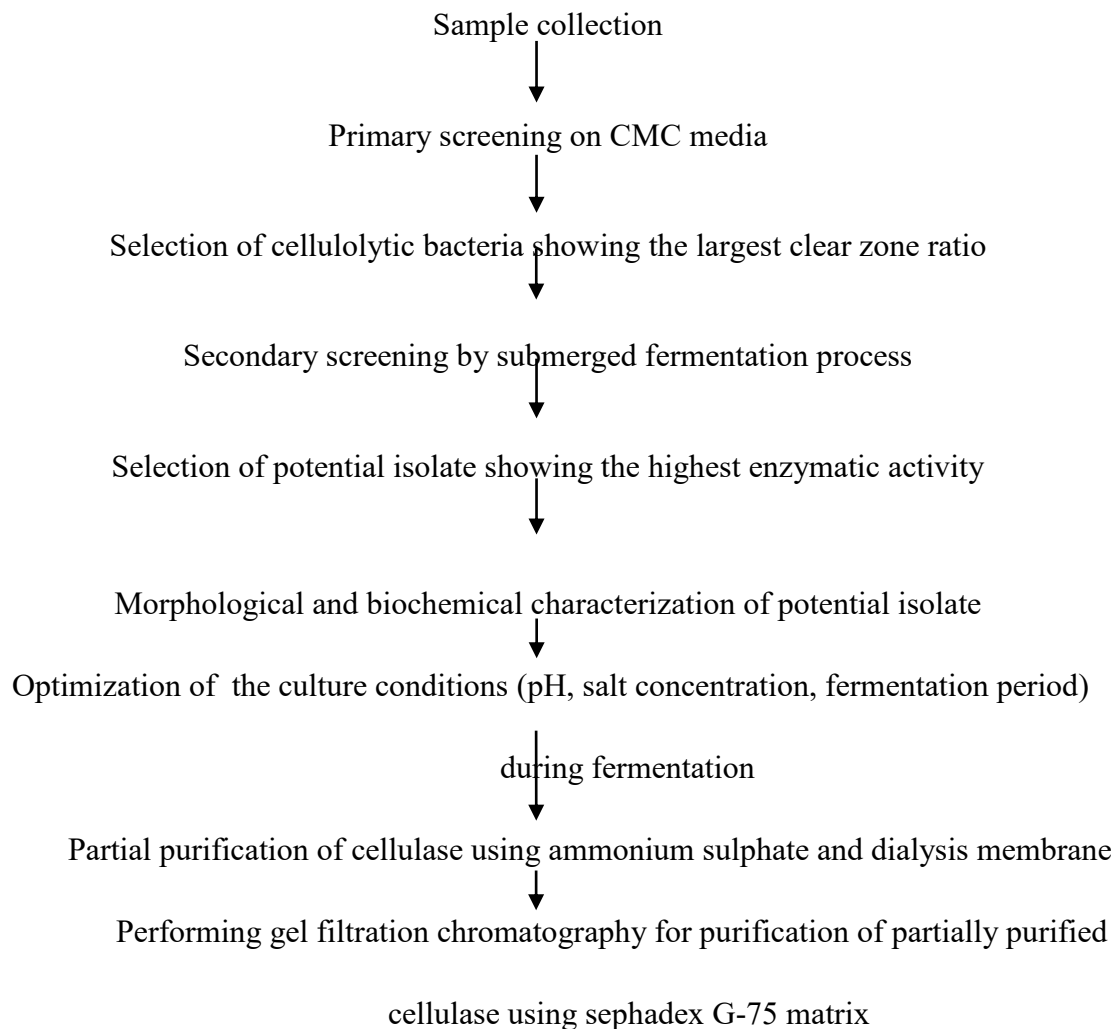
# Chapter 2

## 2. Materials and methods

### 2.1 Research place

This research work was carried out in the Microbiology, Biotechnology and Molecular Biology Laboratory of the Department of Mathematics and Natural Sciences in BRAC University.

### 2.2 Study Design





### **2.3 Sample collection**

The soil sample was collected from Langaljora, Jamalpur. The samples were collected in sterile container and stored at 4°C until use.

### **2.4 Isolation and primary screening of cellulose degrading bacteria**

Ten fold serial dilutions of the soil sample were prepared in autoclaved physiological saline (0.85% NaCl). Initially, 1 g of soil was mixed with 9 ml of saline water in a test tube. This makes a dilution of  $10^{-1}$  which is further diluted in subsequent steps to obtain a final dilution of  $10^{-6}$ . On NA plates 0.1 ml sample from the test tubes labeled  $10^{-4}$ ,  $10^{-5}$  and  $10^{-6}$  were spread plated. The NA plates were then incubated at 37°C for 24 hours. The plates showing discrete colonies were selected for further study. The colonies were transferred using needle to carboxymethylcellulose (CMC) media containing 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 %  $K_2HPO_4$ , 1 % agar, 0.03 %  $MgSO_4 \cdot 7H_2O$ , 0.25 %  $(NH_4)_2SO_4$  and 0.2 % gelatin. After incubation at 37°C for 48 hours, the CMC agar plates were flooded with gram's iodine and allowed to stand at room temperature for about 10 minutes. The ratio of the clear zone diameter to colony diameter of CMC hydrolysis was measured and recorded. For cellulase production in the submerged system the bacterial colonies having largest ratio were selected.

### **2.5 Maintenance of pure culture of the desired isolates**

The colonies showing significant clear zone were streaked on to the NA medium and incubated at 37°C for 24 hours and then stored at 4°C until use (Kuhad *et al.*, 2011).

### **2.6 Secondary screening and production of cellulase**

Isolates showing maximum clear zone were considered for the production of cellulase.

#### **2.6.1 Inoculum development**

From the pure cultures one loopful of bacterial colonies of the selected bacterial isolates were inoculated in inoculum broth containing 0.03%  $MgSO_4$ , 0.2%  $K_2HPO_4$ , 1% glucose, 0.25%  $(NH_4)_2SO_4$  and 1 % peptone and incubated at 37°C for 24 hrs. After 24 hrs these vegetative cells were used as inoculum source for the production medium (Immanuel *et al.*, 2006).

#### **2.6.2 Fermentation process (submerged system)**

CMC broth (50 ml) was prepared containing 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 %  $K_2HPO_4$ , 0.03 %  $MgSO_4 \cdot 7H_2O$ , 0.25 %  $(NH_4)_2SO_4$  and 0.2 % gelatin and

autoclaved at 121°C for 15 minutes. The media were inoculated with 2.5 ml of selected bacterial isolate from the inoculum media and incubated at 37°C for 48 hrs at 150 rpm (Irfan *et al.*, 2012).

### **2.6.3 Crude enzyme preparation**

The fermented broth was centrifuged at 9000 rpm for 20 minutes in order to remove unwanted materials. After centrifugation the clear supernatant was collected to serve as crude enzyme source and utilized for the determination of enzyme activity (Shaikh *et al.*, 2013).

