Isolation and Presumptive Identification of Cellulolytic Bacteria from Soil Sample and Characterization of Crude Cellulase

A DISSERTATION SUBMITTED TO BRAC UNIVERSITY IN PARTIALFULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF BACHELOR OF SCIENCE IN MICROBIOLOGY

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June, 2016

Dedicated

TO

MY

BELOVED FATHER AND MOTHER

Declaration

I, Sazzad Khan declare that this thesis and the work entitled ‘’Isolation and Presumptive Identification of Cellulolytic Bacteria from Soil Sample and Characterization of Crude Cellulase’ submitted to the Department of Mathematics and Natural Sciences, BRAC University in partial fulfillment of the requirements for the degree of Bachelor of Science in Microbiology is a record of work carried out by me under the joint supervision of my supervisors.

I further declare that this thesis has been composed solely by me and it has not been submitted, in whole or in part, in any previous institution for a degree or diploma. Except where states otherwise by reference or acknowledgment, the work presented is entirely my own.

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Acknowledgement

After an intensive period of eight months, today is the day: writing this note of thanks is the finishing touch on my thesis. It has been a period of intense learning for me, not only in the scientific arena, but also on a personal level. Writing this thesis has had a big impact on me. I would like to reflect on the people who have supported and helped me so much throughout this period.

First and foremost, I am grateful to the Almighty for the good health and wellbeing that were necessary to complete this research work and submit this paper.

I would like to express deep my gratitude and appreciation to Prof. Dr. A. A. Ziauddin Ahmad, Chairperson of Department of Mathematics and Natural Sciences, BRAC University, Dhaka for allowing me to continue my research work at BRAC University microbiology laboratory. My indebtedness and deepest gratitude goes to Prof. Naiyyum Choudhury, former Coordinator of the Biotechnology and Microbiology program, MNS department, BRAC University and Chairman of Bangladesh Atomic Energy Regulatory Authority (BAERA); for providing me with his enthusiastic idea about this project, precious advices and experiences that helped me much to carry out the study and eventually succeed.

I express my sincere gratitude and respect to my Co-supervisor Dr. M. Mahboob Hossain, Coordinator of Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University for being a constant support all throughout my student life and guiding me during the research work which have enhanced my capability enormously.

This work was completed under the meticulous guidance of my research supervisor Trosporsha Tasnim Khan, Lecturer, Department of Mathematics and Natural Sciences, BRAC University. She added new directions to my thinking, and acted as a continual stimulus for refinement and depth in the presentation of this work. I am much indebted and would like to express my sincere thanks to her for the expert guidance and constant support throughout the entire period of my research work.

I would like to extend my appreciation to the respective lab officers Asma Binte Afzal and Shamim Akhter Chowdhury for their suggestions and moral support during my work.

I express my heartfelt thanks to my dearest friend and thesis partner Faria Mahjabeen for her constant active and amiable support during the research work. Also, I am very much thankful to another friend of mine Tasnia Islam for her kind cooperation and active support throughout my work.

Finally, I would like to thank my family members for their spontaneous and enthusiastic support.

Sazzad Khan

June, 2016

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LIST OF ABBREVIATION

1. CBMs : Carbohydrate-binding modules
2. CBHs : Cellobiohydrolases
3. BG- β : Glucosidase
4. DNS : 3, 5-dinitrosalicylic acid
5. EG : Endoglucanase
6. CBH : Cellobiohydrolase
7. BG : β-glucosidase
8. CMCase : Carboxy methyl cellulose
9. OD : Optical density
10. NA : Nutrient agar
11. CMCA : Carboxy methyl cellulose agar
12. NB : Nutrient broth
13. LB : Luria Bertani broth
14. DNS : Dinitrosalicylic acid
15. dH_2O : Distilled water
16. MIU : Motility in dole urease
17. TSI :Triple Sugar-Iron Test
18. MR TEST : Methyl red test
19. VP : Voges-Proskaure Test
20. MSW : Municipal solid wastes
21. % : Percentage
22. CFU : Colony forming unit
23. Mg : Milligram
24. µg : Microgram
25. gm : Gram
26. Kg : Kilogram
27. °C : Degree Celsius
28. °F: Degree Ferhenite
29. µm : Micrometer
30. mm : Millimeter
31. ml : Milliliter
32. e.g.: For example
33. et al.: And others
34. spp.: Species
35. rpm : Rotation per minute

Abstract

Microbial cellulases find applications in various industries and constitute a major group of the industrial enzymes. It has attracted attention of many researchers because of its tremendous industrial applications including textile industry, pulp and paper industry, laundry and detergent industry, food and animal feed industry. This study aims to isolate, identify and screen bacteria with high cellulase activity from soil sample. A total of 68 isolates were obtained from soil collected from Bagan Bari Dairy Farm, Keranigonj, Bangladesh. These were screened through clear zone ratio on CMC agar plate with Gram's Iodine. Of the 68 isolates, 31 isolates gave better zone of hydrolysis and finally two of them (isolate 47 and 66) with maximum clear zone ratios were evaluated by secondary screening for enzyme production. Between these two isolates, isolate 66 was selected as most efficient enzyme producer and its enzyme activity along with specific enzyme activity in the crude sample were found to be 0.165 U/ml and 0.333 U/mg respectively. The potential isolate 66 was further presumptively identified to be a *Bacillus* spp. through a series of biochemical tests, colony characteristics and cultural characteristics. It was found to be a gram positive, rod shaped and spore forming bacterium and was facultative anaerobe. The optimum pH and temperature for the activity of crude enzyme was 5 and 65°C respectively .The potential isolate 66 was further checked for filter paper and cotton degradation properties quantitatively. It showed positive result for filter paper degradation and negative result for cotton hydrolysis.

