ISOLATION AND IDENTIFICATION OF CELLULOSE DEGRADING BACTERIA FROM SOIL SAMPLE.

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIRMENTS FOR THE DEGREE OF B.Sc. IN MICROBIOLOGY

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DECLARATION

I Jerin Isnat Abedin, certify that the project report titled –Isolation and Identification of Cellulose degrading bacteria from soil sample" – submitted to the Department of Mathematics and Natural Science, BRAC University in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology is a record of work carried out by me under the supervision of my supervisors.

This thesis is my original work and the contents of this report in full or parts have not been presented for a degree in any other university and Institute.

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This dissertation is dedicated to my supportive mother who has supported me from the beginning of my life. I also dedicate this to my encouraging father and sister who have supported me all the way. Also, this dissertation is dedicated to my professor who has supported, motivated and inspired me throughout my student life. Finally, this dissertation is dedicated to all those kind hearted persons who accompanied me last four years with their kind cooperation, suggestions and encouragement

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List of ABBREVIATION

Cip A …… ….Cellulose intergrating protein A
CMC …… Carboxymethylcellulose
CbP ……Cellobiose phosphorylase
CdP ……Cellodextrin phosphorylase
CV ……Crystal Violet
CMCase ……Carboxymethylcellulase
DNS ……. Dinitrosalicylic acid
MIU…….Motility Indole Urea

XynA ……Xylanase A
XynB ……....Xylanase B
XynV ……Xylanase V
XynY ……Xylanase Y
XynZ…… Xylanase Z
% ……..Percentage
CFU …….Colony forming unit
Mg ……Milligram
µg ……. Microgram
gm ……Gram
°C …….Degree Celsius
°F …….Degree Ferhenite
Km ……. Kilometer
M ……Meter
µm ……Micrometer
mm …….Millimeter
ml ……Milliliter

Abstract

Cellulolytic microorganisms such as fungi and bacteria are responsible for much of the cellulose degradation in soils. Despite this vast number of cellulase producers, there is a deficiency of microorganisms that can produce significant amount of the three cellulase enzyme specifities i.e. endoglucanases, exoglucanases and cellobiases to efficiently degrade cellulose to fermentable products. Little emphasis has been given to cellulase production from bacteria despite their extremely high natural diversity, which endows them with the capability to produce stable enzymes. Soil samples were collected from National parliament area & BRAC nursery. The soil samples were inoculated separately and from each, only a single bacterial isolate was obtained. The three isolates were screened for cellulolytic activity using Congo red stain on Carboxymethylcellulose (CMC) agar plates inoculated with the isolates. All the isolates were found to hydrolyze Carboxymethylcellulose. A Gram stain test carried out identified the three isolates as Gram-positive rods. Morphological and biochemical analysis indicated that they all associated mainly with members of the Bacillus sp. Isolates from BRAC nursery (CDB3, CDB4 & CDB5) selected for further functional studies bore the two enzyme specificities of a cellulase enzyme system. A crude enzyme extract was found to hydrolyze Avicel and CMC with enzyme activities of 0.326ml/mg, 0.374ml/mg, 0.352mi/mg and 0.203ml/mg, 0.206ml/mg and 0.147ml/mg respectively. Optimum temperature for activity measured over 60 minutes was found to be 30°C with relatively high activity at both 37°C and 60°C. The optimum pH at the predetermined optimum temperature was found to be pH 5.5. This

CHAPTER 1: INTRODUCTION

1.1 Introduction to cellulose

Cellulose is the most abundant biological compound on terrestrial and aquatic ecosystem and is the main component of plant biomass (Shankar *et al*., 2011). It is the dominant waste material from agricultural industry in the form of stalks, stems and husk, there has been great interest in utilizing cellulose as an energy resource and feed (Balachandrababu*et al*., 2012). The cellulose is composed of D-glucose units linked together to form linear chain via ß-1, 4-glycosidic linkages (Salmon and Hudson, 1997).

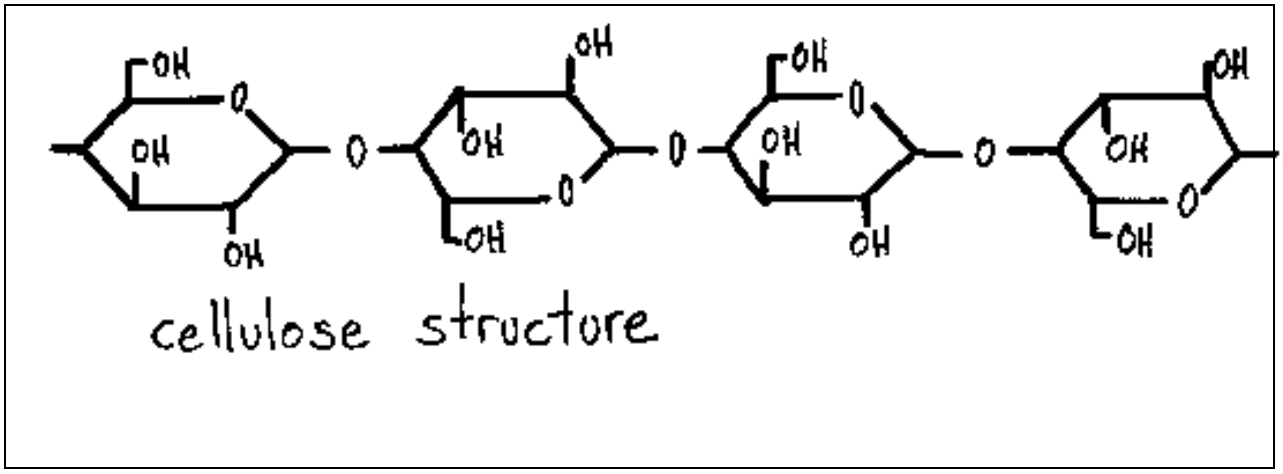


FIGURE 1.1: STRUCTURE OF CELLULOSE

Cellulose is also the most common organic compound on earth. It is well known that plants are the most common source of renewable carbon and energy on the earth. (Yakubu*et al.,* 2011). Cellulose is basically the structural component of the primary cell wall of green plants, many

forms of algae and the oomycetes. Cellulose is the major component of plant biomass. Plants produce 4×10^{9}tons of cellulose annually.

It is also considered as one of the most important sources of carbon on this planet and its annual biosynthesis by both land plants and marine occurs at a rate of 0.85×10^{11} tons per annum (Nowak *et al*., 2005).

There are two types of hydrogen bonds in cellulose molecules: those that form between the C_3OH group and the oxygen in the pyranose ring within the same molecule and those that form between the C_6 OH group of one molecule and the oxygen of the glucosidic bond of another molecule. Ordinarily, the beta-1, 4 glycosidic bonds themselves are not too difficult to break. However, because of these hydrogen bonds, cellulose can form very tightly packed crystallites. These crystals are sometimes so tight that neither water nor enzyme can penetrate them; only *exogluconase*, a subgroup of cellulase that attacks the terminal glucosidic bond, is effective in degrading it. The inability of water to penetrate cellulose also explains why crystalline cellulose is insoluble. On the other hand, amorphous cellulose allows the penetration of *endogluconase*, another subgroup of cellulase that catalyzes the hydrolysis of internal bonds. The natural consequence of this difference in the crystalline structure is that the hydrolysis rate is much faster for amorphous cellulose than crystalline cellulose. The process of breaking the glucosidic bonds that hold the glucose basic units together to form a large cellulose molecule is called *hydrolysis* because a water molecule must be supplied to render each broken bond inactive. In addition to crystallinity, the chemical compounds surrounding the cellulose in plants, e.g. lignin, also limit the diffusion of the enzyme into the reaction sites and play an important role in determining the rate of hydrolysis.

Cellulose degradation and its subsequent utilizations are important for global carbon sources. The value of cellulose as a renewable source of energy has made cellulose hydrolysis the subject of intense research and industrial interest (Bhat*et al.,* 2000). There has been much research aimed at obtaining new microorganisms producing cellulase enzymes with higher specific activities and greater efficiency (Subramaniyan*et al*., 2000).

1.2 Cellulose degrading enzyme

Cellulolytic enzymes play an important role in natural biodegradation processes in which plant lignocellulosic materials are efficiently degraded by cellulolytic fungi, bacteria, actinomycetes and protozoa. In industry, these enzymes have found novel applications in the production of fermentable sugars and ethanol, organic acids, detergents and other chemicals. Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen*et al.,* 2005).

The conversion of cellulose into glucose is now known to consist of two steps in the enzyme system of *Trichodermaviride*. In the first step, beta-1,4glucanase breaks the glucosidic linkage to *cellobiose*, which is a glucose dimer with a beta-1, 4 bond as opposed to maltose, a counterpart with an alpha-1, 4 bond. Subsequently, this beta-1,4glucosidic linkage is broken by beta-glycosidase:

b-1,4 glucanase b-glucosidase

Cellulose ---------->Cellobiose ----------> Glucose

The kinetics of cellulose hydrolysis has been widely studied, and Michaelis-Menten types of rate expressions with substrate or product inhibition terms have been proposed to describe the observed reaction kinetics.

1.3 Cellulose degrading microorganisms

Cellulolytic enzymes are synthesized by a number of microorganisms. Fungi and bacteria are the main natural agents of cellulose degradation (Lederberg, 1992). The cellulose utilizing population includes aerobic and anaerobic mesophilic bacteria, filamentous fungi, thermophilic and alkaliphilic bacteria, actinomycetes and certain protozoa (Alexander, 1961). However, fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd *et al*., 2002).

Microorganisms bring about most of the cellulose degradation occurring in nature. They meet this challenge with the aid of a multi-enzyme system (Aubert*et al*., 1987). Aerobic bacteria produced numerous individuals and extra-cellular enzymes with binding modules for different cellulose conformations, while anaerobic bacteria possess a unique extracellular multi enzyme complex, called cellulase. However, the main cellulose utilizing species are the aerobic and anaerobic hemophilic bacteria, filamentous fungi, basidiomycetes, thermophilic bacteria and actinomycetes (Wright, 2003). At the first step, the microorganisms responsible for cellulose decomposition bring about an enzymatic hydrolysis of the complex polymer, that is, the enzymes system which involves a group of different enzymes, is collectively known as cellulase.

Mainly efficient cellulase activities are observed in fungi but 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. The search for a novel and improved bacterial strain, having hyper cellulase productivity with more activity and high stability against temperature, pH and under non-aseptic conditions might make the process more economical.

The cellulase was first discovered in 1983 from the anaerobic, thermophilic spore-forming *Clostridium thermocellum* (Maki *et al*., 2011). The production of cellulase generally depends on variety of growth parameters which includes inoculums size, pH value, temperature, presence of inducers, medium additives, aeration, growth and time (Immanuel *et al*., 2006) and also the cellulase activity is appear to be depend on the presence of various metal ions as activators and inhibitors (Muhammad *et al.,* 2012).

Cellulose is commonly degraded by cellulase. Cellulolytic enzyme system consists of three major components such as endoglucanases, exoglucanases and ß-glycosidase. Cellulases have a potentiality to use in biotechnology and in industry such as, starch processing, alcoholic beverage, malting and brewing, clarify of juice, pulp bleaching, textile industry and animal feed (Sreeja*et al*., 2013).

Certain cellulase producing bacteria also inhabit the other factors which are responsible for decomposition of organic matter and composting (Shankar *et al.,* 2011*)*. Beyond free bacterial cellulases, is the opportunity for whole cells in bacterial co-culture and strains with multiple exploitable characteristics to reduce the time and cost of current bio-conversion processes. It is also noticeable - as the final product of cellulose degradation by cellulase enzyme is glucose which is soluble sugar. So, isolation and characterization of cellulase producing bacteria will continue to be an important aspect of biofuel research, biodegradation and bioremediation.