### **2.6.4 Optimization of the culture conditions during fermentation**

#### **Effect of pH, Incubation period, salt concentration on cellulase production**

In order to determine all effects on cellulase production, the selected bacterial isolate was grown in CMC broth and incubated at various parameters. The influence of all factors on enzyme activity was determined by measuring cellulase activity at varying pH values from 4 to 9 for 28 hours and incubation period varying from 24 to 120 h at 37°C. Different salt concentrations CaCl<sub>2</sub>(0.2%), ZnSO<sub>4</sub>(0.14%), MnSO<sub>4</sub>(0.16%), NH<sub>4</sub>NO<sub>3</sub>(0.14%) was added in fermentation broth for 48 hours. All factors influence on enzyme activity was determined by measuring cellulase activity.

### **2.7 Cellulase enzyme activity assay**

Cellulase activity was estimated using 1% solution of carboxymethylcellulose (CMC) in 0.05M citrate buffer (pH 4.8).

#### **2.7.1 Glucose standard curve preparation**

At first 0.2- 1.2mg/ml concentrations of glucose solutions were prepared. One milliliters from each of the dilutions were transferred in the test tubes labeled of respective concentration along with 1 extra test tube in which 1 ml of distilled water was transferred which was served as control. Then 1 ml of 0.05M citrate buffer (pH 4.8) was added in all the test tubes followed by 3 ml of Dinitrosalicylic acid (DNSA) reagent. All the test tubes were then placed in boiling water (100°C) for 10 minutes in water bath. After that the test tubes were taken out from the water bath and allowed to cool to room temperature. The absorbance of each of the samples were then measured in a spectrophotometer at 540 nm. A standard curve was prepared plotting the standard glucose concentration (mg/ml) (x-axis) against the respective absorbance (y-axis) in order to achieve a best fit straight line (Shoham *et al.*, 1999).

### 2.7.2 Reducing sugar estimation by DNSA method

The cellulase activities of each of the bacterial strains were assayed in triplicate. The reaction mixtures contained 1ml 0.05 M citrate buffer (pH 4.8), 1 ml substrate solution (1% CMC dissolved in citrate buffer) and 1 ml crude enzyme solution. The blanks contained 1 ml distilled water instead of the enzyme. The reaction was carried out at 50°C for 30 minutes. 1.5 ml of DNS reagent was added after incubation to all the test tubes in order to stop the reaction. After waiting 10 minutes the tubes were boiled at 100°C for 10 minutes in water bath. The absorbance was measured at 540 nm (Islam *et al.*, 2014). enzymatic activity was determined by the following equation (Buchanan & Gibbons, 1974).

$$\text{Enzyme activity (U/ml)} = \frac{\text{Reducing sugar (product concentration)} \times 1000 \times \text{dilution factor}}{\text{Molecular weight of glucose} \times \text{incubation time (minute)}}$$

## 2.8 Identification of the bacterial isolate

### 2.8.1 Morphological characterization

A NA plate was streaked to obtain isolated discrete colonies. The plate was then incubated at 37°C for 24 hours. After incubation, the bacterial colonies were evaluated for size, pigmentation, form, margin and texture (Buchanan & Gibbons, 1974).

### 2.8.2 Microscopic Observation

The potential bacteria were observed under microscope in order to study their properties.

#### 2.8.2.1 Gram staining

A drop of saline was placed on the slide and a small amount of the bacteria were then transferred to the drop of saline with a sterile cooled inoculating loop. A smear was then prepared by mixing and spreading the bacteria by means of a circular motion of the loop. The smear was then allowed to air dry followed by heat fixation. The smear was flooded with crystal violet and let stand for 1 minute. Then, the smear was gently washed with tap water. It was then flooded again with the gram's iodine mordant and let stand for 1 minute followed by gentle wash with tap water. After that, the smear was decolorized with 95% ethyl alcohol and gently washed with tap water. Finally, it was counterstained with safranin for 45 seconds and gently washed with tap

water. The slide was then dried with bibulous paper and examined under oil immersion using microscope (Buchanan & Gibbons, 1974).

### **2.8.2.2 Spore staining**

With 5 mg/L of  $MnSO_4 \cdot H_2O$  NA was prepared in order to incubate the bacteria for 48 hours in a nutrient deficient condition to encourage sporulation. After incubation, using sterile technique, smear was prepared in the usual manner on clean glass slide. It was allowed to air dry and heat fixed in the usual manner. The smear was flooded with malachite green while placed over a water bath and allowed to steam for 2 to 3 minutes. The stain was prevented from drying out by constant application of the dye. The slides were removed, cooled and washed under running tap water. The smear was then counterstained with safranin for 30 seconds and washed with tap water. The slide was then blot dried with bibulous paper and examined under oil immersion (Buchanan & Gibbons, 1974).

### **2.8.3 Biochemical characterization**

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Shoham *et al.*, 1999).

#### **2.8.3.1 Carbohydrate Utilization test**

In separate test tubes phenol red sucrose, fructose, glycerol, maltose and D-xylose broths were prepared by autoclaving at 15 psi 121°C for 15 minutes. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the broths by means of loop inoculation. All the tubes were incubated at 37°C for 24 hours (Buchanan & Gibbons, 1974).

#### **2.8.3.2 Methyl red test**

MR-VP broth of 7 ml test tube was prepared by autoclaving at 15 psi 121°C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tube and the tube was incubated for 24 hours at 37°C. After 24 hours 3.5 ml from the culture tube was transferred to clean test tubes for Voges- Proskauer test and the remaining broth was re-incubated for additional 24 hour. To observe the immediate development of a red color

after 48 hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes (Buchanan & Gibbons, 1974).

### **2.8.3.3 Voges Proskauer test**

In the MR-VP broth after 24 hour incubation, 0.6 ml (12 drops) of 5% alpha naphthol (Barrit's reagent A) was added followed by 0.2 ml ( 4 drops) of 40% KOH (Barrit's reagent B). The tube was gently shaken to expose the medium to atmospheric oxygen (30seconds to 1 minute) and the medium was allowed to remain undisturbed for 10-15 minutes. The test was read, but not beyond, one hour following the addition of the reagents (McDevitt, 2009).

### **2.8.3.4 Citrate utilization test**

Two ml simmon citrate agar slant was prepared by autoclaving at 15 psi, 121°C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the vial by means of a streak inoculation method with an inoculating needle and the vials were incubated at 37°C for 48 hours (Buchanan & Gibbons, 1974).

### **2.8.3.5 Nitrate reduction test**

Six milliliters nitrate broth was prepared by autoclaving at 15 psi, 121°C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tube by means of a loop inoculation method with an inoculating loop and the tube was incubated for 24 to 48 hours at 37°C. After incubation, 5 drops of reagent A and 5 drops of reagent B was added to the broth. If there was no red color development, a small amount of zinc was added to the tube (Buchanan & Gibbons, 1974).