CHAPTER 1

1.0 Introduction

1.1 Background of the study

There has been considerable interest over the past few years in producing microbial enzymes, which has been initiated by expanding concern over preserving resources and environment (Thongekkaew *et al.,* 2014). Bocchini *et al.* (2003) emphasized on the fact that cellulases and xylanases enzymes producing from microorganisms in the most recent decade, have pulled in an incredible consideration due to their biotechnological potential in different modern procedures, for example, waste water treatment, food, feed, and paper-pulp industries (Thongekkaew *et al.,* 2014). In addition to industrial biofuels production has been infuenced by a great deal , in fact, from the cellulosic biomass (Ali &Saad, 2008). Cellulose is the most abundant renewable natural resource and a low-cost energy source in terms of energy content (Lynd *et al.,* 2008; Zhang, 2009). Therefore, bio-based products and bioenergy from less costly renewable lignocellulosic materials would convey advantages to the local economy, environment and national energy security (Zhang, 2008). However, the cost of an enzyme is one of the main factors determining the economics of a process. In fact, the high cost of cellulase enzyme production hinders the commercialization of bioethanol production. (Himmel *et al*., 1999; Wooley *et al*., 1999; Zhang *et al.,* 2006b; Zhu *et al.,* 2009). For example, the estimated cost of the enzymes to the production of lignocellulosic ethanol significantly varies from $0.10/gal to $0.40/gal (Klein-Marcuschamer *et al.,* 2011). Dhillon *et al*. (2000) suggested an alternative way to reduce the high production cost of cellulase enzyme, which can be partially achieved by optimizing fermentation medium and using agricultural wastes for the cultivation of the producer micro-organism.

Cellulases are the enzymes that are produced by a large number of microorganisms such as bacteria, fungi, protozoans, plants and animals are able to produce cellulases (Kulkarni *et al*., 1999; Subramaniyan and Prema, 2002; Henrissat, 1991). Cellulase can convert the world's most abundant biopolymer and renewable biological resource, 'cellulose', into reducing sugars. Although cellulose is commonly degraded by cellulase, bacterial and fungal cellulases are generally differentiated into three classes (Shah and Madamwar, 2005). Moreover, cellulases contain non catalytic carbohydrate-binding modules (CBMs), other functionally known or unknown modules as well, which may be located at the N- or C-terminus of a catalytic module. In nature, complete cellulose hydrolysis is mediated by a combination of three main types of cellulases: (1) Endoglucanases (EC 3.2.1.4), (2) Exoglucanases, including cellobiohydrolases (CBHs) (EC3.2.1.91), and (3) β -glucosidase (BG) (EC 3.2.1.21). In order to hydrolyze and metabolize insoluble cellulose, the microorganisms must secrete the cellulases (possibly except BG) that are either free or cell-surface-bound (Bayer *et al.,* 2007; Himmel *et al.,* 1999; Zaldivar *et al.,* 2001). For instant, endoglucanase is responsible for random cleavage of β-1, 4-glycosidic bonds along a cellulose chain. While, exoglucanase is essential for cleavage of the non-reducing end of a cellulose chain and splitting of the elementary fibrils from the crystalline cellulose, and β-1, 4-glucosidase hydrolyses cellobiose and water-soluble cellodextrin to glucose (Shewale, 1982; Woodward and Wiseman, 1983). In short, the synergism of the three enzymes, mentioned above, allows the complete hydrolysis of cellulose to glucose (Ryu and Mandels, 1980; Wood, 1989; Samdhu and Bawa, 1992) or through an intensive mineralization to H_2O and CO_2.

Cellulase due to its abundant availability and massive applicability makes it an attractive raw material for producing many industrially important commodity products such as biofuels like bioethanol, triphasic biomethanation, agricultural and plant waste management (Gomashe *et al*., 2013). Cellulases are progressively being utilized for a large variety of industrial purposes, such as in the textile industry, pulp and paper industry and food industry. It is also implies as an additive in detergents and improving digestibility of animal feeds. Presently, cellulases represent a significant offer of the world's industrial enzyme market. Apparently, the developing worries about exhaustion of unrefined petroleum (crude oil) and the discharges of greenhouse gasses

have incited the production of bioethanol from lignocellulose, especially through enzymatic hydrolysis of lignocelluloses materials (Bayer *et al.,* 2007; Himmel *et al.,* 1999; Zaldivar *et al.,* 2001). For example, the Oil palm (Elaeisguineensis) meal which is a byproduct of oil palm is acquired from palm after the oil has been extricated. The predominant constituents of oil palm meal are cellulose, hemicellulose and lignin. Oil palm feast comprises around 30% of cellulose (Yan *et al.,* 2009; Rupani *et al.,* 2010; Shahriarinour*et al.,* 2011). These items are sufficiently noteworthy to consider as the bioresources of crude materials for farming such as animal feed and compost (Gomashe *et al*., 2013).

Since, cellulose is the major component of plant biomass and potentially utilizable source of glucose, therefore different microorganisms (bacteria and fungi) are involved in decomposition of cellulose bearing compounds in soil. There are number of factors which influence the cellulose decomposing intensity. Although the temperature and different aeration condition that are involved in Cellulose decomposition, other factors such as ambient pH and the level of available N are affecting on cellulose decomposition as well. Scientists conducted research to find out that cellulose decomposition will progress more rapidly in neutral pH, abundance of N and cellulose decomposing microorganisms utilize cellulose as carbon and energy resource (Hatami, 2008; Ljungdahl *et al*., 2002).

Taking the above points in consideration, it is crucial to gain knowledge of cellulose-degrading microbial taxa, which is of significant importance with respect to nutrition, biodegradation, biotechnology, and the carbon-cycle providing insights into the metabolism, physiology and functional enzyme systems of the cellulolytic bacteria and fungi that are responsible for the largest flow of carbon in the biosphere. Therefore, the aim of the study is to isolate cellulose degrading microbes from soil samples collected from different regions and to identify cellulose degrading microbes including bacteria and fungi and so on (Gomashe *et al*., 2013). The further improvement on cellulase performance needs the better understanding of cellulose hydrolysis mechanisms as well as the relationship of cellulase molecular structure, function, and substrate characteristics. Utilization of cellulase by cellulolytic microorganisms has comprised of two systems regarded as complex and non-complex. This classification is dependent on whether the microorganism is aerobic or anaerobic. The cellulase enzyme systems

are generally classified into either secreted or cell associated enzymes belonging to different classes, which can be categorized based on their mode of action and structural properties. Although cellulases are inducible enzymes, the production of enzymes either induced or repressed only in the presence of substrate and utilizable sugars respectively (Sukumaran *et al.,* 2005; Zhang &Zhang, 2013)

Aim of the study

The aim of the present study is to isolate, identify and screen bacteria with high cellulase activity from soil samples. The present study conducted here to obtain a cellulase producer that would allow cellulase production at low cost; that can be utilized in the industries (biofuel, paper, textile, detergent, agriculture, animal feed etc; a process that would reduce environmental pollution through biodegradation and cellulose decomposing as well. In this investigation, the cellulolytic bacteria isolated from Bagan Bari Dairy Farm, Keranigonj, Bangladesh. For the characterization of the selected isolate, the basic routine laboratory works like morphological, cultural and different biochemical characteristics tests , DNS method (for assaying cellulase enzyme and reducing sugar) and crude enzyme characterization were performed.