The present study was attempted with the following objectives:

- To isolate and screen cellulolytic bacteria from different environmental source.
- Production of cellulase from potential isolates by submerges fermentation process.
- Partial purification of cellulase and determination of its enzyme activity and specific activity.
- Optimization of different parameters for better cultivation and production process.
- Application of potential isolate in biodegradation of cellulosic material

CHAPTER 2: LITERATURE REVIEW

2.1 Cellulose

AnselmePayen (1795-1871) coined the term cellulose and introduced it to scientific literature in 1839 after isolating a fibrous substance mostly found in wood, cotton and other plants (Payen, 1838). Higher plant tissues such as trees, cotton, flax, cereal straw represent the main sources of cellulose i.e. it makes up 35-50% of dry plant weight (Lynd *et al*., 1999). Algae such as *Valoniaventricosa* and *Microdicyan* are representatives of lower plants that synthesize cellulose (Boisset, *et al*., 1999; Fierobe, *et al*., 2002). In addition to plants, non-photosynthetic organisms such as bacteria i.e. aerobic *Acetobacterxylinum*, marine invertebrates from the ascite family i.e. tunicates, fungi, slime moulds and amoebae also produce cellulose (Tomme*et al*., 1995; Lynd *et al*, 2002)

Khianngam*et al*,(2014) studied oil palm (*Elaeisguineensis*) meal, a by-product of palm oil, is rich in fiber and contains lignocelluloses, which inhibits the absorption of the nutrients has been widely used for animal feed. The improvement of the nutrient absorption is required treating with cellulase enzyme. This study was aimed to isolate, screen and characterize the cellulase producing bacteria. Ten strains of cellulolytic bacteria were isolated from 7 oil palm meal samples collected in Phetchaburi, PrachuapKhiri Khan and Pattani provinces, Thailand. They exhibited the ability to degrade carboxymethyl-cellulose (CMC) based on the decolorization of CMC-basal agar medium using Congo red as a color indicator. They showed the cellulase hydrolysis capacity ranged from 1.56 to 4.14. All isolates were Gram positive rod-shaped

bacteria and belonged to *Bacillus* (8 isolates), *Paenibacillus*(1 isolate) and *Lysinibacillus*(1 isolate) based on the phenotypic characteristics and 16S rRNA gene sequence analysis. Their cellulase activity ranged from 0.039±0.002 to 0.233±0.005 IU/ml when they were cultivated in broth.

Kushwaha*et al* (2012), conducted another study where soil samples were obtained (10gm) from Hardoi district, Uttar Pradesh, India. Bacterial colonies were grown over CMC-Agar medium. Maximal cellulase production was obtained after 48 h of incubation at 45 °C in medium containing 1.5% carboxymethyl cellulose (CMC) as substrate. The optimum pH for the enzyme was found to be ranging between 6.5 and 7.5 at which it was found to be most stable. Bacteriological studies indicated *Bacillus*subtilis to be the most frequent cellulolytic bacteria to be found in the agricultural fields. The purpose of the current investigation was to screen *Bacillus* species isolated from soil in order to study its suitability with regard to waste treatment in agricultural fields (bioremediation).

Balamuruga*et al* (2011) performed experiment where cellulose degrading bacteria of tea garden soil were isolated, screened *in vitro* and its characterization, in relation to cellulase activity, was studied. Among the 25 isolates, the five strains showed higher enzyme activity when compared to other strains. Cellulase activity was expressed at a higher level by strain CDB12 when blotting paper was used as a cellulose source in comparison with the other two substrate sources incorporated with minimal salt medium and followed by CDB13 and CDB21 in blotting paper. Maximum growth of cellulose degradation bacteria (CDB) was recorded at 30°C and pH 7.0. Among the carbon sources tested, maximum growth was observed in glucose

amended mineral salts medium followed by fructose and maltose. Ammonium sulphate, ammonium nitrate and potassium nitrate were good nitrogen sources for better survival of CDB isolates. The biomass were continuously removed and placed as such into the tea field, then native and proven CDB strains were applied and they played an important role on the degradation of harvested biomass, which required replenishment to maintain the sustainable productivity of tea.

Barman *et al*, (2011) placed an investigation which was conducted to find out the effective cellulytic bacteria for biodegradation of solid kitchen and agricultural wastes as organic manure or compost. Bacterial strains of *Moraxella* sp., *Cellulomonas* sp. and *Planococcus* sp.were isolated from soil and cultured on nutrient agar media. Changes of temperature and pH, CO_2 release, crude fiber loss, protein, sugar and fat content as well as the activity of endoglucanase and cellobioase of waste were noted for selecting the most effective strain. In comparison to *Cellulomonas* sp. and *Planococcus* sp., inoculation of *Moraxella* sp. enhanced the degradation of kitchen and agricultural waste, shown by the increased CO_2 release (54.3 and 37.62 mg), crude fiber loss (46.86% and 45.11%), total sugar reduction (72.52% and 74.27%), fat reduction (65.20% and 61.22%), endoglucanase (0.097 mg.hr-1.ml-1) and cellobiase (0.82 mg.hr-1.ml-1) activities. Inoculation of *Cellulomonas* sp. strain (53.89% and 77.96%) showed high protein reduction in comparison with inoculation of *Moraxella*sp. strain (20.04% and 63.42%) for kitchen and agricultural wastes. The overall findings of this investigation demonstrate the effectiveness of *Moraxella* sp. as a useful strain for bioconversion of solid organic waste.

In experiment of Shaikh*et al,*(2013) the cellulase producing bacteria were isolated from various region including paper industry waste, municipal waste, sugarcane farm, garden, and wood furnishing region. Total 34 isolates were obtained by the primary screening technique from which 11 isolates were showing maximum cellulase activity. Potential isolates were obtained from wood furnishing region and paper industry waste. These 11 isolates were then evaluated by secondary screening for enzyme production. Among these 11 isolates CDB27 and CDB30 were selected as most efficient enzyme producers and their specific enzyme activity in the crude sample was found to be 6.0U/mg and 8.4 U/mg and of partially purified sample was found to be 6.97 U/mg and 9.3 U/mg respectively. Isolates were tentatively characterized on the basis of their cultural and morphological and biochemical characteristics, CDB27 and CDB30 were identified to be *Pseudomonas sp* and *Bacillus sp* respectively. Further partial purification of the cellulase enzyme was carried out by ammonium sulfate precipitation followed by dialysis. Optimization of different parameters was carried out for the production of cellulase by both efficient isolates. The maximum enzyme producing isolate CDB30 was used to check biodegradation properties at laboratory scale.

2.3. Mechanism of cellulose hydrolysis

A cellulase enzyme system comprises of three classes of enzymes; endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.91) and β glucosidase (EC 3.2.1.21). Exoglucanases are further grouped into glucanohydrolases (cellodextrinases) and cellobiohydrolases. These categories are based on their structural properties and mode of action (Henrissat*et al*., 1998; Henrissat & Davies, 2000). Endoglucanases randomly cleave at the internal sites of cellulose to yield oligosaccharides of various lengths. Exoglucanases on the other hand act on the reducing or non-reducing end of

cellulose to liberate glucose, cellobiose or cellooligosaccharides, which are finally hydrolyzed to glucose by β glucosidases (Sukumaran*et al*., 2005).

This enzyme system exhibits synergy, a phenomenon in which the collective enzyme activity is higher than the sum of activities of individual enzymes. Four forms of synergy have been reported. Exo-exo synergy between exoglucanase attacking the reducing and the non-reducing ends of cellulose; endo-exo synergy between endoglucanases and exoglucanases; Exo-β glucosidase synergy and intramolecular synergy between the catalytic domain and the CBMs (Din *et al*., 1994; Driskill*et al*.,1999).

Cellulolytic anaerobes have an extra cytoplasmiccellodextrinase for hydrolyzing cellodextrins and intracellular cellodextrin and cellobiosephosphorylases (CdP and CbP). These phosphorylasescatalyse Pi mediated phosphorylation of cellodextrins and cellobiose respectively to yield glucose 1 monophosphate (G-1-P) which is converted to Glucose 6 Phosphate (G-6-P), the entry point to Embden-Meyerhoffpathway (Lynd *et al*., 2002). Other bacteria produce intracellular β glucosidases which cleave cellobiose and cellodextrins to produce glucose which is assimilated by the microbes (Karmakar& Ray, 2011). Simultaneous presence of extracellular cellodextrinases, intracellular CbP and CdP activities, and intracellular β glucosidases in cellulolytic microorganisms suggest that metabolism of cellobiose and cellodextrins probably occurs through several pathways. (i) Extracellular hydrolysis of the substrates i.e. cellobiose and cellodextrins and subsequent uptake and metabolism. (ii) Direct uptake followed by intracellular phosphorolytic cleavage and subsequent catabolism. (iii) Direct uptake by the organism followed by hydrolytic cleavage and metabolism (Lynd *et al*., 2002). Cellulosic substrates occurring in nature contain hemicellulose and lignin which impedes the access of cellulase components to β

(1-4) glucosidic linkages thus other hydrolytic enzymatic activities distinct to those of cellulases are required. Enzymatic cleavage of the β 1 - 4-glucosidic linkages in cellulose proceeds through an acid hydrolysis mechanism, using a proton donor and nucleophile or base (Lynd *et al*., 2002).

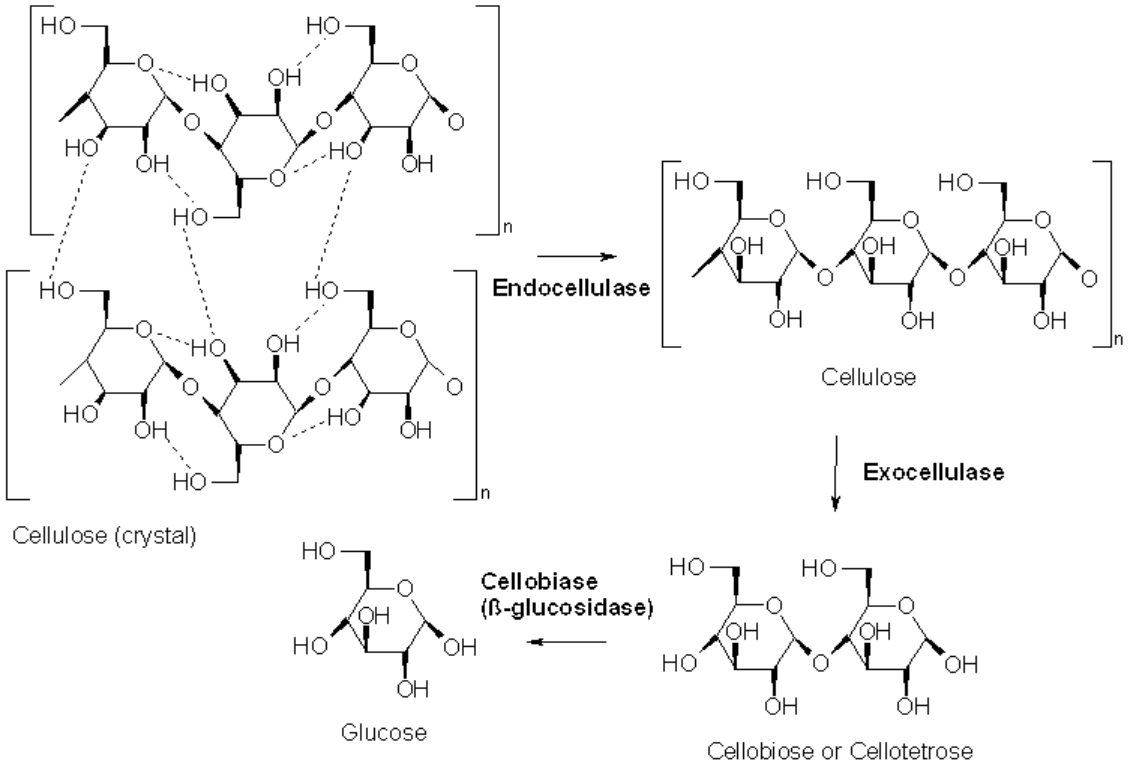


Figure 2.1: A schematic representation of cellulolysis

The three types of reactions catalyzed by cellulases: (1) Breakage of the non covalent interactions present in the amorphous structure of cellulose by endoglucanase (2) Hydrolysis of chain ends to break the polymer into smaller sugars by exoglucanase (3) Hydrolysis of disaccharides and tetrasaccharides into glucose by β-glucosidase (Adapted from Karmakar& Ray, 2011).

2.4 Cellulase enzyme systems

Cellulose utilization takes place in both aerobic and anaerobic microorganisms. Members of the genus *Cellulomonas*are the sole facultatively anaerobic degraders reported so far (Bagnara, *et al* 1985; Bagnara*et al*., 1987; Clemmer& Tseng, 1986; Dermoun&Belaich, 1988). Cellulase enzyme systems are generally classified into two; complexed (Shoham*et al*.,1999; Schwarz, 2001) and non complexed (Stutzenberger, 1990; Teeri, 1997). This classification is dependent on whether the microorganism is aerobic or anaerobic (Lynd *et al*., 2002).