*Note: Caution was maintained during the use of powdered zinc as it is hazardous.*

### **2.8.3.6 MIU (Motility- Indole- Urease) test**

The MIU media was prepared by autoclaving at 15 psi, 121°C. the media was cooled to about 50-55°C and 100ml of urease reagent was added aseptically to 900 ml base medium. After that, 6 ml solution was transferred to the sterile test tube and allowed to form a semi solid medium. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tube by means of a stab inoculation method with an inoculating needle and the tube was then incubated at 37°C for 24 hours (Buchanan & Gibbons, 1974).

### **2.8.3.7 Catalase test**

A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24 hour pure culture was placed onto the microscopic slide. One drop of 3% H<sub>2</sub>O<sub>2</sub> was placed onto the organism on the microscopic slide using a dropper and observed for immediate formation of bubble (Reiner, 2010).

### **2.8.3.8 Oxidase test**

In Gaby and Hadley oxidase test reagent a small piece of filter paper was soaked and let dry. Using an inoculating loop, a well isolated colony from pure 24 hour culture was picked and rubbed onto filter paper and observed for color change (Shil *et al.*, 2014).

### **2.8.3.9 Gelatin hydrolysis test**

All the ingredients of the nutrient gelatin medium were mixed and gently heated to dissolve. 3 ml from the media was dispensed in glass vials. The glass vials with the medium were then autoclaved at 121°C, 15 psi. The tubed medium was allowed to cool in an upright position before use. Using sterile technique, a heavy inoculum of 24 hour old bacterial culture was stab inoculated into the tubes with an inoculating needle. The glass vials were then incubated at 37°C and observed up to 1 week (Cruz & Torres, 2012).

### **2.8.3.10 Triple Sugar Iron Agar test**

Triple sugar iron slants were prepared in the test tubes and autoclaved at 15 psi 121°C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab and streak inoculation method. The tubes were incubated for 24 hours at 37°C (Buchanan & Gibbons, 1974).

### **2.8.3.11 Mannitol Salt Agar test**

A plate of MSA agar was streaked by picking a loopful colony of 24 hour old pure culture to obtain isolated colonies. The plate was then incubated at 37°C for 24 hours (Shil *et al.*, 2014).

### **2.8.3.12 Starch hydrolysis test**

A starch agar plate was streaked by picking a loopful colony of 24 hour old pure culture with an inoculating loop. The plate was then incubated at 37°C for 48 hours and using gram's iodine the hydrolysis was observed (Buchanan & Gibbons, 1974).

### **2.8.3.13 Blood agar test**

In a conical flask nutrient agar was prepared and autoclaved at 121°C, 15 psi. The nutrient agar medium was allowed to cool at 45-50°C and 5% (vol/vol) sterile defibrinated sheep blood that had been warmed to room temperature was added and gently mixed avoiding air bubbles. The media was then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a blood agar plate was streaked by picking a loopful colony of 24 hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 37°C for 24 hours. After incubation, the plates were observed for gamma, beta and alpha hemolysis (Aryal, 2015).

## **2.9 Purification of crude cellulase**

The crude enzyme was purified by partial purification and gel filtration chromatography using sephadex G-75(Sigma Aldrich).

### **2.9.1 Partial purification of Cellulase**

Partial purification was performed by ammonium sulphate and then dialysis was performed using dialysis membrane.

#### **2.9.1.1 Ammonium sulphate precipitation**

About 20 ml of the crude enzyme prepared was brought to 60% saturation with solid ammonium sulphate (Lee *et al.*, 2006). The mixture was left overnight at 4°C in a magnetic stirrer. Centrifuge the mixture and the pellet was dissolved in 10 ml of 50 mM sodium acetate buffer (pH-5.5) for further purification.

### **2.9.1.2 Dialysis**

The dialysis bag was used for the dialysis of the enzyme collected after the ammonium sulphate precipitation (Lee *et al.*, 2006). Eight milliliters of the partially purified enzyme was dialyzed against 30 mM sodium acetate buffer (pH-5.5) at 4°C with three changes of buffer. The partially purified sample was assayed for protein content and enzyme activity.

### **2.9.2 Gel filtration chromatography**

At first the glass column was washed several times using sodium acetate buffer (pH 5.5). After washing and removing the buffer sephadex G-75 matrix solution was first loaded into the column. After setting the matrix the partially purified enzyme was loaded into column and allowed to diffuse. The passed enzyme through the matrix was collected in different test tubes. The elute was collected in 2-mL fractions and monitored for protein content enzyme activity using spectrophotometer. The protein content was measured in spectrophotometer at 270 nm using sodium acetate buffer as control and enzyme activity was measured by DNSA method.

### **2.9.3 Analysis of Purification by SDS-PAGE**

To check the purity of enzyme, SDS-PAGE was performed following the method of Laemmli (1970). The purified enzyme sample was mixed with 5x loading dye buffer in a ratio of 4:1. The sample mixture was subjected to heat denaturation for 5 min and centrifuged at 12,000 rpm for 1 min. The crude cellulase and column-purified cellulase from different steps of purification were loaded on 10% acrylamide gels and the electrophoresis was carried out using 1x running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl, pH 8.3) with a current of 2.5 mA per lane. The first two gels were loaded with same samples of crude supernatant and ammonium sulfate-purified cellulose. The remaining gel was loaded with column-purified cellulase fractions.

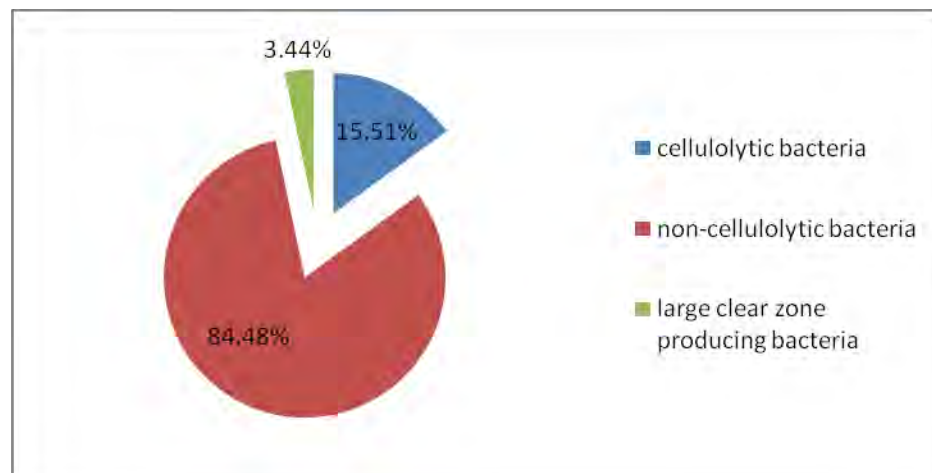


# Chapter 3

### 3. Results

#### 3.1 Primary screening of cellulolytic bacteria

Fifty eight colonies were obtained from the dilution  $10^{-4}$  and  $10^{-5}$  plate and their ability to produce cellulase was determined by primary screening on Carboxy Methyl Cellulose (CMC) agar to select the potential isolates showing best yellow clear zone of hydrolysis. Only 9 isolates showed cellulolytic activity. Of them, 2 isolates (27 and 37) showed significantly large clear zone.