The present study was attempted with following objectives:

- To isolate cellulolytic bacteria from different soil
- Determination of the abundance of the cellulase producers in the collected sample
- Screening for the isolate with highest cellulase activity through :
    - determination of clear zone ratio of cellulose hydrolysis on CMC agar
    - production of cellulase in submerged fermentation by determining cellulase activity
    - determination of specific activity
- Presumptive identification of the best isolate by cultural, morphological and biochemical characterization
- Optimization of different parameters for better cultivation and production process by :
    - determination of optimum pH
    - determination of optimum temperature

- Application of potential isolate in biodegradation of cellulosic material by :
  - quantitative for filter paper degradation
  - quantitative for filter paper degradation

1.2 Literature Review

1.2.1 Enzyme:

Life relies upon a very much coordinated arrangement of chemical reactions. A considerable lot of these reactions, however, continue too slowly on their own to sustain life. Thus nature has designed catalysts, which we now allude to as compounds called 'Enzyme', to greatly accelerate the rates of these chemical reactions. Basically, the life processes in essentially all life forms; from viruses to man, is facilitated by the catalytic power of enzymes (Copeland, 2000). Simply, an enzyme is a protein that functions as a biological catalyst to speed up a chemical reaction in the body. One of the most important characteristics of an enzyme that it is not used up in the chemical reaction, rather it is recycled and used over and over again. Generally, it is only needed in small amount. Hence chemically recognizes, binds and modifies substrates and is highly selective about its substrate. Enzymes are highly specific because they catalyze only one chemical reaction, having a specific substrate.

There are two classes of enzymes: intracellular and extracellular. Intracellular enzymes functions in living cells, where they catalyze and manage responses of biochemical pathways key to the presence of the living system. In the other hand, the extracellular compounds are initially characterized as proteins, which are outside to the cell well and in contact with the encompassing medium. At present, a large portion of the industrial enzymes used are extra-cellular and hydrolytic based on their important characteristics. For example, it allows simple separation and purification, fairly broad tolerance to pH in activity and stability, good long-term storage stability and diversified uses.

In terms of, manufacturing of cheeses, breads, alcoholic beverages and for the tenderizing of meats, the earliest known references to enzymes are from ancient texts. For instance, at present enzymes continue to play key roles in many food and beverage manufacturing processes and are ingredients in numerous consumer products, such as laundry detergents (which dissolve protein-based stains with the help of proteolytic enzymes). In fact, enzymes are also of

fundamental interest in the health sciences, since numerous sickness procedures can be connected to the deviant exercises of one or a few enzymes. Hence, much of modern pharmaceutical research is in light of the quest for intense and particular inhibitors of these enzymes. Nevertheless, it is the study and the action of enzymes that have subsequently fascinated researchers since the beginning of history, to fulfill erudite enthusiasm as well as utility of such knowledge for some reasonable needs of society (Copeland, 2000).

1.2.2 Cellulose

Shankar *et al.* (2011) casted the term 'cellulose', which is the most abundant biological, compound both on the terrestrial and aquatic ecosystem and is the main component of plant biomass. There has been great interest in utilizing cellulose as an energy resource and feed because it is the dominant waste material from agricultural industry in the form of stalks, stems and husk (Khianngam *et al.,*2014). Sukumaran *et al.* (2005) stated that being the most common organic polymer, cellulose representing about $1.5X10^{12}$ tons of the total annual biomass production through photosynthesis especially in the tropical zone.

Cellulose is a linear polysaccharide which consists of 8000-12000 D-glucose units that are linked through ß-1, 4-glycosidic linkages Studies shows that biosynthesis of cellulose by both plants and marine algae at a rate of $8.5x10^{11}$ tons per annum, ensuring the earth's most abundant polysaccharides as well as an inexhaustible source of renewable bio energy (Sohag *et al.,* 2013). Approximately 70% of plant biomass is locked up in 5- and 6-carbon sugars (D-xylose, D- arabinose, D- glucose, D- galactose, D-mannose) which are found in lignocellulosic biomass comprised of mainly cellulose, lesser hemicelluloses and least of all lignin (Shaikh *et al.,*2013). The cellulose net of plant tissues is not solely constructed from polysaccharide glucose. There are also some polysaccharides found as complex with pure cellulose. These polysaccharides are called as cellulosan. In general, this compound is 20-30% plant cellulosic structure (Hatami *et al., 2008).*

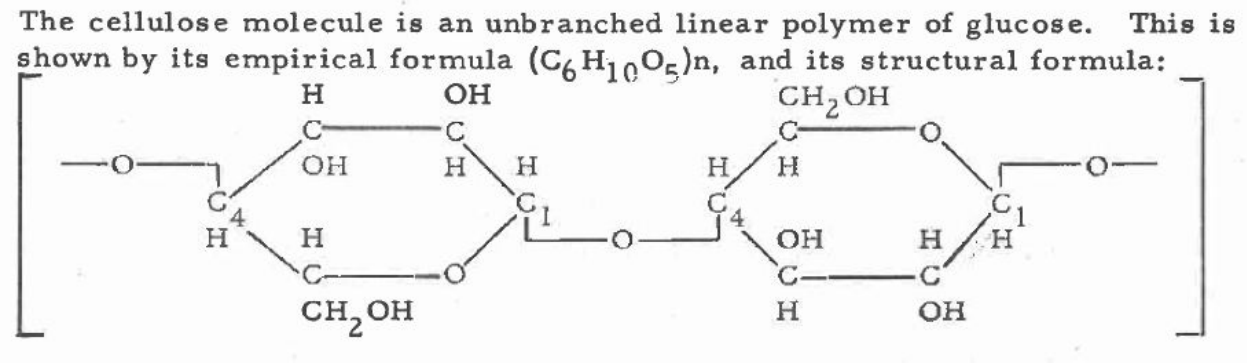


Figure 1.2: Structure of Cellulose molecules (Cowling, 1958)