2.4.1 Non complexed systems

In non complexed cellulase systems components are free and mostly secreted thus can be recovered from the culture supernatant. These are normally found in aerobic cellulose degraders i.e. both fungi and bacteria (Rapp & Beerman, 1991). Cellulases from aerobic fungi are by far the most studied group (Lynd *et al*., 2002). Representatives in this category include *Trichoderma reesei* previously *Trichoderma viride. T. reesei* produces cellobiohydrolases CBHI and CBHII, eight endoglucanases EGI-VIII and seven β- glucosidases BGI-VII (Pakula&Penttila, 2005). The cellulase enzyme system from *Humicolainsolens* is homologous to that of *T. reesei* with at least seven cellulases i.e. Two cellobiohydrolases CBHI and CBHII and five endoglucanases EGI, EGII, EGIII, EGV and EGVI (Schülein, 1997).

Most aerobic bacteria species are found in soil. They fall in genera that are known for non growth associated metabolism (secondary metabolism) that include formation of dormant states (*Bacillus, Miromonospora* and *Thermobifida* and production of secondary metabolites such as antibiotics *(Bacillus* and *Micromonospora*(Lynd *et al.,* 2002). Most aerobic bacteria adhere to

cellulose but the physical contact is not necessary for cellulose hydrolysis (Kauri & Kushner, 1985).

2.4.2 Complexed system

Anaerobic cellulose degraders degrade cellulose via a complexed system; a cellulosome (Schwarz, 2001). Cellulosomes are protuberances on bacterial cell wall that harbor enzyme complexes. These enzyme complexes are firmly bound on to the cell wall but flexible enough to bind cellulose. Cellulosomes from different *Clostridia* (*Clostridium thermocellum, Clostridium cellulolyticum*, *Clostridium cellulovorans*, and *Clostridium josui*) and *Ruminococcus* species in the rumen have been studied. Cellulosome enzyme sub units are not any different from free cellulases. Both have catalytic domains from the same glycosylhydrolase families. The major difference between these two enzyme types is that all cellulosomal enzymes have a dockerin domain which mediates the integration of the enzyme into the cellulosome complex. Free cellulases however lack a dockerin domain but have a catalytic binding module that helps binding of a given catalytic domain to the substrate (Bayer *et al*.,1994; Tomme*et al*., 1995b; Be´guin&Lemaire, 1996).

The cellulosome structure from *C. thermocellum* was resolved through a combination of biochemical, immunochemical, ultra structural and genetic techniques. It consists of a large non catalytic and multi modularscaffoldin protein (CipA) of 197kDa which is anchored to the cell wall via type II cohesin domains. A total of 22 catalytic modules have dockerin moieties that can associate with the cohesins of the CipA protein to form the cellulosome. Nine of these catalytic modules exhibit endoglucanase activity (CelA, CelB, CelD, CelE, CelF, CelG, CelH, CelN, and CelP), four exhibit exoglucanase activity (CbhA, CelK, CelO, and CelS), five exhibit

hemicellulase activity (XynA, XynB, XynV, XynY and XynZ), chitinase activity is exhibited by ManA and lichenase activity is exhibited by LicB (Bayer *et al*., 1994).

The cellulosome is thought to bring enzyme activity in close proximity to the substrate thus facilitating optimum synergy by the cellulases present in the cellulosome and also to minimize the distance over which hydrolysis products diffuse thus allowing for efficient uptake of oligosaccharides by the cell (Bayer *et al*., 1994; Schwarz, 2001).

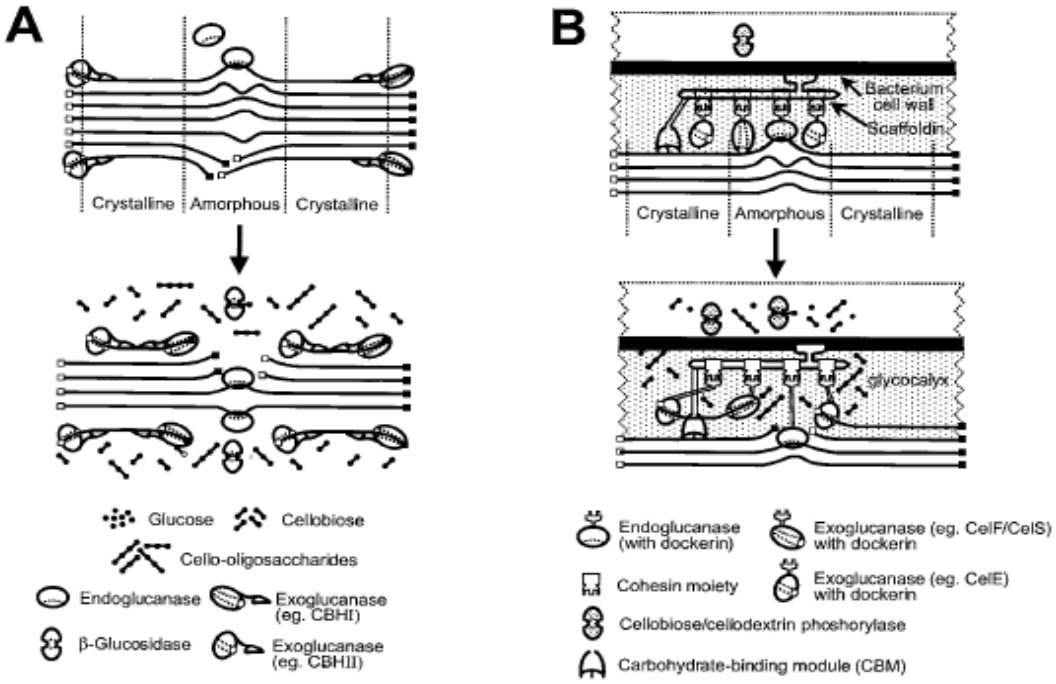


Figure 2.2: A schematic representation of the hydrolysis of amorphous and crystalline cellulose by (A) non complexed (B) Complexed cellulase systems (Adapted from Lynd *et al*., 2002).

2.5 Taxonomic diversity of cellulolytic microorganisms

Ability to degrade cellulose is widely distributed in several fungal and bacterial genera. In addition to these two, the domain eubacteria has a considerable distribution of cellulytic capability. Members in the aerobic order *Actinomyctes*and anaerobic order *Clostridiales*. Fungi are the main agents of decomposition of organic matter in general and especially cellulosic substrates (Lynd *et al*., 2002; Montegut *et al*., 1991) and it‘s no surprise that cellulolytic capability is distributed across the entire kingdom from the advanced *Basidiomycetes*to the primitive *Chytridomycetes* (Lynd *et al*., 2002). Chytridomycetes are known to degrade cellulose in gastrointestinal tracts of ruminant animals (Orpin, 1977).

Cellulolytic capability is however not exclusive to microorganisms. Species such as termites and cray fish produce their own cellulases that are different from those produced by their indigenous micro flora (Orpin, 1977). There is a broad distribution of cellulolytic capability and it‘s possible that a primordial ancestor acquired it early in the evolutionary development. This however may not be the case because cellulose biosynthesis capability evolved much later with the development of land plants, algae amongst others (Lynd *et al*., 2002).

Table 2.1: Fungi and bacteria with cellulolytic capability (Adapted from Kuhad *et al.*, 2011).

Fungi	**Soft rot fungi** *Aspergillus niger, A. nidulans, A. oryzae, A. terreus; Fusarium solani, F.oxyspourm; Humicola insolens, H.grisea; Melanocarpus albomyces; Penicillium brasilianum, P.occitanis, P.decumbans, P. janthinellum ; Trichoderma reesei, T. harzianum, T. longibrachiatum, T.atroviride; Chaetomium cellulyticum, C. thermophilum; Thermoascus aurantiacus; Mucorcircinelloides; Paelomyces inflatus, P. echinolatum.*
	White rot fungi *Phanerochaete chrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantea.* *Brown rot fungi* *Coniophora puteana; Lanzites trabeum; Poria placenta, Tyromyces palustris; Fomitopsis sp*
Bacteria	**Aerobic bacteria** *Acinetobacter junii, A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp; Bacillus subtilis, B.pumilus, B. licheniformis, B. amyloliquefaciens, B. circulans, B. flexus; Bacteroides sp; Cellulomonas biazotea; cellvibrio gilvus; Eubacterium cellulosolvens, Geobacillus sp; Microbispora bispora; Paenibacillus curdlanolyticus; Pseudomonas cellulose; Salinivibrio sp; Rhodothermus marinus.* **Anaerobic bacteria** *Acetovibrio cellulolyticus; Butyrivibrio fibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogenes; Ruminoccus albus*

2.6 Regulation of cellulase production

Cellulase is an inducible enzyme system where enzyme production is regulated by activation and repression mechanisms (Sukumaran *et al*., 2005). In *T. reesei*, production of cellulase genes is

regulated at the transcriptional level. The expression of cellulase genes (cbh1, cbh2, egl1, egl2 and egl5 in *T. reesei* strain QM9414 is coordinated by transcriptional factors (Ilme´n, *et al*., 1997). Cellulase genes have binding sites for both transcriptional activators i.e. Activator of cellulase expression proteins I & II (ACEI & ACEII) and catabolite repressor protein I (CRE I) in addition to the CCAAT sequence which binds general transcription activator complexes designated ‗HAP' proteins (Narendja*et al*., 1999). Genes encoding the transcriptional factors ACEI (Saloheimo*et al*, 2000) and ACEII (Aro*et al*., 2001) were identified due to their ability to bind to the *T. reesei*cbh1 promoter region.

Several carbon sources have been tested to find the best inducer (Mandels& Reese, 1960; Mandels, 1975). Substrates are known to induce the synthesis of the enzymes that catalyze their hydrolysis (Reese *et al*., 1969). Products of hydrolysis can often induce their respective polysaccharasses; galacturonic acid for polygalacturonases in *Penicillium chrysogenum(*Phaff, 1947), xylose for pentonase in several molds (Simpson, 1954), maltose for amylase in *Aspergillusniger*(Mandels& Reese, 1957), N-acetylglucosamine for chitinase in *Aspergillusfumigatus* and *Myrotheciumverrucaria*(Mandels& Reese, 1957). The use of a product as an inducer often leads to lower enzyme yields than what is obtained with the substrate (Mandels*et al*, 1957).

Production of cellulolytic enzymes in *T. reesei*, production is induced in the presence of cellulose and repressed by the availability of easily utilizable sugars (Sukumaran*et al*., 2005). In order to serve as an inducer, a substance must access the site of enzyme production (Mandels*et al*, 1960). The insolubility and size of cellulose makes it hard to enter the cell. This probably implies that

the hypothesis that an inducer has to access the enzyme production site is not sufficient toexplain why cellulose is the best inducer (Mandels*et al*, 1960). Other inducers are sophorose, cellobiose, δ-cellobiose-1-5- lactone and lactose (Mandels*et al*, 1957; Vaheri*et al*., 1979; Nogawa*et al*., 1979). When organisms are grown on dimmers like cellobiose, enzyme yields are much lower than those obtained from growth on the polymeric cellulose because the soluble sugars are rapidly metabolized and therefore repress enzyme formation. Similar results can be obtained by using metabolites that are not inducers (Mandels*et al*, 1960). Repression can be avoided when using soluble induces to obtain high enzyme yields. This is achieved by supplying the inducer continuously in low quantities, retarding metabolism by unfavorable growth conditions such that the inducer is slowly consumed, supplying a modified soluble substrate which is slowly broken down by the organism to release the inducer (Mandels*et al*, 1960). Production of cellulases was found to be increased by use of non ionic surfactants (Reese *et al*, 1969).

2.7. Applications of cellulases in the industries

For years, cellulases have been a target for academic and industrial research and are currently being applied in many industries (Singh *et al.,* 2007).Many researches has been conducted to find out industry beneficiary strain to increase the yield of cellulase production.

2.7.1. Cellulases in the textile industry

The textile industry has been revolutionized by introduction of enzymes that are slowly replacing the conventional chemical processes, which are generally severe and lead to fiber damage (Bhat, 2000; Kuhad *et al*., 2011). Cellulases have the ability to modify cellulosic fiber in a controlled

and desired manner thus improving the fabric quality (Mojsov, 2012). They are mostly used during wet processing to improve fabric properties. Processes that involve cellulase activity include biostoning of jeans and biopolishing cellulosic fibers**.** Denim is heavy grade cotton and when dyed, the dye is mainly adsorbed on the surface of the fiber. When cellulases are used during the biostoning process, they break off small fiber ends on the yarn surface to loosen the dye, which is consequently easily removed during the wash cycle by mechanical abrasion. This enzyme based treatment replaced pumice stone biostoning hence less damage to the fiber, increased productivity and a safe working environment (Christian *et al*., 2006; Karmakar*et al*, 2011).