**Figure 3.1: The percentage of the types of bacteria in the soil sample**

Theses 2 isolates were selected for further studies.



**Figure 3.2: Zone of hydrolysis of isolate 27 (left) and 37 (right)**

### 3.2 Secondary screening of cellulolytic bacteria

For cellulase production in submerged fermentation process these two bacterial isolates (37 and 27) were screened using CMC broth. A glucose standard curve was prepared for the estimation of reducing sugar concentration.

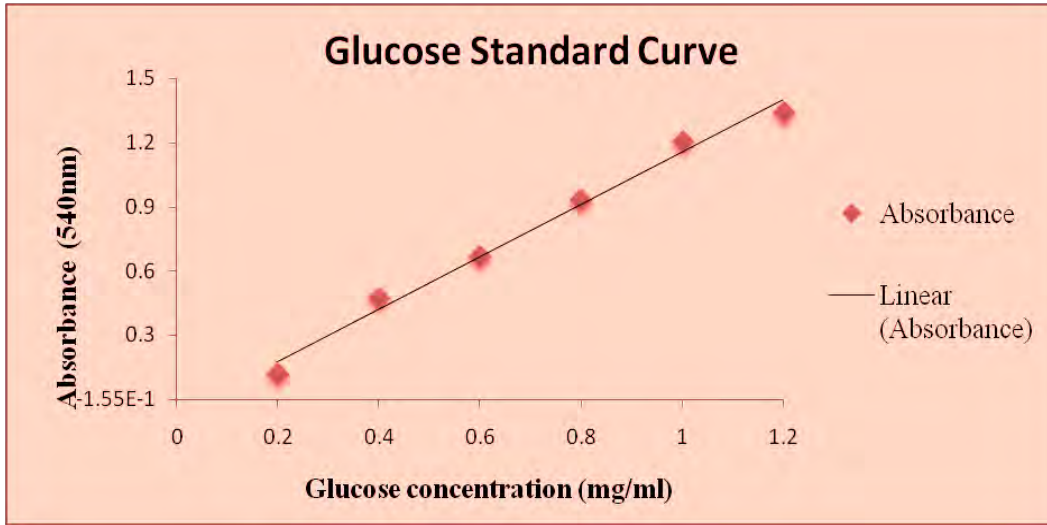


Fig 3.3: Glucose standard curve

The cellulase activities of the isolate 27 and 37 were found to be 0.052 U/ml and 0.076 U/ml respectively. This is shown in figure 3.4.

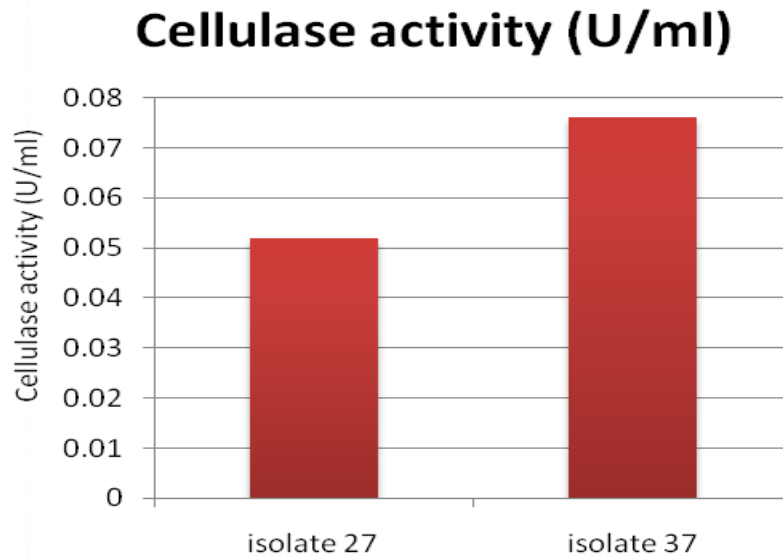
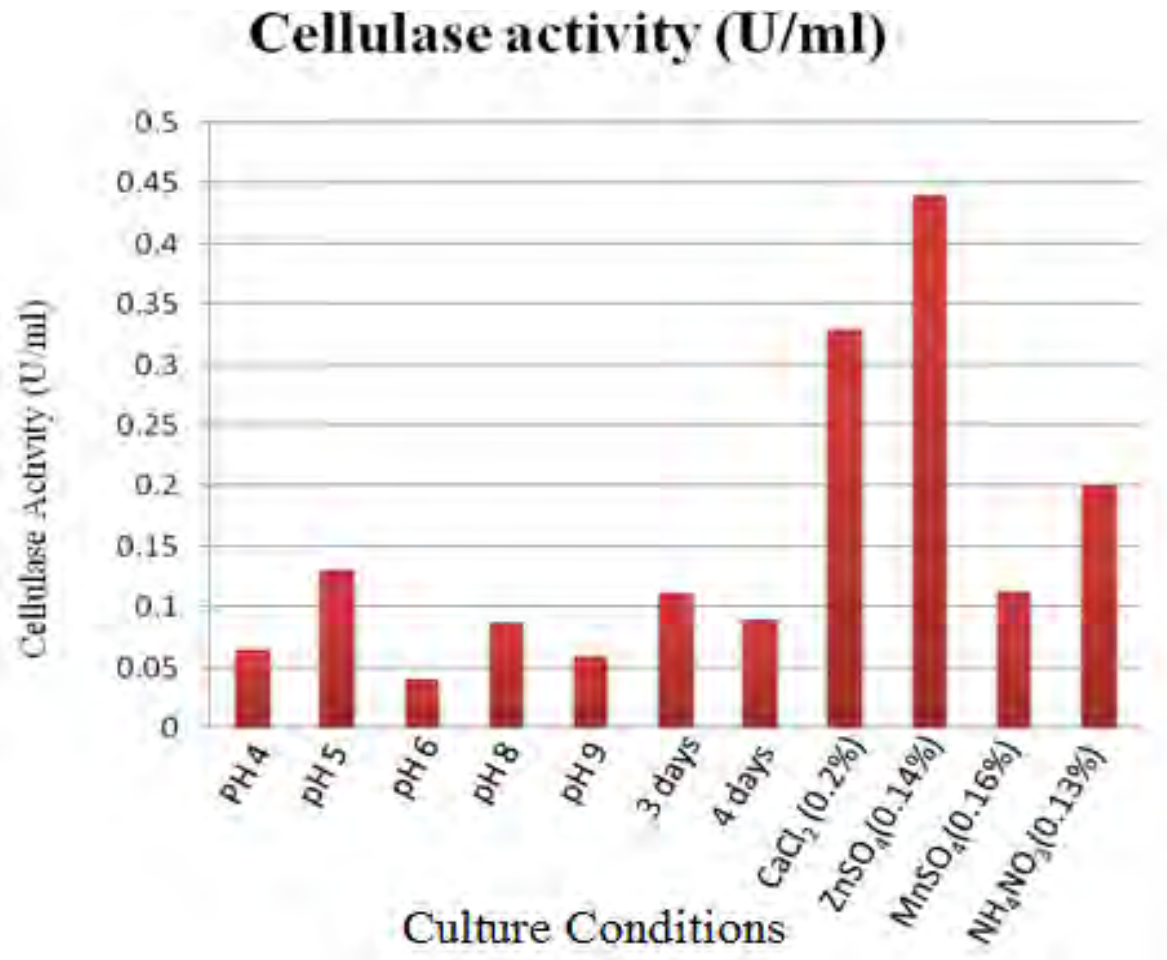