Besides, cellulose, which is the major chemical component of fiber wall, contributing 40-45% of the wood's dry weight with the degree of polymerization from 10,000 in native wood to 1,000 in bleached Kraft pulps. Each D-anhydroglucopyranose unit possesses hydroxyl groups at C2, C3, and C6 positions, capable of undergoing the typical reactions known for primary and secondary alcohols. The molecular structure imparts cellulose with its characteristic properties: hydrophylicity, chirality, degradability, and broad chemical variability initiated by the high donor reactivity of hydroxyl groups (Klemm *et al.,* 2005).

1.2.3 Crystallinity of Cellulose

Generally, two types of hydrogen bonds can be seen in cellulose molecules. First of all, those that forms between the C_3OH group and the oxygen in the pyranose ring within the same molecule. And those that form between the C_6OH group of one molecule and the oxygen of the glucosidic bond of another molecule. In fact, 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 (Abedin, 2015). Cellulose has a strong tendency to form intra- and inter-molecular hydrogen bonds by the hydroxyl groups on these linear cellulose chains, which stiffen the straight chain and promote aggregation into a crystalline structure and give cellulose a multitude of partially crystalline fiber structures and morphologies (Klemm *et al.,* 2005). Cellulose presents in nature partially in pure state only at a small amount and in most cases, the cellulose fibers are embedded in a matrix of other structural biopolymers for example, hemicelluloses or lignin. The crystalline array has an important feature of relative impermeability which is like large enzyme as well as the small water molecules. There are crystalline and an amorphous region, in the polymeric structure and in addition there exits

several types of surface irregularities. This heterogeneity makes the fibers capable of swelling when partially hydrated, with the results that the micro-spores and cavities become sufficiently large enough to allow penetration of larger molecules including enzymes. If we look at the molecular level, cellulose which is a linear polymer of glucose composed of anhydoglucose units coupled to each other by ß-1, 4-glycosidic bonds. The number of glucose units in the cellulose molecules varies and degree of polymerization ranges from 250 to well over 10,000 depending on the source and treatment method. An essential element of this crystalline cluster is the relative impermeability of water. Inside the polymeric structure there are crystalline and amorphous regions; in fact, additionally there exits several types of surface irregularities. This heterogeneity makes the strands equipped for swelling when somewhat hydrated, with the outcomes that the micro-spores and cavities become sufficiently large enough to permit penetration of larger molecules including enzymes (Sukumaran *et al.,* 2005)

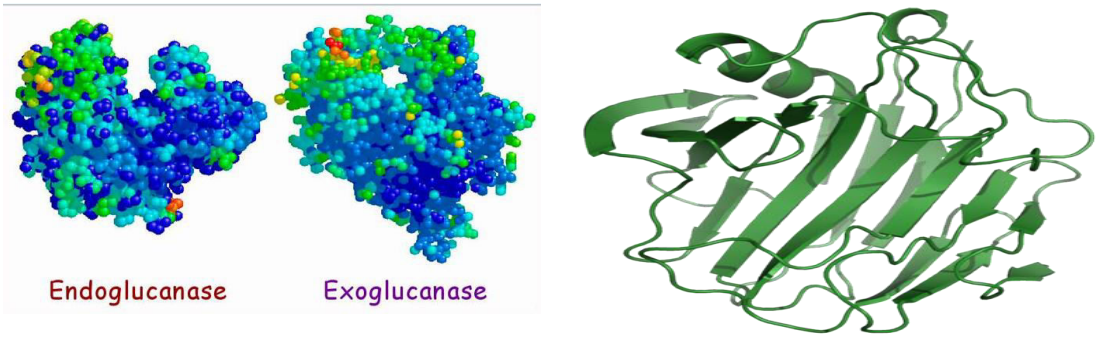
Table 1.2.1: X-Ray Crystallinity of Some Cellulose Materials (Adapted from Klemn *et al.,* 1998)

Sample	X-ray crystallinity (%)
Cotton linters	56-63
Sulfite dissolving pulp	50-56
Prehydrolyzed sulfate pulp	46
Viscose rayon	27-40
Regenerated cellulose film	40-45

1.2.4 Enzymatic degradation of cellulose

With the help of cellulolytic system, cellulose can be converted to glucose which is a multi-utility product, in a much cheaper and biologically favourable process. Cellulolysis is basically the biological process controlled and processed by the enzymes of cellulase system

(Gomashe , 2013). Complete enzymatic hydrolysis by cellulase enzyme requires the synergistic action of three enzymes, namely 1. 1,4-β-endoglucanase (EG) [EC 3.2.14] or carboxy methyl cellulose (CMCase); 2. Cellobiohydrolase (CBH) [EC 3.2.1.91] or 1,4-β-exoglucanase and ; 3. β-glucosidase (BG) [EC3.2.1.21] or (β-D-glucoside glucohydrolase or cellobiase). These enzymes act sequentially in the synergistic system and subsequently convert cellulose into an utilizable energy source and glucose and hence cellulases provide a key role in biomass utilization (Gopinath *et al.,*2012; Sukumaran *et al.,* 2005; Shaikh *et al.,*2013; Gomashe *et al.,*2013) Enzymes within these classification can be separated into individual components, such as microbial cellulase compositions may consist of one or more CBH components, one or more EG components and possibly b-glucosidases. The complete cellulase system comprising CBH, EG and BG components synergistically act to convert crystalline cellulose to glucose. The exocellobiohydrolasesand the endoglucanases act together to hydrolyze cellulose to small cellooligosaccharides. The oligosaccharides (mainly cellobiose) are subsequently hydrolyzed to glucose by a major b-glucosidase (Gopinath *et al.,* 2012).



a b

Figure 1.2.1: A typical cellulase structure of endoglucanase vs exoglucanase (a), Ribbon representation of the *Streptomyces lividans* beta-1,4-endoglucanase catalytic domain - an example from the family 12 glycoside hydrolases (b).