Fading can be achieved without loss of fabric strength. Fabrics made from cellulosic fibers such as cotton, linen, ramie, viscose and lyocell are normally characterized by short fibers protruding from the surface (fuzz formation) and _pilling' i.e. loosened fuzz attached to the surface. This often decreases their market value and in order to prevent this, a process called biopolishing is done. Biopolishing is usually done during the wet processing stage and includes scouring, bleaching, dyeing and finishing. Cellulase mixtures usually rich in endoglucanases are used in this process to remove the small protruding fibers from the fabric surface without using chemicals. The fabric attains a smooth and a glossy appearance, improved brightness and uniformity. Biopolishing is a key procedure in the production of high quality garments (Bhat, 2000; Kuhad*et al*., 2011).

2.7.2. Cellulases application in the wine and brewing industry

Enzyme technology plays a crucial role in the beer and wine industries. Wine making requires the extraction of juice from grapes and subsequent fermentation by yeast while beer brewing involves malting of barley and fermentation of the resulting wort (Bhat, 2000). Brewing of beer

is based on activity of the enzymes activated during malting and fermentation stages. Malting is dependent on seed germination, which initiates biosynthesis and activation of α and β amylases, carboxy peptidases and β glucanase that hydrolyses the seed reserves (Bamforth, 2009). Under optimal conditions all the three enzymes act in synergy to produce high quality malt. However due to seasonal variations and or poor harvest, brewers end up using poor quality barley which contains low levels of endogenous β glucanase activity. This results in the presence of a 6 -10 % of non starch polysaccharide mainly soluble β glucan which forms a gel during the brewing process leading to poor filtration of the wort, slow run off time, low extract yield and development of a haze in the final product (Galante*et al*., 1998b). The viscosity of the wortis usually reduced by addition of microbial β glucanases, which hydrolyse β glucan. Commonly used microbial β glucanases are obtained from *Penicilliumemersonii, Aspergillusniger*, *Bacillus subtilis* and *Trichoderma reesei*(Galante*et al*., 1998b).

A study carried out by Oksanen*et al*., (1985) observed that endoglucanase II and cellobiohydrolase II of the *Trichorderma* cellulase system were responsible for most activity in reduction of the degree of polymerization and wort viscosity thus they are best suited for the production of high quality beer from low quality barley. In wine making, pectinases, β glucanases and hemicellulases comprise the main exogenous enzymes added. These enzymes give a better skin maceration, improved colour extraction, easy must clarification and filtration and improved wine quality and stability (Galante*et al*., 1998b). In order to improve the wine‘s aroma, β glucosidase is added to modify glycosylated precursors that are naturally present (Caldini*et al*., 1994; Gunata, *et al*., 1990).

2.7.3. Cellulases in the detergent industry

Recent innovations in the detergent industry have seen the incorporation of enzymes such as cellulases, proteases and lipases in detergents (Singh *et al*., 2007). Due to repeated washing, cotton and cotton blend fabrics become dull and fluffy due to the presence of detached micro fibrils. Cellulase containing detergents are capable of degrading the cellulose micro fibrils to restore a smooth surface and original color to the garment. In addition, the degradation softens the fabric and removes dirt particles trapped in the micro fibril network (Sukumaran*et al*., 2005; Singh *et al*., 2007). Cellulase preparations from *H. insolens* that are active under mild alkaline conditions (pH 8.5 - 9) and temperatures over 50oC are added to detergents. Such cellulases active under alkaline conditions increase the cleaning capacity of detergents by selective contraction fibers hence facilitating the removal of oil from inter fiber space (Karmakar& Ray, 2011).

2.7.4. Cellulases in pulp and paper industry

Application of enzyme preparations comprising cellulases, xylanases and lignases in the pulp and paper industry has increased in the last decade (Mai *et al*., 2004; Karmakar& Ray, 2011). Pulping starts with the conversion of woody raw material into a flexible fiber that can be made into paper. Depending on the application of the paper, various methods of pulping can be used (Bajpai, 2012). Mechanical pulping usually involves mechanical grinding of the woody material to give fibers that can be used in the production of different grades of paper. This method is usually characterized by high energy consumption and gives paper with incompletely ground fiber bundles, low strength and tends to yellow with time due to little removal of lignin a weakness associated with the process (Bhat, 2000).

Bio pulping using cellulases and allied enzymes reduces the energy required to achieve the desired strength and freeness of the pulp hence it's a better alternative to mechanical pulping (Karmakar& Ray, 2011). Cellulases containing enzyme mixtures are also useful in the hydrolysis of _fines' small particles produced during refining of primary or secondary fibers. These particles usually reduce the drainage rate of pulp during the paper making process. Hydrolysis of these particles improves the pulps drainage property which in turn determines the paper mill's speed. Addition of these preparations before refining is either done to improve the beatability response or modify the fiber properties (Noe*et al*., 1986; Pommier*et al*., 1989; Pommier*et al*., 1990).

Deinking process is crucial during paper recycling. Enzymatic deinking using cellulases reduces the need for deinking chemicals and also results to little or no loss in paper strength. Enzymatic deinking is usually combined with mechanical agitation in order to improve the efficacy of the process (Karmakar& Ray, 2011).

2.7.5. Cellulases in Agriculture

Cellulolytic fungi including, *Trichoderma sp*, *Geocladium sp*, *Chaetomium sp* and *Penicillium sp* are known to play an important role in agriculture by facilitating enhanced seed germination, rapid plant growth and flowering, improved root system and increased crop yields (Bailey &Lumsden, 1998; Harman &Kubicek, 1998).

Cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens hence controlling plant diseases. The β-1, 3- glucanase and N-acetyl- glucosaminidase

From *Trichoderma harzianum* were reported to synergistically inhibit the spore germination and germ tube elongation of *B. cinerea*(Loritoet *al*.,1994; Bhat, 2000). The β- 1, 3- glucanase from *Trichoderma harzianum* CECT 2413 induced morphological changes such as hyphal tip swelling, cytoplasm leakage and the formation of numerous septae and also inhibited the growth of *R. solani*and *Fusarium sp* (Benitez *et al.,*1998).

Cellulases are also important in soil quality improvement. In order to reduce overreliance on mineral fertilizers, farmers incorporate straw in soil. Microbial routes to hasten straw decomposition were using organisms such as *Aspergillus*, *Chaetomium*and *Trichoderma* and *actinomycetes* have shown promising results (Ortiz Escobar & Hue, 2008; Tejada, *et al*., 2008).

2.7.6. Cellulases in the food industry

Industries producing fruit juices in the 1930s encountered challenges such as low yield and a poor clarity of the product (Uhlig, 1998). Research on industrially suitable enzymes such as cellulases, hemicellulases and pectinases from food grade microorganisms such as *Aspergillusniger, Trichoderma sp* and increased knowledge of fruit components led to the overcoming of these challenges and led to improved methods of extraction, clarification and stabilization (Singh *et al.,* 2007). Cellulases along with xylanases and pectinases are the macerating enzymes that serve to increase the yield and process performance without any additional cost. Macerating enzymes are usually used in two steps; after crushing, the fruit pulp is macerated to either partial or complete liquefaction. After the extraction, pectinases are then used for its clarification and this lowers viscosity of fruit juice prior to its concentration and further increases the filtration rate and the stability of the juice. Macerating enzymes also

improve the cloud stability, texture, decrease viscosity and facilitate easy concentration of nectars and purees (Grassin &Fauquembergue, 1996a).

There is a growing demand for natural pigments for food colorants such as carotenoids. In their natural state, carotenoids remain bound to proteins thus preventing pigment oxidation. When solvents are used to extract carotenoids, they disrupt that association thus making the pigments insoluble in water and oxidation. This can be prevented by use of enzymatic methods. Cellulases hydrolyze cellulose in the cell walls hence the structural rigidity is interfered with exposing intracellular materials for extraction. These pigments remain bound to proteins and are more stable than those obtained through traditional methods that involve use of solvents (Bassi*et al*., 1993).

2.7.7. Cellulases in the Bio-refinery

Bioconversion of lignocellulosic biomass to produce biofuel is the most popular area of cellulase application being investigated recently (Sukumaran *et al*., 2005). Potential lignocellulosic feedstocks sources include agricultural crop residues such as straw, the perennial prairie grass, municipal waste, packaging and construction debris, agricultural or forest processing by products e.g. food processing residues, pulping liquor from paper mills and forest woody biomass either logging residues from conventional harvest operations or removal of excess biomass from timberlands (NREL, 2006). Lignocellulosic biomass consists of cellulose tightly linked to lignin and hemicellulose (Kuila *et al*., 2011).

Cellulose must be separated from lignin and hemicelluloses in order to make it more accessible to the hydrolytic enzymes through pretreatment. Pretreatment methods used can be physical, chemical or biological but the latter are not yet intensely developed as the physical and chemical methods (Kuila *et al*., 2011). Conversion takes place in two phases; hydrolysis of cellulose into fermentable reducing sugars by cellulases and fermentation of the sugars to ethanol, a process carried out by yeast or bacteria (Sun & Cheng, 2002). The cost of ethanol production from lignocellulosic materials is relatively high with the main challenges being low yield and a high cost of hydrolysis. Studies on optimization of the cellulase enzymes and enzyme loading can be done in order to improve the hydrolytic process (Sun & Cheng, 2002). Screening for cellulolytic microorganisms from extreme environments will also enrich the current databanks.

CHAPTER 3: MATERIALS AND METHODS

The soil samples were collected from different areas such as garden soil, soil from different nurseries around Dhaka. Tenfold serial dilutions of each soil sample were prepared in sterilized distilled water and 0.1 ml of that diluted sample was spread on Carboxymethylcellulose medium.

3.1 Isolation of cellulytic bacteria

Cellulolytic bacterial isolates were isolated from soil by using serial dilutions and pour and spread plate technique. The medium used for isolation of cellulolytic bacteria contains 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25%$(NH_4)_2SO_4$ and 0.2 % gelatin at pH 7 for 48 hours of incubation at 30˚C. Bacterial colonies were purified by repeated streaking. The purified colonies were preserved at 4°C for further identification and screening for cellulase production.

3.2 Screening of cellulolytic bacteria

Pure cultures of bacterial isolates were individually transferred in CMC agar plates. After incubation for 48 hours, CMC agar plates were flooded with 1 % Congo red and allowed to stand for 15 min at room temperature. One molar NaCl was thoroughly used for counterstaining the plates. Clear zones were appeared around growing bacterial colonies indicating cellulose hydrolysis. The bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system.

3.3 Identification of cellulolytic bacteria

Identification of cellulolytic bacteria was carried out, which was based on morphological and biochemical tests.

3.3.1 Morphological characterization

Gram stain test was carried out on the bacterial isolates as described in the manual of veterinary laboratory techniques in Kenya (1981). A bacterial smear from a pure culture was prepared and fixed on a clean glass slide. The slide was flooded with crystal violet for 1 minute, and rinsed with running tap water. The slide was then flooded with Gram‘s iodine for 1 minute, and again rinsed with running tap water. This was followed by decolorization with 95% ethanol and rinsing with tap water. The slide was counter stained with Safranin for 1 minute, rinsed with running tap water and allowed to air dry. The dry slide was covered with immersion oil and viewed under a microscope.

3.3.2 Biochemical characterization

1) Oxidase test

Soak a small piece of filter paper in 1% Kovac‘s oxidase reagent and let dry.

Use a loop and pick a well-isolated colony from a fresh (18- to 24-hour culture) bacterial plate and rub onto treated filter paper and observed for color changes.

Microorganisms are oxidase positive when the color changes to dark purple within 5 to 10 seconds. Microorganisms are delayed oxidase positive when the color changes to purple within 60 to 90 seconds. Microorganisms are oxidase negative if the color does not change or it takes longer than 2 minutes.

2) Catalase test:

The enzyme catalase converts hydrogen peroxide into water and oxygen, thus helping an organism copes with toxic O_2 species. The catalase test is used to detect an organism‘s ability to produce catalase.

3) Indole test

Indole test is used to determine the ability of an organism to spilt amino acid tryptophan to form the compound indole.

Methods

a. Inoculate the tryptophan broth with broth culture or emulsify isolated colony of the test organism in tryptophan broth.

b. Incubate at 37°C for 24-28 hours in ambient air.

c. Add 0.5 ml of Kovac‘s reagent to the broth culture.