Fig 3.4: Cellulase activity of isolated bacterial strains in submerged fermentation

The result of optimizing the culture conditions (pH, incubation time and salt concentration) are shown in the figure. The fermentation time was 2 days in all cases.



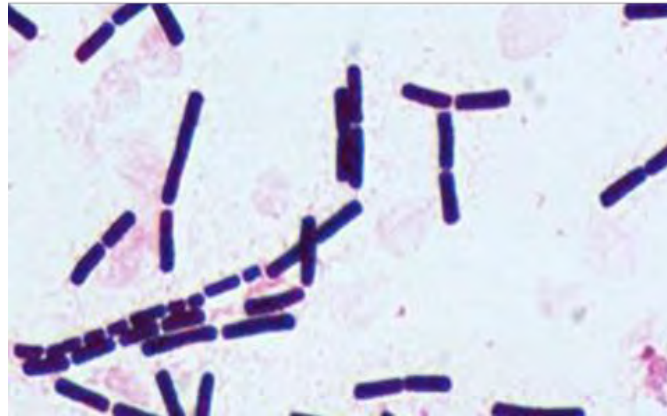
**Fig 3.5 : Cellulase activity of bacterial isolate 37 after optimizing culture conditions**

Since, isolate 37 showed higher enzyme activity compared to isolate 27, it was selected for further studies: biochemical characterization, cellulase purification (partial purification and gel filtration chromatography).

### 3.3 Microscopic observation of bacterial isolate

#### 3.3.1 Gram stain

The cells were observed under light microscope (Krüss, Germany). The cells were found to be Gram positive.



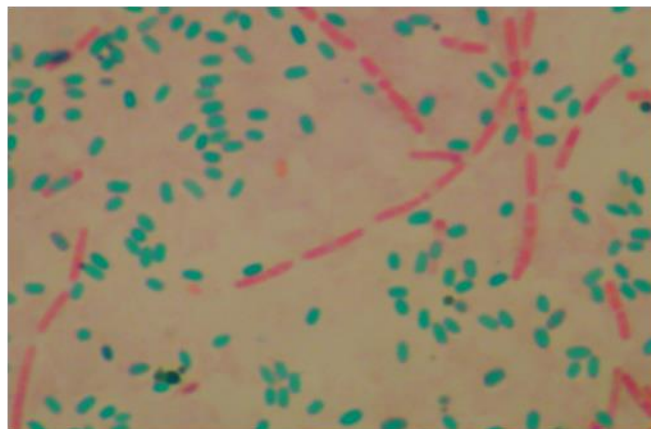
**Fig 3.6 : Gram stain result of isolate 37.**

**Table 3.1: Gram staining results of isolate 37**

<b>Parameters</b>	<b>Isolate 37</b>
Gram reaction	Positive
Cell shape	Rod
Cell color	Purple
Cell arrangement	Single bacillus

### 3.3.2 Spore stain

Under the light microscope, the cells were observed. The addition of  $MnSO_4 \cdot H_2O$  in the nutrient agar media encouraged the sporulation creating nutrient deficient condition for the bacteria. Spores were visible under immersion oil.



**Fig 3.7 : Spore stain result of isolate 37.**

Parameters	Isolate 37
Color of spores	Green
Color of vegetative cells	Red

### 3.4 Biochemical characterization

In order to have presumptive identification of the bacteria biochemical tests were carried out. Out of these glycerol, sucrose, fructose, triple sugar iron agar test, VP (Voges Proskauer) test, citrate utilization test, nitrate reduction test, catalase test, starch hydrolysis test, blood agar test and growth in anaerobic test gave positive results while oxidase test, mannitol salt agar test, gelatin hydrolysis test, methyl red test, Indole and Urease test gave negative results. The results of the tests are given in the following table 3.2.

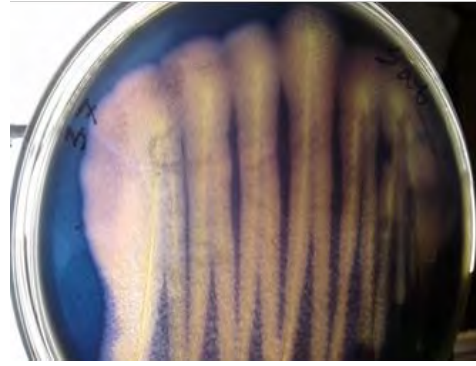
**Table 3.2: Biochemical test results**

<b>Biochemical tests</b>	<b>Results</b>
Glycerol utilization test	+
Sucrose utilization test	+
Fructose utilization test	+
Maltose utilization test	-
Triple sugar iron test	+
VP (Voges Proskauer) test	+
Citrate utilization test	+
Nitrate reduction test	+
Catalase test	+
Oxidase test	-
Gelatin hydrolysis test	-
Starch hydrolysis test	+
Methyl red test	-
MIU test	Motility(+), Indole(-), Urease(-)
Mannitol salt agar test	-

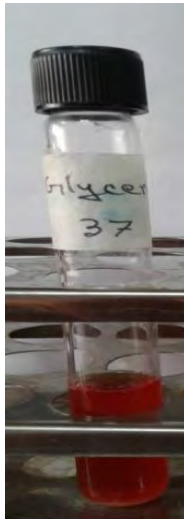
The biochemical test results suggests that the organism is *Bacillus cereus*.



Catalase test



Starch hydrolysis test



Glycerol utilization test



VP test



Citrate utilization test



Methyl red test



Nitrate reduction test

**Fig 3.8: Biochemical tests performed with the isolate 37.**



Since, it is hard to fully determine the species only on the basis of performances of biochemical tests given by isolate 37, the bacteria were tentatively identified to be *Bacillus cereus*.