(Source:https://www.weizmann.ac.il/Biological_Chemistry/scientist/Bayer/images/research/Enzime/enzime2_B.jpg; https://upload.wikimedia.org/wikipedia/commons/thumb/f/fd/1NLRribbon.png/400px-1NLRribbon.png)

The conversion method of cellulose into glucose presently can be classified into two steps in the enzyme system of *Trichoderma viride*. In the first step, beta-1,4-glucanase breaks the glucosidic linkage to *cellobiose*, which is a glucose dimer with a beta-1,4 bonds as opposed to maltose, a counterpart with an alpha-1, 4 bond. And this beta-1,4glucosidic linkage is broken by beta-glycosidase (Abedin, 2015):

β-1,4 glucanase β-glucosidase

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

As Ronald *et al.* (2001) performed experiment where he found that the distinction between exoglucanases and cellobiohydrolases is not always clear due to differences in the methods. Although these three classes of enzymes have been identified in *Aspergilli*, the number of iso-enzymes produced by different species or even strains of the same species can differ. After experimentation and analysis of the production of endoglucanases by *A. terreus*isolates revealed different electrophoretic mobilities for the enzymes of the different isolates as well as indicated the absence of endoglucanase-I in a number of the isolates. Furthermore, the enzyme endoglucanases and glucosidases are also able to degrade the backbone of xyloglucan. For example an endoglucanase produced form *A. aculeatus*has been purified that is specific for the substituted xyloglucan backbone. This enzyme was not able to hydrolyze cellulose, in fact, can be utilized as a nitrogen source instead of nitrate, whereas the production of glucosidase in *A. terreus* was higher on nitrate than on ammonia.

Enzymatic degradation of cellulose by fungi is of great biological as well as economical importance. It constitutes one of the necessary steps in the balance of opposing synthetic and degradative forces in the carbon cycle, and is a major limitation to the usefulness of wood, paper, pulp, cotton, rayon, cellophane, and a host of other cellulosic materials of great and diverse utility. It is a subject of considerable practical as well as fundamental interest, particularly for plant pathologists. Some enzymes are secreted by the cells in which they formed and act extracellular; others are active within the cells that produce them and called intracellular enzymes. The degradation of cellulose by fungi involves enzymes of both types. Digestion of

cellulose takes place outside the cell by hydrolysis of its large molecules into water-soluble sugars of low molecular weight. These sugars are then taken within the cell and then transformed by intracellular enzymes to give various degradation products from which the organism derives the energy and substance needed for its growth (Ronald *et al.,* 2001).

1.2.5 Factors Affecting Cellulose Decomposition:

Cellulose is more insoluble and less available than the compound called cellulosan and microorganisms are more prefer to decompose them due to their availability leads to rapid decomposing of microbial population. The decomposition of cellulose is referred as an indispensable process naturally for carbon cycle in nature. One of the common problems after harvesting agricultural products such as grains, beans, is that a great amount of plant residues are left in each hectare. As a result, during the second farming in many agricultural systems, it's a very time consuming process to decompose the residues as well as to manage the process of soil tillage and prepare and appropriate bed for the second farming. In fact, most of soils don not consist of more than 0.5% organic matter. Hence, cellulose hydrolysis is the key process for biological conversion of cellulosic materials. However, one of the most useful and beneficial biological process in which different microorganisms such as bacteria and fungi can participate is the decomposition of cellulose bearing compound in soils. There are some factors which are responsible for affecting the number and activity of decomposing intensity. For instance, soil moisture and aeration also influence cellulose decomposition. Wide range of temperature and different aeration conditions such as aerobic, micro aerobic, facultative and obligate anaerobic microorganisms (which may be psychrophilic, thermophilic or mesophilic) are involved in cellulose decomposition. The maximum rate of decomposition has occurred by mesophilic microorganisms in aerobic condition, similarly other factors such as the ambient pH and the level of available N (nitrogen) affecting on cellulose decomposition. As Ljungdahl *et al.* (2002) placed an investigation, which was conducted to find out that cellulose decomposition progress more rapidly in neutral pH and abundance of N and showed cellulose decomposing microorganisms utilize cellulose as carbon and energy source. Therefore, to assess cellulase enzyme activity, Kluepfe performed experiment, where he mixed the agar gel with filter paper or microcrystalline cellulose called as Avicel. A clear zone was observed around the colonies after a definite time.

The clear zone diameter was related to bacterial activity in cellulose decomposition (Hatami *et al., 2008)*.

1.2.6 Cellulose Degrading Microorganism

As Gomashe *et al.* (2013) stated in his study that, it is very important to gain knowledge of cellulose-degrading microbial taxa, in fact, the noteworthy significance due to nutrition, biodegradation, biotechnology, and the carbon-cycle, providing insights into the metabolism, physiology, functional enzyme systems of the cellulolytic bacteria and fungi that are in charge of the biggest stream of carbon in the biosphere. However, one of the biggest material streams in the biosphere is the microbial cellulose utilization. Therefore, this study is to isolate cellulose degrading microorganisms from soil sample gathered from various areas and to distinguish cellulose degrading microbes including bacteria and fungi. Numerous microorganisms have been accounted for with cellulosic activities including many bacterial and fungal strains both aerobic and anaerobic. For example, *Chaetomium, FusariumMyrothecium, Trichoderma.Penicillium, Aspergillus* and so on is a part of the reported fungal species responsible for cellulosic biomass hydrolysis. Whereas, cellulolytic bacterial species including *Trichonympha, Clostridium, Actinomycetes, Bacteroides succinogenes, Butyrivibrio fibrisolvens, Ruminococcus albus, and Methanobrevibacter ruminantium* (Schwarz, 2001; Milala *et al*., 2005). Because of the massive applicability of cellulase, it has been utilized as a part of different industrial procedures, for example, biofuels like bioethanol (Ekperigin, 2007; Vaithanomsat *et al*., 2009), triphasic biomethanation (Chakraborty *et al*., 2000), chiral separation (Mswaka and Magan, 1998; Lu *et al*., 2004) and ligand binding studies (Nutt *et al*., 1998).