Expected results:

Positive: Pink colored rink after addition of appropriate reagent

Negative: No color change even after the addition of appropriate reagent.

4) MR-VP test

Methyl red test and Voges-Proskauer test both are done in methyl red–Voges-Proskauer (MR-VP) broth, but the reagents that we add differs in terms of reaction.

Methyl Red (MR) Test:

- Positive methyl red test are indicated by the development of red color after the addition of methyl red reagent.
- A negative methyl red test is indicated by no color change after the addition of methyl red

Voges-Proskauer (VP) test:

1. Negative test is indicated by lack of color change after the addition of Barritt‘s A and Barritt‘s B reagents.
2. A positive Voges-Proskauer test is indicated by the development of red-brown color after the addition of Barritt‘s A and Barritt‘s B reagents.

5) Citrate Utilization Test

Citrate utilization test is performed on Simmons citrate agar:

A. Negative citrate utilization test is indicated by the lack of growth and color change in the tube
B. A positive citrate result as indicated by growth and a blue color change.

6) Carbohydrate Utilization Test

When carbohydrates are fermented by bacteria, they produce acidic products. A change in pH can be detected when fermentation of a given carbohydrate has occurred. Acids lower the pH of the medium which will cause the pH indicator of Phenol Red to turn Yellow. When bacteria do not ferment the carbohydrate, the media remains red. Sometimes during fermentation, gas is produced. The Durham tube will then have a gas bubble trapped within it. They are the Glucose

(Dextrose) test, Lactose Test and the Sucrose Test. In all of these tests, the bacteria will be inoculated to the medium using a transfer loop. The results obtained will be similar to that illustrated in the picture (Figure9).

7) Motality Utilization Test

Motality utilization test is a used to determine whether an organism is equipped with flagella and thus capable of swimming away from a stab mark. The results of motility agar are often difficult to interpret. Generally, if the entire tube is turbid, this indicates that the bacteria have moved away from the stab mark (are motile).

8) Nitrate reduction test

This test determines whether the microbe produces the enzymes nitrate reductase and nitrite reductase. The two enzymes catalyze two reactions involved in converting starting compound nitrate into end product nitrogen gas. If a bacterium producing nitrate reductase is grown in a medium containing nitrate, the enzyme converts the nitrate to *nitrite*. *Nitrite* reacts with certain chemicals to yield a red-colored product. If the bacterium also produces *nitrite reductase*, nitrogen gas will be liberated. Bubbles collecting in an inverted Durham tube indicate that nitrogen has been produced.

9) Triple Sugar Iron

The Triple Sugar Iron or TSI test is a microbiological test roughly named for its ability to test microorganism's ability to ferment sugars and to produce hydrogen sulfide. It is often used in the selective identification of enteric bacteria. Bacteria that ferment any of the three sugars in

the medium will produce byproducts. These byproducts are usually acids, which will change the color of the red pH-sensitive dye (phenol red) to a yellow color. Position of the color change distinguishes the acid production associated with glucose fermentation from the acidic byproducts of lactose or sucrose fermentation. Many bacteria that can ferment sugars in the anaerobic butt of the tube are enterobacteria.

10) Blood agar

Blood agar is an enriched, bacterial growth medium. Fastidious organisms, such as streptococci, do not grow well on ordinary growth media. Blood agar is a type of growth medium (*trypticase soya agar enriched with 5% Sheep blood*) that encourages the growth of bacteria, such as streptococci, that otherwise wouldn‘t grow well at all on other types of media.

Blood contains inhibitors for certain bacteria such as *Neisseria* and *Haemophilus* genera and the blood agar must be heated to inactivate these inhibitors. Heating of blood agar converts it into chocolate agar (heated blood turns a chocolate agar) and supports the growth of these bacteria. Certain bacterial species produce extracellular enzymes that lyse red blood cells in the Blood agar (hemolysis). These hemolysin (extotoxin) radially diffuses outwards from the colony (or colonies) causing complete or partial destruction of the red cells (RBC) in the medium and complete denaturation of hemoglobin within the cells to colorless products.

11) Casein Hydrolysis Test

Milk agar contains skim milk (lactose and casein), peptone, and agar. Many organisms can grow on this medium. This medium is used to detect the production of proteases/caseases that digest casein to soluble peptides. This results in a clear zone. Soluble peptides can then be absorbed by

the cell. Casein is responsible for the white color of milk. When digested by exoenzymes, the white agar turns clear and colorless. Bacterial pigments can be seen distinctly on this agar.

12) Lecithinase Test

Bacterial lecithinases are of special interest because of the possible role of these enzymes in pathogenicity. Lecithinases or phospholipases are enzymes released by bacteria that have the ability to destroy animal tissues. Phospholipid complexes are usually emulsifying agents occurring in tissues, serum and egg yolk. Lecithin is a normal component of the egg yolk. Bacterial lecithinases break down this lecithin to an insoluble diglycerides resulting in an opaque halo, surrounding the colony when grown on the egg yolk agar medium.

In egg yolk agar, the lipoprotein component lecithovitellin can also be split by lecithinase into phosphorylcholine and an insoluble diglyceride, which results in the formation of a precipitate in the medium. This precipitate occurs as a white halo, surrounding the colony that produces lecithinase enzyme. The opalescence created is due to the release of free fat. Lecithinase activity is used to characterize several gram positive and gram negative bacteria.

Egg Yolk Agar, Modified is a differential and enriched medium used in the isolation and presumptive differentiation of different species based on their lecithinase and lipase production and proteolytic activity. The egg yolk suspension in the medium allows the detection of lecithinase and lipase activity of the microorganism. The degradation of lecithin present in the egg yolk results in the formation of opaque precipitate around the colonies. The Lipase enzyme hydrolyzes the fats within the egg yolk, which results in an iridescent sheen on the colony surface. Another common reaction observed is proteolysis of the egg yolk as indicated by a clearing of the medium around the colonies. Enzymatic digests of casein and soybean meal

supply amino acids and other complex nitrogenous substances. Yeast extract primarily provides the B-complex vitamins. Hemin improves the growth of anaerobic microorganisms. L-cystine is a reducing agent and an essential amino acid.

3.4. Inoculums' development

Pure cultures of selected bacterial isolates were individually maintained on CMC supplemented minimal agar slants at 4˚C, until used. Pure cultures of selected bacterial isolates were inoculated in broth medium containing 0.03 % $MgSO_4$, 0.2 % K_2HPO_4, 1 % glucose, 0.25% $(NH_4)_2SO_4$ and 1 % peptone at pH 7 for 24h of fermentation period. After 24h of fermentation period these vegetative cells were used as inoculum source.

3.5 Secondary screening and production of cellulase enzyme:

The potential isolates were then evaluated for enzyme productivity. Those isolates showing maximum cellulase production were then considered for the further study.

3.6 Cellulase enzyme production

Newly isolated isolates were screened for cellulase enzyme production in submerged fermentation process. Fermentation medium was prepared using 1% potato waste (as cellulose substrate), 0.2% K_2HPO_4, 0.03 % $MgSO_4$, 1 % peptone, 0.25 % $(NH_4)_2SO_4$ and autoclaved at 121°C for 15min. After sterilization, the medium was allowed to cool at room temperature. The medium was inoculated with 1 ml of selected bacterial isolates and incubated in a shaker at 35˚C for 24 h of fermentation period with agitation speed of 140 rpm. After termination of the fermentation period the fermented broth was centrifuged at 1400× g for 10 min at 4˚C to remove

the unwanted material. The clear supernatant thus obtained after centrifugation served as crude enzyme source.

3.7 Estimation of cellulase activity

Cellulase activity was assayed using dinitrosalisic acid (DNS) reagent by estimation of reducing sugars released from CMC solubilized in 0.05 M phosphate buffer at pH 8. The culture broth was centrifuged at 14000 × g for 10 min at 4˚C and the clear supernatant served as crude enzyme source. Crude enzyme was added to 0.5 ml of 1 % CMC in 0.05 M phosphate buffer and incubated at 50˚C for 30 min. After incubation, reaction was stopped by the addition of 1.5ml of DNS reagent and boiled at 100˚C in water bath for 10 min. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1μmol of glucose per minute under standard assay conditions.

3.8 Optimization of Cultivation Conditions for CMCase Production by the selected isolates-

The effect of initial pH and temperature on CMCase production was determined by cultivating the strains in medium, same medium used in submerged fermentation at various pH (ranging from 3.0 to 11.0 with an interval of 0.5).

The effect of carbon sources on cellulase production by the strains was determined by using 4 different carbon sources (Filter paper, Cotton, Avicell, CMC). The different carbon sources were used at a concentration of 10 g/L, instead of the core carbon source in the basal medium.

3.8.1. Determination of optimum pH for endoglucanase activity

The effect of pH on enzyme activity was determined by mixing 500µL of the crude enzyme extract with 500µl substrate at various pH values (1% CMC prepared in 0.05)using sodium phosphate buffer(pH6.0 to 8.0), sodium citrate (pH 3.0 - pH 6.0) and Glycine-NaOH (pH 9.0-11.0) buffer solutions(Lin *et al*., 2012). The reaction mixture was incubated in water bath at pre-determined optimum temperature of 98°C for50 minutes. Amount of glucose produced was assayed by carrying out a DNase test. Using a standard curve, amount of glucose produced was calculated and values obtained used to determine specific enzyme activity.

CHAPTER 4: RESULT

4.1 Sampling:

The study was conducted by two soil samples one from National Parliament residential area &another from BRAC nursery.

4.2 Isolation of soil bacteria

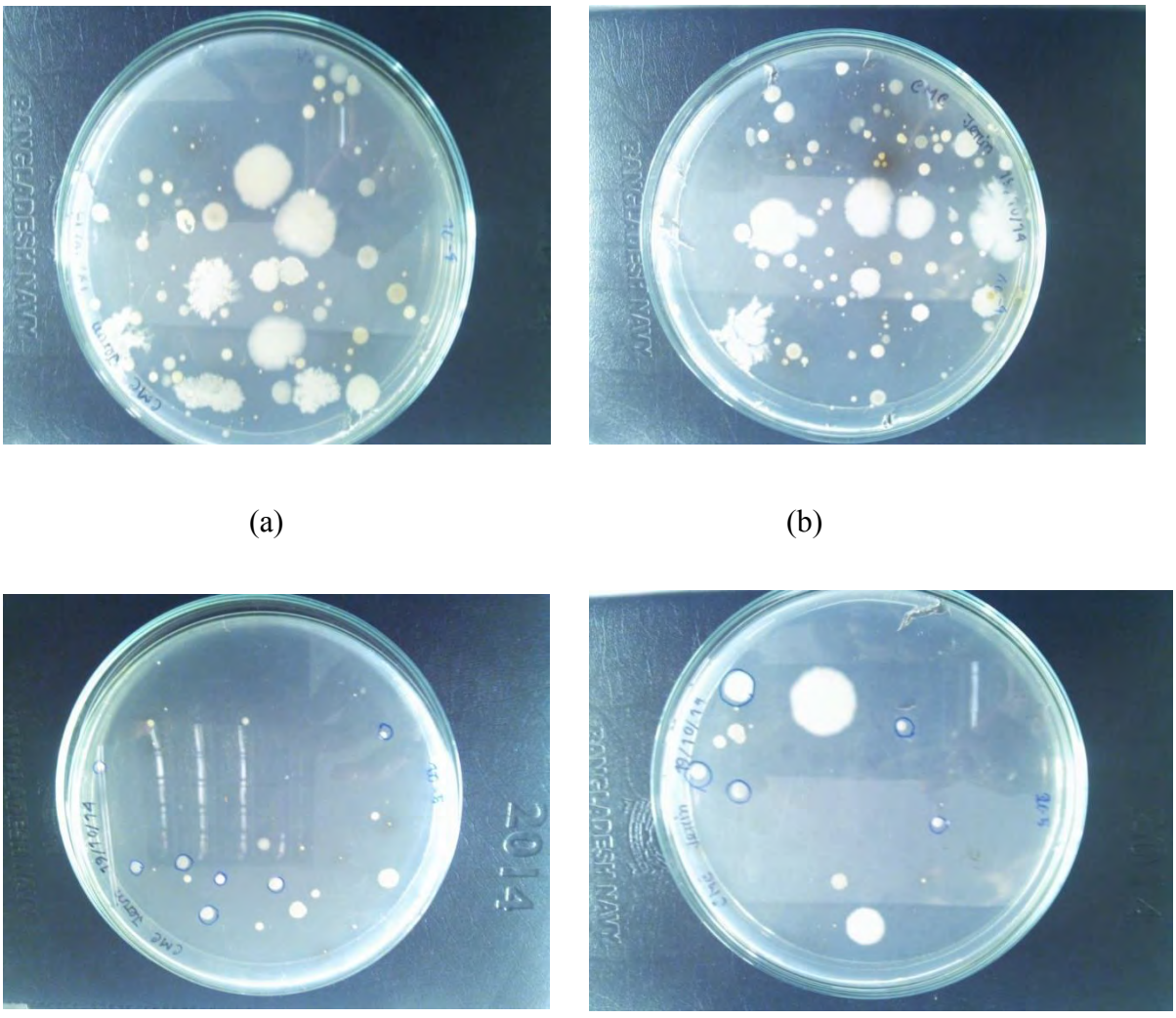
Figure (4) showed four isolates were isolated from soil samples collected from National Parliament Residential area & BRAC Nursery. The isolates were treated as different isolates and denoted as NCDB1 - NCDB12& CDB1- CDB6respectively.