### 3.5 Enzyme activity after partial purification

The crude enzyme produced from isolate 37 (*B. cereus*) was partially purified using ammonium sulphate and dialysis membrane and their enzyme activity was measured.



Fig 3.9: Cellulase after  $(\text{NH}_4)_2\text{SO}_4$  precipitation



Fig 3.10 : After dialysis

The enzyme activity after ammonium partial purification was found 0.132 U/ml. This is shown in the fig 3.11.

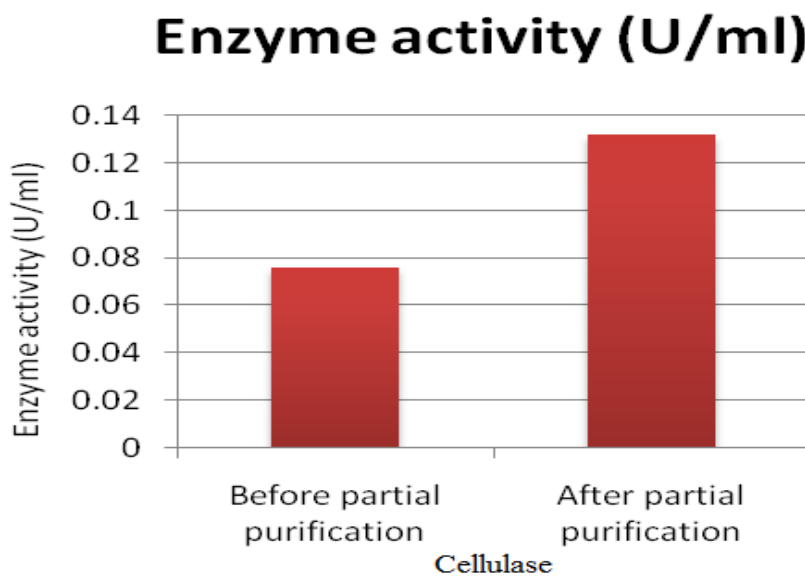


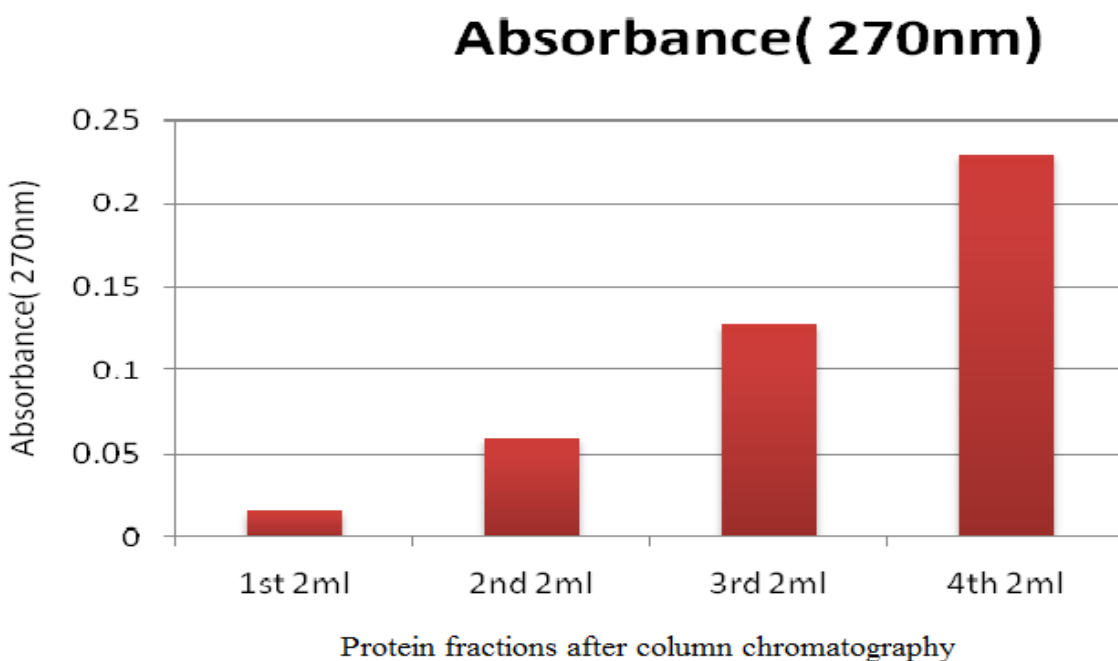
Fig 3.11 : Cellulase activity of *B. cereus* after partial purification.

### 3.6 Protein content and enzyme activity after column chromatography



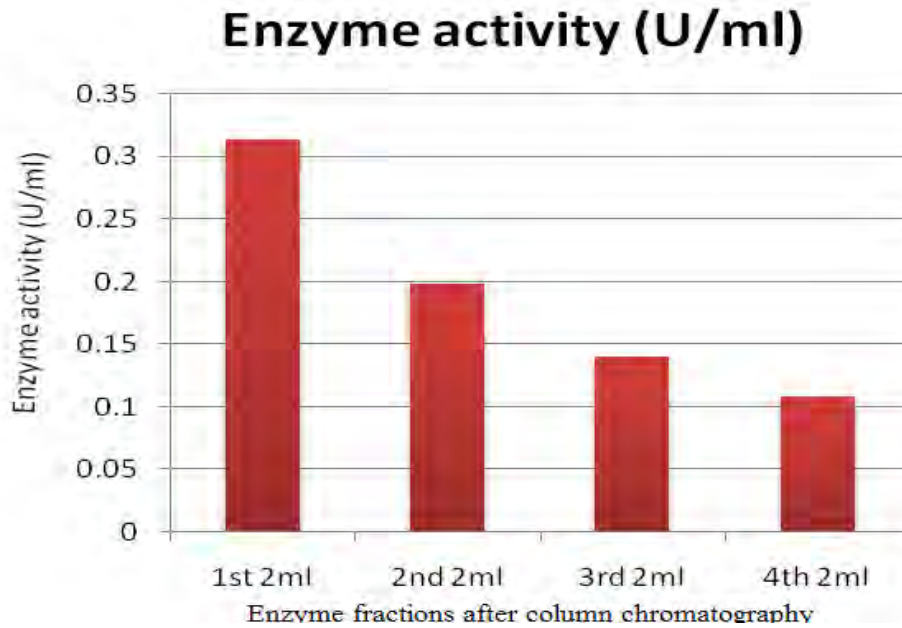
**Fig 3.12 : Gel filtration using sephadex G-75**

Lowest (0.016) absorbance was observed in 1<sup>st</sup> 2 ml drops of purified protein fractions after gel filtration chromatography and it was gradually increased (0.059, 0.128, 0.230).