For the most part effective cellulase activities are seen in fungi however there is expanding enthusiasm for cellulase production by bacteria since these microbes have high growth rate when contrasted with fungi and can possibly be utilized as a part of cellulase manufacturing. In order to boost cellulase productivity, the research is going on more frequently 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 may make the procedure more economical. Moreover, bacteria and fungi have been found to create and secrete these enzymes freely in solution, as well as, a few microorganisms have additionally been found

to produce cell-bound enzymes and multi-protein complexes expressing cellulases and hemicellulases called cellulosomes (Shaikh *et al.,* 2013) .

As Maki et al. (2011) discussed that the cellulosome was first discovered in 1983, from the anaerobic, thermophilic spore-forming *Clostridium thermocellum.* Thermotolerant and/or thermophilic microorganisms are quite useful for certain industrial processes. The production of biological materials at high temperatures rather than room temperature makes it possible to reduce the risk of contamination and the cost of maintaining low growth temperatures in large-scale systems. It also increases the productivity rate. Moreover, the organisms are valuable sources of thermostable enzymes that are often stable also in solvents and detergents used in many biotechnological and industrial applications (Lasa and Berenguer, 1993; Haki and Rakshit, 2003). Cellulolytic microbes are primarily carbohydrate degraders and generally unable to use proteins or lipids as energy sources for growth. Cellulolytic microorganism such as the bacteria. *Cellulomonas, Cytophaga* and most fungi can utilize a variety of other carbohydrates in addition to cellulose. The anaerobic celluloytic species have a confined carbohydrate range, limited to cellulose and /or its hydrolytic products. Nevertheless, there are certain microorganisms, such as fungi that have the characteristic ability to secrete large amounts of extracellular protein and there are such strains which are most suited for production of higher levels of extracellular cellulases (Sukumaran *et al.,* 2005).

Table 1.2.2: 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; Trichoderma atroviride F. oxysporum; Humicola insolens; H. grisea; Melanocarpus albomyces; Penicillium brasilianum, P. decumbans; Trichoderma reesei; T. longibrachiatum; T. harzianum; Chaetomium cellulyticum; C. thermophilum; Neurospora crassa; P. fumigosum; Thermoascus aurantiacus; Mucor circinelloides; P. janthinellum; Paecilomyces inflatus; ; P. echinulatum;
	Brown rot fungi
	Coniophora puteana; Lanzites trabeum; Poria placenta; Tyromyces palustris; Fomitopsis sp.
	White rot fungi
	Phanerochaete chrysosporium; Sporotrichum thermophile; Trametes versicolor; Agaricus arvensis; Pleurotus ostreatus; Phlebia gigantea
Bacteria	**Aerobic bacteria**
	Acinetobacter junii; A. amitratus; Acidothermus cellulolyticus; Anoxybacillus sp.; Bacillus subtilis; B. pumilus; B. amyloliquefaciens; B. licheniformis; B. circulan; B. flexus; Bacteriodes sp.; Cellulomonas biazotea; Cellvibrio gilvus; Eubacterium cellulosolvens; Geobacillus sp.; Microbispora bispora; Paenibacillus curdlanolyticus; *Pseudomonas cellulosa; Salinivibrio sp.; Rhodothermus marinus*
	Anaerobic bacteria
	Acetivibrio cellulolyticus; Butyrivibrio fibrisolvens; Clostridium thermocellum; C. cellulolyticum; C. acetobutylium; C. papyrosolvens; Fibrobacter succinogenes; Ruminococcus albus
Actinomycetes	Cellulomonas fimi; C. bioazotea; C. uda; Streptomyces drozdowiczii; S. lividans; Thermomonospora fusca; T. curvata

1.2.7 Factors Affecting Cellulase Production:

Since cellulose is the major component of plant biomass and potentially utilizable source of glucose, therefore, the process of microbial degradation of cellulose can be considered as financially viable and seems to be the wise choice. Variety of culture system such as the solid or submerged culture including batch, fed batch and continuous flow process has been widely used for the commercial production of cellulases. These types of media that are used in cellulase fermentations contain either cellulose in different level of purity or raw lignocellulosic substrates which is especially true in terms of solid-state fermentation. As an inducible enzyme, cellulase production is very problematic in sense of high production cost and requirements of the appropriate inducer. Cellulase production in cultures is growth associated and is influenced by various factors and their interactions can affect cellulase productivity. Among known inducers of cellulase genes, lactose is the only economically feasible additive in industrial fermentation media. Therefore, production of cellulase on a commercial scale is induced by growing the fungus on solid cellulose of a disaccharide inducer such as lactose (Sukumaran *et al.,* 2005).A number of growth parameters such as inoculum size, pH value, and temperature, presence of inducers, medium additives, aeration, growth and time which are responsible for cellulase production. Besides presence of various metal ions which works both as activators and inhibitors are responsible for cellulase activity. Beyond free bacterial cellulases, is the open door for entire cells in bacterial co-culture and strains with multiple exploitable characteristics to reduce the time and cost of current bio-conversion processes. Glucose which is a soluble sugar produced by degradation of cellulose by cellulase degrading enzyme. Hence, isolation and characterization of cellulase producing microorganisms allows an imperative part of biofuel examination, biodegradation and bioremediation (Shaikh *et al.,* 2013).

1.2.8 Past, Present and Future Aspects of Cellulase Enzyme

It is the enormous potentiality of cellulases to convert lignocelluloses that leads a dynamic research on cellulase and related polysaccharidases, began in the early 1950s. In fact, cellulase is the most abundant and renewable source of energy on earth in terms of glucose and other soluble sugars. However, the period from 1970s-1980s, there was an extensive basic and applied research made by many scientists, as they demonstrated that the enzyme-induced bio-conversion of lignocellulose to soluble sugars was rather difficult and uneconomical (Coughlan, 1985a; Ladisch *et al.,* 1983; Mandels, 1985; Ryu and Mandels, 1980). Due to the continuous

research on cellulases, hemicellulases and pectinases their biotechnological potential in various industries like food, brewery and wine, animal feed, textile and laundry, pulp and paper, agriculture, as well as research and development was revealed (Bhat, 2000).