A total of 14 cellulose-degrading aerobic bacterial isolates were isolated from different natural reserves in the Dhaka & nearest places of Dhaka, which were cultured in agar medium containing CMC as the sole carbon source.

Table-4.1 Different locations selected for sample collection to identify cellulase producers.

Site	Sample no	Total no isolate	Labeled as
National Parliament Area	NS1- NS12	12	NCDB1- NCDB12
BRAC Nursery	BS1-BS6	6	CDB1-CDB6

Among the samples, soil sample from national parliament area had shown less clear zone &cellulase activity. The soil sample collected from BRAC nursery, showed more cellulase activity so the further study was conducted by using isolates from BRAC nursery which are identified as CDB1- CDB6.



(a) (b)

(c) (d)

Figure 4.1: Soil Sample from BRAC Nursery collected & incubated ay 30°C in selected media which is CMC media for 48 hrs.

Figure 4.2: Clear zone was formed after applying Congo red & followed by counter staining NaCl in elected plates for further study.

Out of these strains, five isolates showed hydrolyzing zones on agar plates containing CMC as core carbon source, after Congo-red staining the hydrolyzing zone diameter and colony diameter were listed & given below in Table 3.

Table 4.2- Zone of CMC hydrolysis of different isolates

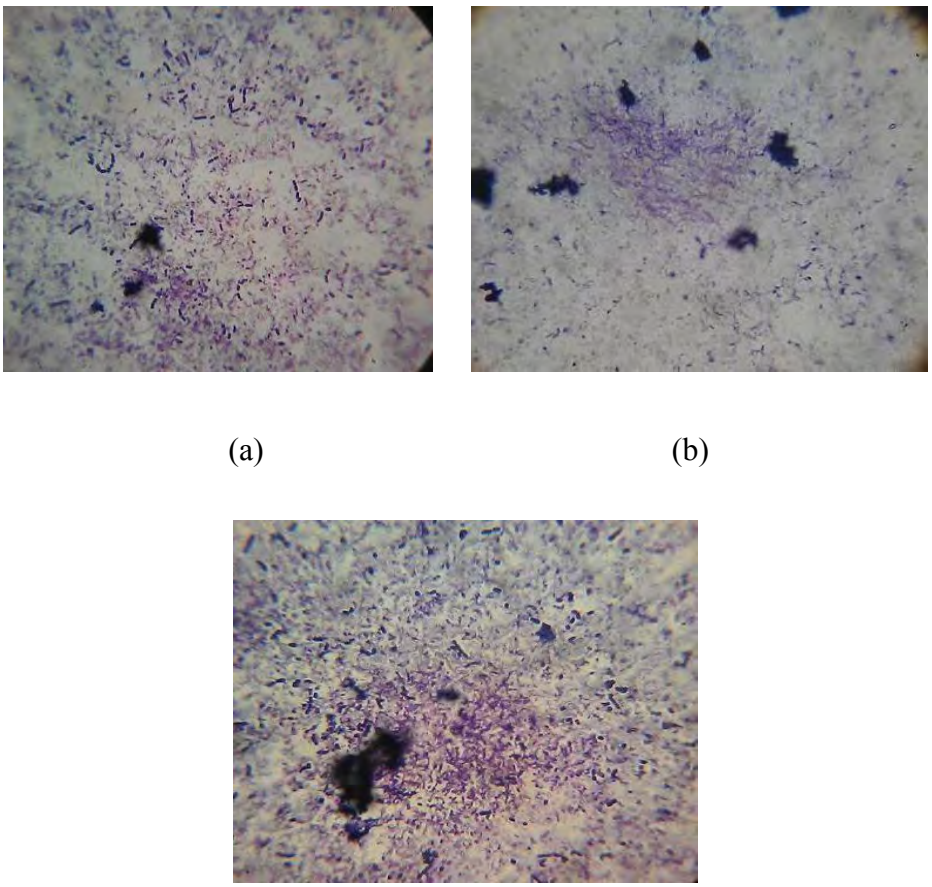
Serial No	Isolate No	Colony Diameter(z)(mm)	Zone Diameter(z)(mm)	(z/n)(mm)
1	CDB1	8.7	10.0	1.3
2	CDB2	7.5	9.0	1.5
3	CDB3	7.0	9.4	2.4
4	CDB4	9.6	12.0	3.6
5	CDB5	7.6	9.0	2.0

CBD - 3, CDB - 4 and CDB-5 showed largest clear zone & selected for further identification.

4.3. Characterization of bacteria isolates

4.3.1 Morphological characterization

The isolates‘ morphological features were determined by Gram stain test. All the isolates retained the purple color of crystal violet stain implying that they were Gram positive rods as shown in Figure 6.



(a) (b)

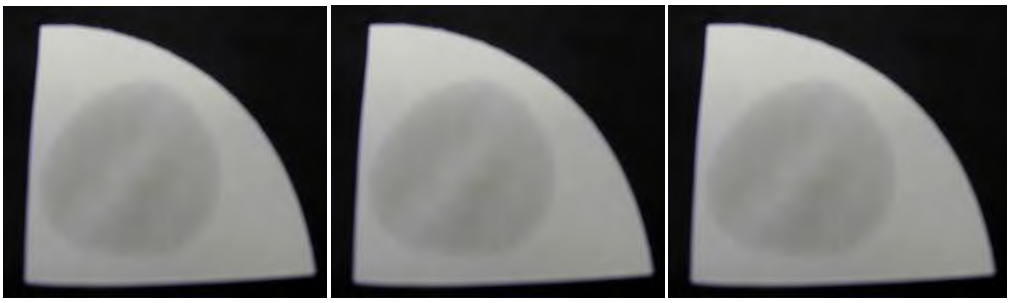
(c)

Figure 4.3 - Gram staining of CDB3, CDB4, and CDB5 isolates respectively

In figure 4.3 all three isolates showed positivity in Gram staining test. It indicates that all three isolates are Gram positive (G+) organism.

4.3.2 Biochemical characterization:

1. Oxidase test-



(a) (b) (c)

Figure 4.4- Oxidase test is negative for all three isolates.

In figure 4.4 showed that there were no color change in filter paper which clearly indicates that all three isolates were oxidase negative.

2. Catalase Test

Figure 4.5: Catalase test is positive for CDB4, CDB5, and CDB6 respectively

The formation of bubble in figure 4.5 indicated that the isolates were catalase positive and CDB3, CDB4& CDB5 all three isolates were able to produce enzyme catalase &converts hydrogen peroxide into water and oxygen.

3. Indole Test

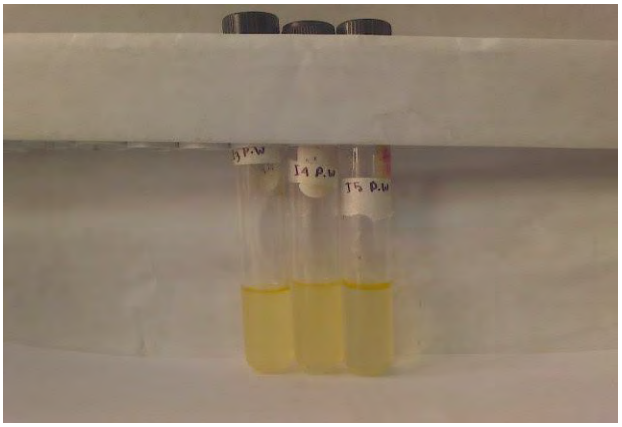


Figure 4.6: Indole test for CDB3, CDB4 & CDB5

There was no visible change in the solution showed above in figure 4.6. CDB3, CDB4 & CDB5 all this isolates remain unchanged after incubation which indicated that all this isolates were indole negative.

4. MR-VP

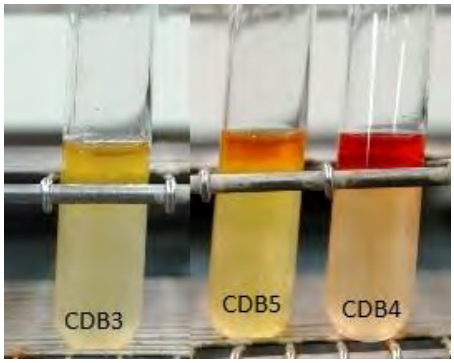
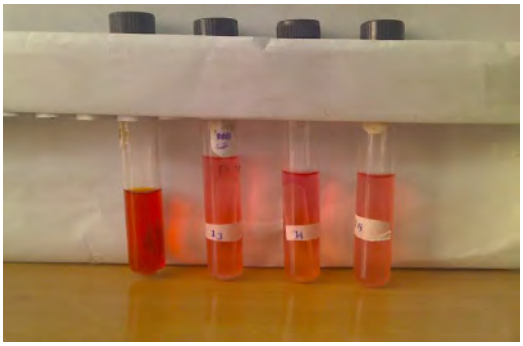


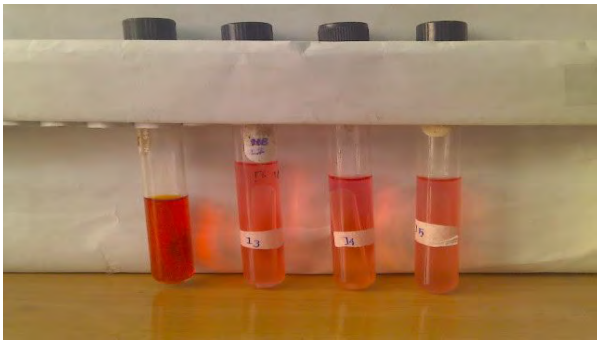
Figure 4.7: MR-VP test

In MR-VP test the isolates showed different result as showed in figure 4.7. CDB 5 & CDB 4 changed the color of solution, where CDB3 remain unchanged. So it indicated that CDB5 & CDB4 is positive & CDB3 is negative.

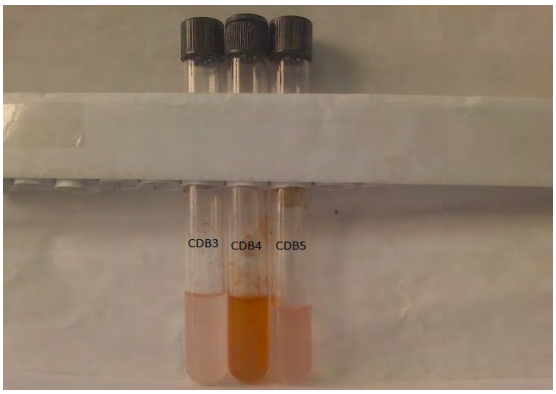
6) Carbohydrate Utilization Test



Glucose



Sucrose



Lactose

FIGURE 4.8: Carbohydrate utilization test

In figure 4.8 , the results of carbohydrate utilization test is presented. CDB3 produced acid & gas in glucose solution, no acid production in sucrose & no visible change in lactose solution. For CDB4 there was no visible change in glucose solution, in sucrose solution it produces only acid no gas in produced & in lactose solution the result is positive. In CDB5, in glucose solution only acid produced as the color changed while no gas produced, in sucrose solution both acid & gas produced as the color changed & lactose remain unchanged.