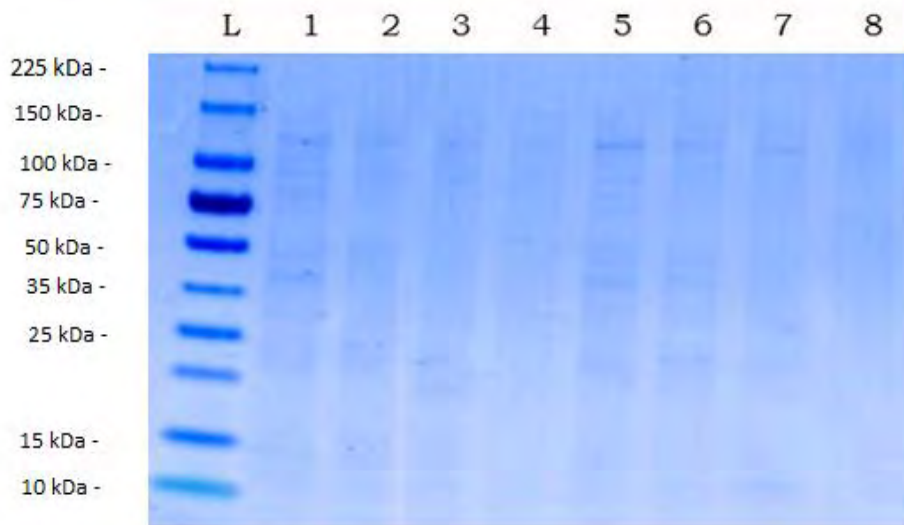
**Fig 3.13 : Absorbance of purified protein fractions after gel filtration chromatography**

Here 1st 2 ml filtrates showed highest enzyme activity (0.313U/ml) and it gradually decreased to next filtrates (0.198 U/ml, 0.140 U/ml, 0.108 U/ml).



**Fig 3.14 : Enzyme activity after gel filtration chromatography**

SDS-PAGE results shows no secretion of protein.



**Fig 3.15. SDS-PAGE gel and a protein ladder. From left to right: protein ladder (Promega), following show the sample fractions.**

# Chapter 4

## 4. Discussion

The present study aims for isolation of potent cellulose degrading bacteria that can hold a high demand in the industry, optimization of production conditions to determine the most suitable conditions and purify the crude cellulase by partial purification and gel filtration chromatography.

Degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes. Territories that contain these substrates are the best sources in which to discover these microorganisms (Lee, 2006). Cow waste was chosen as a hotspot for getting attractive cellulose creating creatures, since it is a rich wellspring of a various gathering of cellulolytic microorganisms inferable from eating regimen of the ruminants which comprises of high measures of cellulosic matter. Further, its wide accessibility, simplicity of preparing and cost adequacy additionally assumes an essential part for its determination (Haung and Priest, 2004). The disengaged bacterial states were portrayed for their morphological and biochemical qualities. Further, chose provinces were screened for their cellulase movement by Congo red test.

At the beginning of this study, out of 58 colonies that were isolated from the sample soil mixed with cow manure, 9 colonies had the capability of cellulose hydrolysis. Just two of them were later chosen to be the powerful cellulose hydrolyzer as indicated by their most noteworthy clear zone proportion. After the protein examine, at long last one of them (37) was chosen to complete further examination contrasting their chemical movement along and particular action and the one with the better outcome was chosen. The explanation behind better clear zone was because of the assortment of microorganism display in the dirt and the sort of natural issue in these dirt. Cow compost is a rich wellspring of cellulose debasing microscopic organisms because of the eating routine of ruminant which comprises of high measures of cellulosic material. Hence, the soil mixed with cow dung refers to the superior strains with more ability for survival and production of cellulase.

The level of cellulolytic microbes gathered from this example was 18.95%. In a portion of the past investigations, the rate was 34.7% in cultivating soil, 42.86% in dairy animals excrement, 32.35% in soil of wood outfitting area and sugar stick ranch and water tests that were gathered from paper industry squander and civil waste (Shaikh et al., 2013). Contrasted with these outcomes, the gathered soil turned out to be a decent wellspring of cellulase maker.

Media advancement is a critical perspective to be considered in the improvement of maturation innovation. The segregated *Bacillus* spp. was vaccinated in maturation medium and production of cellulase was examined. Catalyst generation was tried with various

pH, diverse temperature, distinctive carbon sources and nitrogen sources (Lynd et al., 2002). In view of the outcomes, the aging media has been planned and the generation of cellulase was completed. The way of life utilized for vaccination in the aging medium must be in sound, dynamic state and of ideal size, conceivably in the log stage, along these lines it will be in its high rate for substrate transformation.

The cellulase exercises of the segregate 27 and 37 were observed to be 0.052 U/ml and 0.076 U/ml separately by DNSA technique after submerged maturation procedure of 48 hours at 150 rpm and 37°C.

Since cellulases are dynamic at pH scope of 5.0-6.5 for *Bacillus* strains (Mawadza et al., 2000). In this examination subsequent to upgrading pH for the separate 37 the subsequent action were observed to be 0.065, 0.13, 0.041, 0.088 and 0.06 individually for the pH 4, 5, 6, 8 and 9. The greatest movement was found at pH 5.

Time was moreover upgraded for 48 h, 72 h, 96 h and 120 h the compound activity were found 0.076, 0.06, 0.112 and 0.09 separately. The most outrageous activity was found at 96 h. Some chemicals (CaCl<sub>2</sub>, ZnSO<sub>4</sub>, MnSO<sub>4</sub> and NH<sub>4</sub>NO<sub>3</sub>) were included and greatest movement was found by including ZnSO<sub>4</sub>. The exercises were 0.33, 0.44, 0.113 and 0.2 individually.

From that point forward, biochemical portrayal alongside assurance of province morphology and cell morphology was done for the provisional recognizable proof of the confine. Considering the result of spore recoloring, it could be watched that the segregate 37 is fit for creating spore. The biochemical qualities of the seclude 37 was in this way observed to be certain for glycerol, sucrose, fructose, triple sugar press agar test, VP (Voges Proskauer) test, citrate use test, nitrate diminishment test, catalase test, starch hydrolysis test, blood agar test and development in anaerobic test while oxidase test, mannitol salt agar test, gelatin hydrolysis test, methyl red test, Indole and Urease test gave negative outcomes. The strain was distinguished to be *Bacillus cereus*. there are still odds of variable outcomes to be discovered relying upon the strain write. Additionally, there are dependably conceivable outcomes of microorganisms from class, for example, *Bacillus* to raise transformative changes because of ecological pressure effectively amid ensuing sub refined, hatching and refrigeration period.