At present, the enzymes are commonly used due to their numerous industrial applications and the demand for more stable, highly active and specific enzymes is growing rapidly day by day. In 1995, world sale of industrial enzymes was expected to be >1.0 billion US dollars comparing to the world market for industrial enzymes in 2005, which was somewhere expected to be around 1.7 and 2.0 billion US dollars (Godfrey and West, 1996a). A recent investigation made by a publication where they claimed that the industrial enzymes have already reached a market of 1.6 billion US dollars (Demain, 2000). It is surprising that 60% of the total world's industrial enzymes are produced in Europe, and the remaining 40% from USA and Japan. About 75% of the industrial enzymes are hydrolases, therefore, carbohydrolases being the second largest group. Concerning the development of food applications such as animal feed, allow the biotechnology of cellulases and hemicellulases which began in early 1980s (Chesson, 1987; Thomke *et al*., 1980; Voragen, 1992; Voragen *et al.,* 1980). In this way, these enzymes were utilized as a part of the textile, laundry as well as in the pulp and paper industries (Godfrey and West, 1996b; Harman and Kubicek, 1998; Saddler, 1993; Uhlig, 1998). Today, these enzymes account for approximately 20% of the world enzyme market for the most part from *Trichoderma and Aspergillus* (Mantyla *et al.,* 1998; Godfrey and West, 1996b; Uhlig, 1998). Currently, several commercial enzyme producers are marketing tailor-made enzyme preparations suitable for biotechnology, and the redesigned points of interest can be found in respective company's web pages (Bhat, 2000).

For future purposes, the scientists have already started designing cellulolytic enzymes with enhanced catalytic efficiency; and improved thermostability is essential to market lignocelluloses biorefinery. Individual cellulase can be improved by utilizing either reasonable plan or coordinated advancement. However, changes in the cellulase execution have been incremental, and no extreme movement upgrade has been accounted for to date. The further changes on cellulase performance need the better comprehension of cellulose hydrolysis mechanisms and in addition the relationship of cellulase molecular structure, function, and substrate characteristics (Zhang *et al.,* 2013). Therefore, the difficulties in cellulase production

include creating reasonable bioprocesses and media for cellulase maturation, other than distinguishing proof of less expensive substrates and inducers. The genetic modification of the cellulase producers has to be improved. However, cellulase activity of the producer improved a lot to give high enzyme titers (28-30), thus, at the same time the financial aspects of cellulase production needs facilitate change for commercial manufacturing of ethanol from biomass (Sukumaran *et al.,* 2005).

1.2.9 Applications of Cellulases In various Industry

For the last several decades, the enzyme cellulase has been investigated for the bioconversion of biomass which offered a way to research, in the industrial applications of the enzyme, in animal feed, food, textiles and detergents and in the paper industry. Because of the shortage of fossil fuels as well as the arising need to find alternative source for renewable energy and fuels, there is a renewal of interest in the bioconversion of lignocellulosic biomass using cellulases and other enzymes. In alternate fields, nonetheless, the advancements and products utilizing cellulases have reached the stage where these enzymes have become irreplaceable (Sukumaran *et al.,* 2005). Therefore, the microbial cellulases find applications in various industries as described below:

1.2.9.1 Textile Industry

Since, just a decade ago after initial entry, cellulases have turned into the third largest group of enzymes used in the industry applications. Cellulases are utilized as a part of the biostoning of denim garments of clothing for producing softness and the faded look of denim garments replacing the use of pumice stones which were traditionally employed in the industry. They follow up on the cellulose fiber to discharge the indigo color utilized for shading the fabric, producing the faded look of denim. Cellulase obtained from *H. insolens,* is most commonly employed in the bio-stoning. However, utilization of acidic cellulase from *Trichoderma* alongside proteases is found to be similarly good. Cellulases are used for processing off the small fiber ends projecting from the fabric bringing about a superior finish. Cellulases have additionally been utilized as a part of softening, defibrillation, and in processes for giving confined variation in the color density of fibers (Sukumaran *et al.,* 2005).

The bio-polishing is typically completed amid the wet handling stages which include desizing, scouring, blanching, coloring, and wrapping up, acidic cellulases improving softness and water

absorbing property of fibers, strongly reducing the tendency for pill formation, and provide a cleaner surface structure with less fuzz. Cellulase arrangements rich in endoglucanases are most appropriate for biopolishing improving fabric look, feel, and shading without the need of any substance to cover the fibers. The activity of cellulases expels short filaments, surface fluffiness, makes a smooth and reflexive appearance, enhances glossy shading and brilliance appearance, hydrophilicity and dampness absorbance in an environment friendly procedure. In general, the use of cellulase additionally helps in softening the pieces of clothing and in removal of dirt particles trapped within the microfibril network (Kuhad *et al.,* 2011).

1.2.9.2Laundry and Detergents Industry

Cellulases, specifically EGIII and CBH I, are commonly utilized as a part of cleansers for cleaning materials. A few reports reveal that EG III variations, specifically from *T. reesei*, are reasonable for the utilization in detergents. There are other organisms like *T. viride*, *T.harzianum* and *A. niger* that are also used industrially as natural sources of cellulases. The cellulases, mainly from species of Humicola (*H. insolens* ,*H. grisea, Var. thermoidea*) that are active under mild alkaline conditions and at hoisted temperatures are commonly added in washing powders, and in detergents as well (Sukumaran *et al.,* 2005). Nowadays, for the improvement of the stability of cellulases various mixture of liquid laundry detergent has used containing anionic or nonionic surfactant, citric acid or a water-soluble salt, protease, cellulose, and a mixture of propanediol and boric acid or its derivative as well. Hence, cellulases are applied to cellulase-based detergents, shows superior cleaning action without damaging fibers, improved color brightness and removes dirt, removes of rough protuberances in cotton fabrics and anti-redeposition of ink particles (Kuhad *et al.,* 2011).