7) MOTALITY TEST

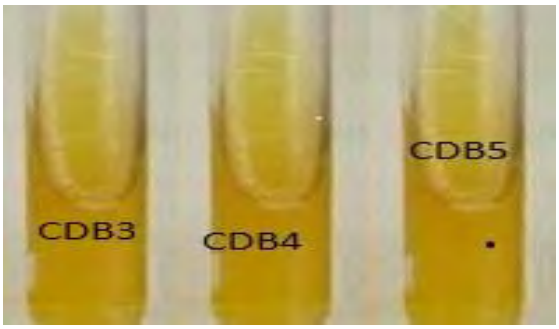


Figure 4.9: Motality test

Motility test for all three isolates were negative. It declares that isolates were non- motile.

8) Nitrate reduction test

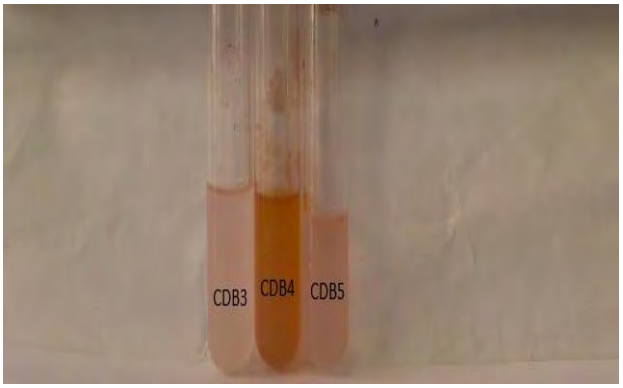


Figure 4.10 - Nitrate reduction test

This test determines whether the microbe produces the enzymes nitrate reductase and nitrite reductase. In figure 4.10, it showed only CDB 4 is able to produce the enzymes nitrate reductase and nitrite reductase while CDB 3 & CDB 5 was unable to produce the enzymes.

9) Triple Sugar Iron

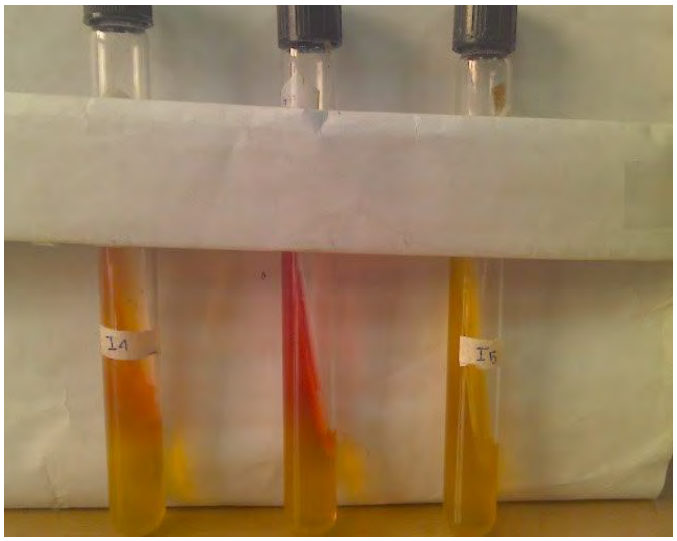


Figure 4.11 –Triple Sugar Iron (CDB4, CDB3 & CDB5)

In figure 4.11, the reactions in TSI media slants indicated the ability of isolates to utilize the media. CDB3 had acidic butt (yellow) & alkaline slant (red).CDB4 had acidic butt (yellow) & alkaline slant (red).CDB5 had both acidic butt & slant (yellow).

Table 4.3 - The result of Biochemical test –

Test		CDB3	CDB4	CDB5
Glucose		A,G	NC	A,G-
Sucrose		$A^{\pm}$	A,G-	A,G+
Lactose		-	+	-
M		-	-	-
I		-	-	-
U		-	-	-
TSI	Slant	A	A	A
	Butt	K	K	A
MR		+	+	-
VP		+	+	-
Oxidase		-	-	-
Catalase		-	+	+
Nitrate		-	+	-

*Suc=Sucrose, lac=Lactose, Mot= Motility,Ind=Indole,U=Urease, VP= Vogues proskauer

Cit =citrate, Ox=oxidase, Cat = catalase, Nit= nitrite, TSI= triple sugar iron A= acid, AG= acid/ Gas, K= alkaline, NC= Not Countable

9) Blood agar

FIGURE 4.12 –Blood Agar

The clear zone in the media plate containing blood agar showed the ability of isolates to utilize the blood agar media.

11) Casein Hydrolysis Test

FIGURE 4.13 –Skim Milk Agar

In figure 4.13, skim milk agar medium was used to detect the production of proteases/caseases that digest casein to soluble peptides. The clear zone indicated the ability of all three isolates able to produced proteases/caseases & soluble peptides that can then be absorbed by CDB3, CDB4& CDB5.

12) Lecithinase Test

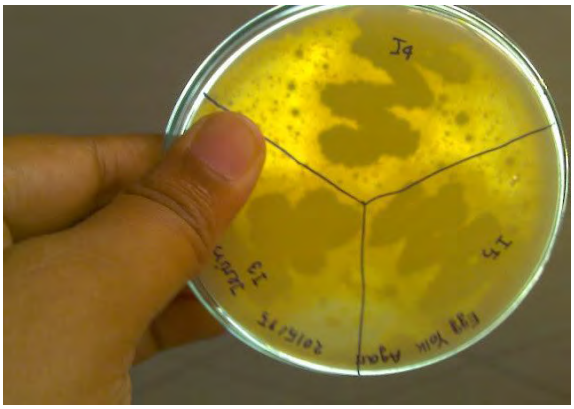


FIGURE 4.14 –Egg Yolk Agar

In figure 4.14 it was showed that CDB3, CDB4 & CDB5 were able to produce lecithinases (enzymes released by bacteria that have the ability to destroy animal tissues). The colonies in the egg yolk agar indicated that all three isolates were positive in this test.

Table 4.4 - Table of Media Utilization test by isolated strains.

Medium	CDB3	CDB4	CDB5
Nutrient Agar	(+ve)	(+ve)	(+ve)
Skim Milk Agar	(+ve)	(+ve)	(+ve)
Blood Agar	(+ve)	(+ve)	(+ve)
Egg Yolk Agar	(+ve)	(+ve)	(+ve)
Gelatin Agar	(+ve)	(+ve)	(+ve)

4.4 Optimization of cellulase production:

The optimum parameters were determined for cellulase production from the efficient isolates. After fermentation at the different parameters the crude enzyme product was collected for determination of enzyme activity. Enzyme activity was determined by DNase method. Data analysis clearly indicated that the highest enzyme activity of CDB4isolate was found to be0.206 at 540nm absorbance at 30°C. The enzyme activity of CDB3 isolate & CDB5 isolate was 0.203& 0.147 respectively at same temperature. The enzyme activity fluctuates while placed in different pH and at pH 5.5 the isolates showed highest activity.

Cellulase production was estimated by using glucose calibration curve. One unit (U) of enzyme activity is expressed as the quantity of enzyme, which is required to release 1μmol of glucose per minute under standard assay conditions.

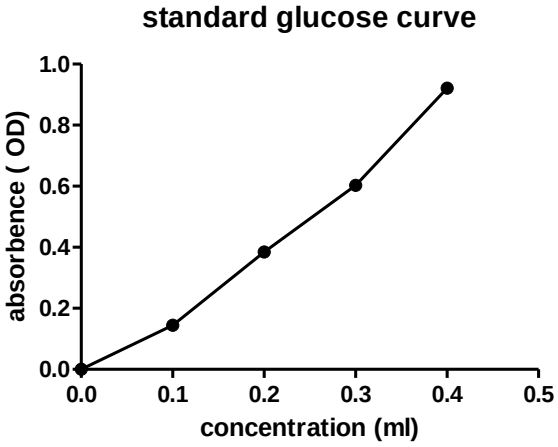


Figure 4.15- Glucose standard curve for the determination of the quantity of reducing sugar produced from enzyme assays and for determination of cellulose activity. Absorbance's were read at 540 nm.

4.4.1. Determination of cellulase specificities produced by Isolate

Cellulase activities were determined using an aliquot of the crude enzyme sample and 1% of substrate (Filter paper, Cotton, Avicell & CMC respectively) at pH 5 and 30°C. There was a significant difference in enzyme activities for the four enzyme specificities showed in figure 20 to figure 23.

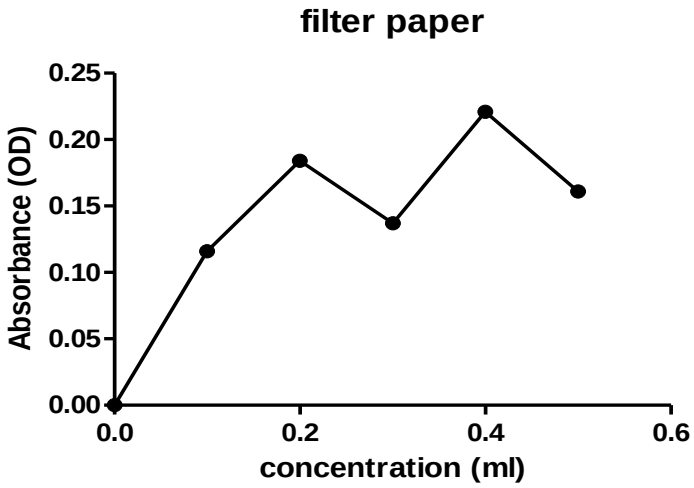


Figure 4.16- The enzyme activity of five isolates showed different absorbance capacity in solution where filter paper used as carbon source.

In the figure CDB4 showed highest cellulase activity which was 0.221 ml/mg. The other isolates were respectively CDB1 - 0.116, CDB2- 0.184, CDB3- 0.137, and CDB5 - 0.161.

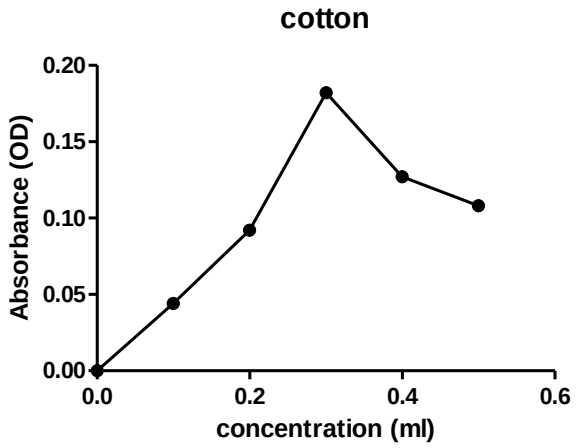


Figure 4.17 - The enzyme activity of five isolates showed different absorbance capacity in solution where cotton was used as carbon source.

In the figure CDB3 showed highest cellulase activity which was 0.182 ml/mg. The other isolates were respectively CDB1 -0.44, CDB2- 0.092, CDB4- 0.127, and CDB5 – 0.108.

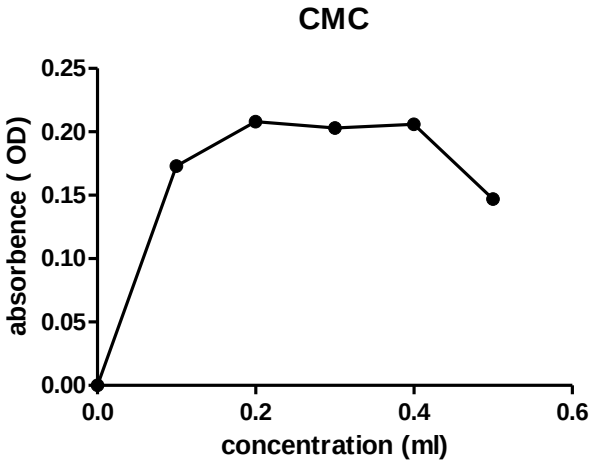


Figure 4.18 - The enzyme activity of five isolates showed different absorbance capacity in solution where CMC was used as carbon source.

In the figure CDB2 showed highest cellulase activity which was 0.208 ml/mg. The other isolates were respectively CDB1 -0.173, CDB3- 0.203, CDB4- 0.206, and CDB5 – 0.147.

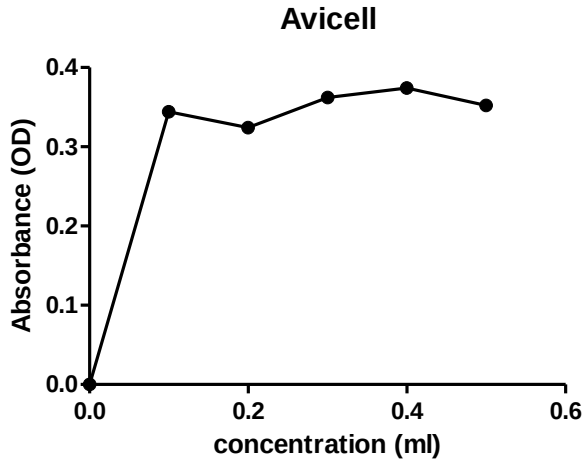


Figure 4.19 - The enzyme activity of five isolates showed different absorbance capacity in solution where Avicell was used as carbon source.