The extracellular cellulase delivered by the *Bacillus* spp. segregate was halfway decontaminated by ammonium sulfate precipitation and dialysis (Wood and Bha, 1988). The catalyst movement after fractional decontamination was discovered 0.132 U/ml which was 0.076 U/ml before halfway cleaning. Cleansed cellulase was isolated from a business unrefined cellulase readiness by gel filtration chromatography utilizing sephadex G-75. Most minimal (0.016) absorbance was seen in first 2 ml drops after gel filtration chromatography and it was step by step expanded (0.059, 0.128 and 0.230). first 2 ml filtrates indicated most elevated compound action (0.313U/ml) and it bit by bit diminished to next filtrates (0.198 U/ml, 0.140 U/ml, 0.108 U/ml). It demonstrates the most astounding cellulase focus in first drops and it step by step diminished. This diminishing in protein yield may be because of denaturation of chemical amid the sanitization steps or other reasons.(Olama et al. 1993). filtered cellulase from *Trichoderma viride*

by DEAE-Sephadex A-50 chromatography strategy took after by CM-SephadexC-50 and watched 99.8% loss of protein. Comparable chromatographic strategies were utilized for the cleansing of cellulase from *Bacillus subtilis* YJ1 (Yin et al., 2010). SDS/PAGE uncovered that the sub-atomic mass of the cleansed CMCase was 58 kDa. Comparable outcome was acquired with *Bacillus amyoliquefaciens* DL-3 (Lee et al., 2008).

Great movement of protein was seen after fractional cleaning and cleansing yet no band was watched when stacked on SDS PAGE. It could be a basic affectability issue - the protein focus may be beneath as far as possible, yet at the same time all around ok to cause the movement.

In this examination, the species had been picked upon its most elevated compound movement contrasted with others and afterward completed with the distinguishing proof of the species. The ID of the living being would control the maturation as indicated by the necessity of the particular creature. Other than cellulase creation, a superior comprehension of the physiology of the microorganisms, pre treatment of cellulosic biomass for better microbial assault, forms for practical generation of cellulases, treatment of biomass for creation of hydrolytic items which can fill in as substrates for downstream fermentative generation of different important metabolites lastly use of the learning of metabolic and protein designing standard is an absolute necessity for this reason (Sukumaran et al., 2005). The decontamination of cellulose utilizing gel filtration chromatography other than cellulose generation was the principle motivation behind the investigation.



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## **Appendices**

## Appendix – I

### **Buffers and reagents**

#### **Gram's iodine**

To 300 ml distilled water, 1 g iodine and 2 g potassium iodide was added. The solution was mixed on a magnetic stirrer overnight and transferred to a reagent bottle and stored at room temperature.

#### **3,5-Dinitrosalicylic acid**

To make 100 ml DNS, 1 g of DNS was added to 50 ml distilled water. Then, 20 ml of 2 M NaOH was added along with 28.2 g of sodium potassium tartarate. The volume was adjusted to 100 ml by adding distilled water and mixed well. To prevent exposure to light the solution was stored at room temperature in an amber bottle.

#### **Folin reagents:**

**Reagent A:** To make 100 ml of reagent A, 50 ml of 2% sodium carbonate was mixed with 50 ml of 0.1 N NaOH solution (0.4 gm in 100 ml distilled water.)

**Reagent B:** To make 20 ml of reagent B, 10 ml of 1.56% copper sulphate solution was mixed with 10 ml of 2.37% sodium potassium tartarate solution.

**Reagent C (analytical reagent):** To make 100 ml of reagent C, 2 ml of reagent B was mixed with 100 ml of reagent A. This was prepared prior use.

**Folin - Ciocalteu reagent solution (1N):** To make 4 ml of Folin - Ciocalteu reagent solution 2 ml of commercial reagent (2N) was diluted with an equal volume (2 ml) of distilled water on the day of use.

#### **Safranin (100ml)**

To 10 ml 95% ethanol, 2.5 g safranin was dissolved. Distilled water was added to the solution to make a final volume of 100 ml. The final solution was stored in a reagent bottle at room temperature.

#### **Crystal Violet**

To 29 ml 95% ethyl alcohol, 2 g crystal violet was dissolved. To 80 ml distilled water, 0.8 g ammonium oxalate was dissolved. The two solutions were mixed to make the stain and stored in a reagent bottle at room temperature.

#### **Malachite green (100 ml)**

To 20 ml distilled water, 5 g malachite green was dissolved in a beaker. The solution was transferred to a reagent bottle. The beaker was washed two times with 10 ml distilled water separately and a third time with 50 ml distilled water and the solution was transferred to the reagent bottle. The remaining malachite green in the beaker was washed a final time with 10 ml distilled water and added to the reagent bottle. The stain was stored at room temperature.

#### **Methyl Red (200 ml)**

In a reagent bottle, 1 g of methyl red powder was completely dissolved in 300 ml of ethanol (95%). 200 ml of distilled water was added to make 500 ml of a 0.05% (wt/vol) solution in 60% (vol/vol) ethanol and stored at 4°C.

#### **Barrit's Reagent A (100 ml)**

5% (wt/vol) a-naphthol was added to 100 ml absolute ethanol and stored in a reagent bottle at 4°C.

#### **Barrit's Reagent B (100 ml)**

40% (wt/vol) KOH was added to 100 ml distilled water and stored in a reagent bottle at 4°C.

#### **Oxidase Reagent (100 ml)**

To 100 ml distilled water, 1% tetra-methyl-*p*-phenylenediamine dihydrochloride was added and stored in a reagent bottle covered with aluminum foil at 4°C to prevent exposure to light.

#### **Catalase Reagent (20 ml 3% hydrogen peroxide)**

From a stock solution of 35 % hydrogen peroxide, 583  $\mu$ l solution was added to 19.417 ml distilled water and stored at 4°C in a reagent bottle.

#### **Urease Reagent (50 ml 40% urea solution)**

To 50 ml distilled water, 20 g pure urea powder was added. The solution was filtered through a HEPA filter and collected into a reagent bottle. The solution was stored at room temperature.

#### **Nitrate Reagent A (100 ml)**

5N acetic acid was prepared by adding 287 ml of glacial acetic acid (17.4N) to 713 ml of deionized water. In a reagent bottle, 0.6 g of N,N-Dimethyl- $\alpha$ -naphthylamine was added along with 100 ml of acetic acid (5N) and mixed until the colour of the solution turned light yellow. The reagent was stored at 4°C.

#### **Nitrate Reagent B (100 ml)**

In a reagent bottle, 0.8 g of sulfalinic acid was added along with 100 ml acetic acid (5N)<sup>a</sup> to form a colourless solution and stored at 4°C.

#### **Citrate Buffer (0.05 M 50 ml)**

To make 1 M 50 ml citrate buffer, 14 g citric acid was added to 50 ml distilled water. From there, 2.5 ml buffer was added to 47.5 ml distilled water to make 50 ml of 0.05 M buffer. The pH was adjusted to need (3,4,5) using NaOH and HCL. The buffer was autoclaved and stored at 4°C.

#### **Sodium acetate buffer**

Dissolve 4.4g of sodium acetate trihydrate in about 0.8 L of distilled water. Adjust the pH to 5.5. Then transfer into 1L of volumetric flask and bring to volume with distilled water.