In the figure CDB4 showed highest cellulase activity which was 0.374 ml/mg. The other isolates were respectively CDB1 -0.344, CDB2- 0.324, CDB3 - 0.326, and CDB5 –0.352.

4.4.2. Determination of optimum pH for maximum cellulase activity

Enzyme assays were carried out using buffer solution with carboxymethylcellulose at pH values ranging from 3 to 11 in order to determine the optimum pH.There was a significant change in enzyme activity with change in pH (Figure 22- Figure 24).

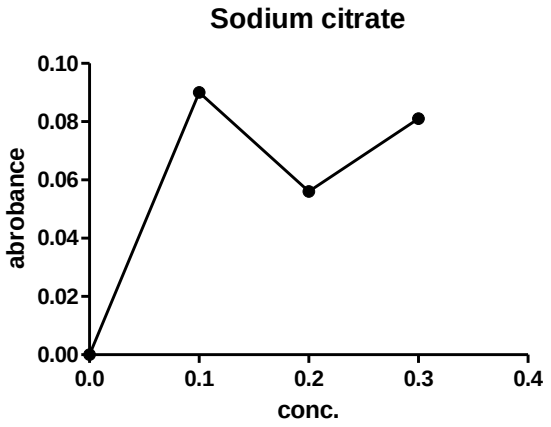


Figure 4.20 - The enzyme activity of three isolates showed different absorbance in buffer solution of sodium citrate pH 5.7, where CMC was used as core carbon source.

In the figure CDB3 showed highest cellulase activity which was 0.090 ml/mg. The other isolates were respectively, CDB4 - 0.056 and CDB5-0.081.

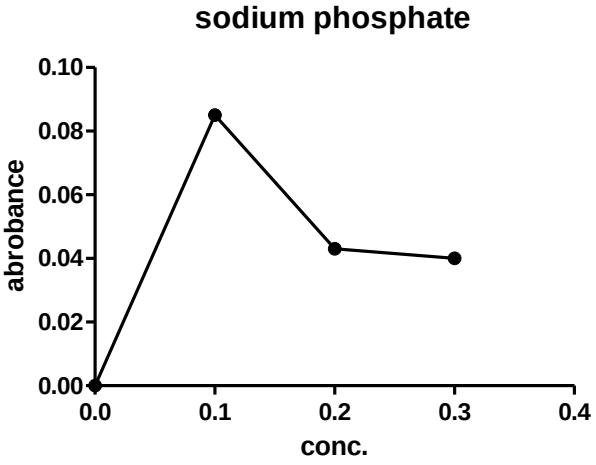


Figure 4.21 - The enzyme activity of three isolates showed different absorbance in buffer solution of sodium phosphate pH 6.7, where CMC was used as core carbon source.

In the figure CDB3 showed highest cellulase activity which was 0.085 ml/mg. The other isolates were respectively, CDB4 - 0.043 and CDB5-0.040.

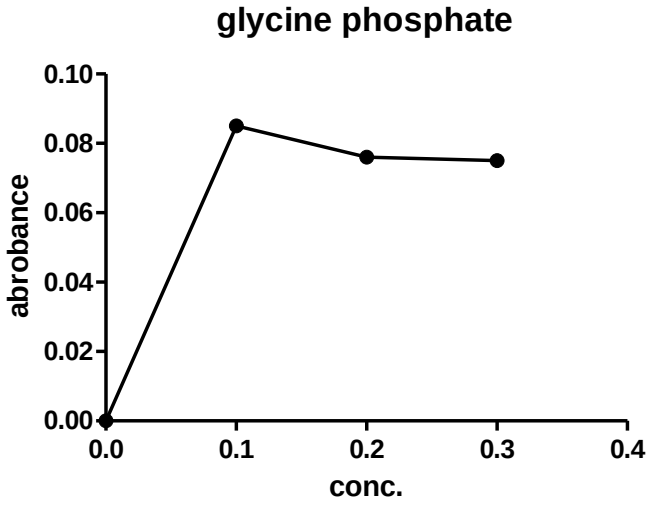


Figure 4.22 -The enzyme activity of three isolates showed different absorbance in buffer solution of sodium phosphate pH 9.9, where CMC was used as core carbon source.

In the figure CDB3 showed highest cellulase activity which was 0.085 ml/mg. The other isolates were respectively, CDB4 - 0.070 and CDB5-0.075.

CHAPTER 5: DISCUSSION

Cellulose is the most abundant plant biomass on earth with an estimated annual production of 4.0×10^7 units (Bakare *et al*., 2005). This abundance confers to cellulose an immense potential as a renewable source of energy. In order to convert cellulose into soluble sugars for fermentation, various bioconversion methods such as pyrolysis, acid hydrolysis and enzymatic hydrolysis can be applied (Cooney *et al*., 1978). The latter is more environmental friendly, and gives a pure product with consumption of less energy (Bakare, 2005; Fennington *et al*., 1982).

Cellulolytic microorganisms such as fungi and bacteria are responsible for much of the cellulose degradation in soils, though some insects, crayfish and mollusks produce their own cellulases to utilize cellulose (Watanabe &Tokuda 2001; Ohkuma, 2003; Breznak, 1982). Despite this vast number of cellulase producers, there is a deficiency of microorganisms that can produce significant amount of the three cellulase enzyme specifities like cellulase to efficiently degrade cellulose to fermentable products (Maki *et al*., 2011). In addition to this, majority of the studies have been focused on fungi with less emphasis on bacterial sources for cellulase production. Due to their extremely high natural diversity, bacteria have the capability to produce stable enzymes that can be applied in industries (Bhat, 2000; Bischoff *et al*., 2006; Camassola*et al*., 2004; Haakana *et al*., 2004). These two factors were the prime motivation in this study; to isolate and characterize cellulolytic bacteria from environments.

5.1 Isolation and characterization of cellulolytic bacteria

Three cellulolytic bacteria isolates were isolated in soils excavated from BRAC nursery. Attempts to isolate cellulolytic microorganisms from various places have led to the isolation of strong cellulase producers such as *Trichoderma*, *Aspergillus*, *Pellicularia*, *Penicillium*,

Acremonium and *Humicola*. However, these microorganisms were found to be heat sensitive (Fujimoto *et al*., 2011). The isolates in the current study are mesophilic cultured at 30°C.

Identification of the three isolates was carried out using various biochemical tests. All isolates that were found to be Gram-positive rods hence were likely to belong to be *Bacilli* genus. Gram-positive bacteria have a thick mesh like cell wall comprising of 50-90% peptidoglycan while Gram-negative bacteria on the other hand have a thinner cell wall and an additional outer membrane composed of lipids. Gram-positive bacterium remains purple while the Gram negative ones pick the positively charged Safranin counter stain to stain pink.

5.2 Enzyme production

The selected isolates which were named CDB3, CDB4 & CDB5and was cultured in a medium containing 1% CMC; a carbon source to induce the production of cellulases which are inducible enzymes synthesized during the bacteria‘s growth on cellulosic materials (Sang-Mok& Koo, 2001; Kubicek, 1993). The supernatant was found to bear cellulolytic enzymes, supporting other previous studies that indicated the ability by known members of the *Bacillus* genus to secrete proteins extracellularly (Schallmey *et al*., 2004; Lin *et al.,* 2012).

5.2.1. Determination of cellulase enzyme specificities produced by isolates

Cellulase activities are mainly evaluated using a reducing sugar assay to measure end products of the hydrolysis of substrate thus assay results are expressed as the hydrolysis capacity of the enzyme (Dashtban *et al*., 2010). The isolate showed significantly different levels of specific enzyme activities ($P<0.05$).

5.2.1.1. Enzyme activity determined using CMC as carbon source

The structural complexity of pure cellulose and difficulty of working with insoluble substrates has led to the wide use of CMC for endoglucanases studies (Lynd *et al*., 2002). CMC is a soluble cellulose derivative with a high degree of polymerization (DP). The isolate was found to bear CMCase activity. This was in line with other studies that have reported on *Bacillus licheniformis* strains degrading amorphous substrates such as CMC amongst them (Fujimoto *et al*., (2011) and Bischoff *et al*., (2006). DNase is reduced to 3-amino-5-dinitrosalicylic acid an aromatic compound that absorbs light strongly at 540nm (Miller, 1959). The disadvantage of using this method is loss of some reducing sugars during the analysis but despite that it‘s a more convenient test compared to other sugar tests.

5.2.1.2 Enzyme activity determined using Avicell as carbon source

Exoglucanases cleave β - (1-4) glycosidic bonds from side chains to release cellulase or glucose molecules (Béguin & Aubert, 1994). Commercial Avicel (microcrystalline cellulose) is used for measuring exoglucanases activity because it has a low degree of polymerization and inaccessible to attack by endoglucanases despite having some amorphous regions. Avicel contains some quantities of amorphous cellulose and cellodextrins which act as substrate for both exoglucanases and endoglucanases. This implies that there is no highly specific substrate to test exoglucanase activity in cellulase mixtures (Sharrock *et al*., 1988; Wood & Bhat, 1988). However enzymes that show relatively high activity on Avicel and less activity on CMC can be identified as exoglucanases (Maki *et al*., 2009; Makky, 2009).

The isolate was found to display avicelase activity contrary to some previous research work that indicated that members of the genus *Bacilli* are unable to degrade Avicel (Makky, 2009; Bischoff *et al*., 2006). However during the experiment Avicel might settle at the bottom of the flask if the incubation is carried out without agitation. This also lowered the quantity of reducing sugar production. This can be attributed to substrate availability i.e. not all substrate in the reaction mixture was available for attack by the exoglucanases in the cellulase mixture.

5.4 Determination of optimum pH for enzyme activity

Enzyme activity assays to determine the optimum pH were carried out in reaction mixtures at varying pH values (3-11) at the predetermined temperature (60°C) using water bath. Each enzyme has its own optimum pH and if the pH increases or decreases beyond the optimum, the ionization groups at the active site may change slowing or preventing the formation of an enzyme substrate complex (Eijsink *et al.,* 2005).

Optimum pH values of 4.5-8.0 have been reported for different microbial cellulase (Bakare*et al*., 2005; Immanuel *et al*., 2007; Dutta *et al*., 2008). For this isolates, there was a significant change in enzyme activity with change in pH ($P<0.05$). The highest activity was recorded at pH 5.5 (Figure 16) suggesting that the enzyme is an acid cellulase. Acid cellulases act at a pH range of 3.8 and 5.8 (Mosjov, 2012). Similar observations have been made by Bajaj *et al.,* (2009). There was no significant difference in enzyme activity at pH 6, 7 and pH 3, 11 ($P>0.05$). This finding suggests that these pH pairs have more or less the same effect on enzyme activity.

CONCLUSION

Five cellulolytic bacterial isolates were obtained from soils excavated from BRAC nursery. The Gram-positive rods were deduced to be *Bacillus acidiceler*(CDB3) , *Bacillus mycoides*(CDB4) & *Paenibacillus illinoisensis* (CDB5) from both morphological and molecular analysis.

Crude cellulase mixture from CDB3, CDB4 & CDB5, selected for further studies was shown to bear the two types of cellulolytic activities. From the functional tests carried to determine the optimal conditions for cellulolytic activity, the isolate‘s enzyme activity was found to be high over a range of temperatures i.e. from 20°C to 45°C with the optimum temperature being 30°C. Similarly, enzyme activity was found to be high at the range of pH 4 to pH 7 with an optimum of pH 5.5 suggesting that the isolate bears acid cellulases.

Application of these three isolates in industries may have several advantages such as high growth rate and ability to secrete proteins extracellularly; features of the *Bacillu*s species.

More studies are however needed before industrial application of this isolates. These include enzyme activity assays of the purified specific cellulases for comparison with the results in this study and with those that have been purified. These studies would shed more light on whether to use the whole organism in the industry or harvest the enzymes and carry out downstream processes or purify the gene to know whether the gene is to be added to the genetic pool for protein engineering and directed evolutionary studies to come up with super enzymes. Similar studies should be extended to other environments in the country.

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