1.2.9.3 Food and Animal Feed

There is an extensive variety of potential applications of cellulase in food biotechnology. For the production of fruit and vegetable juices, it is necessary to follow some improved methods including extraction, clarification, and stabilization. Cellulases likewise have a critical application as part of macerating chemicals complex (cellulases, xylanases, and pectinases) utilized for extraction and clarification of fruit and vegetable juices to increase the yield of juices. However, use of macerating compounds increases both yield and process execution without extra capital venture, in fact, it improves cloud stability ,texture and decreases viscosity of the nectars and purees from tropical fruits, for example, mango, peach, papaya, plum, apricot,

and pear. Many properties of fruits and vegetables such as the texture, flavor, and aroma can be improved by reducing excessive bitterness of citrus fruits by infusing pectinases and β-glucosidases enzymes. There are some enzyme mixtures containing pectinases, cellulases, and hemicellulases that can be used for improved extraction of olive oil (Kuhad *et al.,* 2011).In carotenoid extraction, cellulases can also be used as a coloring agent. In order to improve the nutritive quality of forages, a mixture of cellulase containing enzymes including hemicellulase and pectinase are usually used. For the betterment in food absorbability and animal feed, are accounted for the utilization of cellulases in food processing. Bedford *et al.* (2003) claimed that the use of *Trichoderma* cellulases in feed additive can improve the feed conversion ratio and/or increase the digestibility of a cereal based feed (Sukumaran *et al.,* 2005). The pretreatment of agricultural silage and grain feed by cellulases or xylanases can improve its nutritional value, the enzymes can also eliminate anti-nutritional factors that are present in the feed grains. Besides, it can degrade certain feed constituents to improve the nutritional value, as well as provide supplementary digestive enzymes such as proteases, amylases, and glucanases. For instance, the dietary fiber consists of non-starch polysaccharides such as arabinoxylans, cellulose, and many other plant components including resistant dextrins, inulin, lignin, waxes, chitins, pectins, *β*-glucan, and oligosaccharides, which can act as anti-nutritional factor for several animals such as swine. In this case, the cellulases effectively hydrolyse the anti-nutritional factor, cellulose in the feed materials easily absorb into ingredient and also improves animal health and performance (Kuhad *et al.,* 2011).

1.2.9.4 Pulp and Paper Industry

Since the last decade, the application of cellulases has increased significantly due to the interest in the pulp and paper industry. The mechanical pulping procedures, for example, refining and grinding of the woody crude material lead to pulps with high substance of fines, mass, stiffness etc. Interestingly, biomechanical pulping utilizing cellulases came about as a part of sustainable energy saving funds (20–40%) amid refining and upgrades close by sheet quality properties. Mixtures of cellulases for example endoglucanases (I & II) and hemicellulases have additionally been utilized for bio-modification of fiber properties with the point of de-inking of recycled fibers, hand sheet strength properties as well as enhancing drainage and run-ability in the paper mills before or subsequent to beating of pulp (Kuhad *et al.,* 2011). In terms of removing of inks, coating and toners from paper, cellulases are often largely employed.

Microbial cellulases are also employed in bio characterization of pulp fibers which is another application of pulp and paper industry. Moreover, cellulases are not only utilized as a part of easily biodegradable cardboard but also employed in the manufacture of soft paper including paper towels and sanitary paper. Therefore, preparations containing cellulases are used to remove cling paper (Sukumaran *et al.,* 2005).

1.2.9.5 Bioethanol Industry

For biofuel production, enzymatic saccharification of lignocellulosicmaterials, for example, sugarcane bagasse, corncob, rice straw, *Prosopisjuliflora*, *Lantana camara*, switch grass, saw tidy, and backwoods deposits by cellulases, are perhaps the most prevalent application currently being explored. Bioconversion of lignocellulosic into helpful and higher quality products typically requires multistep processes including pretreatment (mechanical, chemical, biological), hydrolysis of the polymers to produce readily metabolizable molecules (e.g., hexose and pentose sugars) and so on. The utility expense of enzymatic hydrolysis might be low contrasted with acidic or alkaline hydrolysis since enzyme hydrolysis is generally occurs at mild conditions (pH 4–6 and temperature 45–50◦C) and does not have erosion issues. There are several technologies currently available for all steps in the bioconversion of lignocellulosics to ethanol, like other chemical products. But, some of these technologies must be improved to produce renewable biofuel and other byproducts at prices, which competes with more conventional production systems. Moreover, two aspects are widely addressed in order to reduce the enzyme cost in the production of bioethanol from lignocellulosic biomass. These are the: optimization of the cellulase production and development of a more efficient cellulase-based catalysis system. Protein engineering and directed evolution are powerful tools that can facilitate the development of more efficient thermophiliccellulases. There are some strategies that applied for recycling and reusage of the enzymes that may also reduce enzymatic hydrolysis costs. Enzymes recovery can be largely influenced by adsorption of the enzymes onto the substrate, particularly to lignin and enzyme inactivation. There are a few reports where the nonspecific and irreversible adsorption of cellulase to lignin has been observed (Kuhad *et al.,* 2011).

1.2.9.6 Wine and Brewery Industry

In fermentation processes microbial glucanases and related polysaccharides play an important role in terms of producing alcoholic beverages including beers

and wines. In fact, enzymes such as pectinases, glucanases, and hemicellulases play a significant role in wine production by improving color extraction, skin maceration, clarification, filtration, and finally the quality and stability of wine. β-Glucosidases can enhance the fragrance of wines by adjusting glycosylated precursors. On the other hand, macerating enzymes also improve pressability, settling, and juice yields of grapes used for wine fermentation. As a matter of fact number of commercial enzyme preparations is now available to the wine industry. Various business catalyst arrangements are currently accessible to the wine business. The principle advantages of utilizing these chemicals amid wine making incorporate better maceration, enhanced shading extraction, simple illumination, simple filtration, enhanced wine quality, and enhanced steadiness. Lager fermenting depends on the activity of enzyme while malting and maturation. Malting of grain depends on seed germination, which starts the biosynthesis and activation of α-and β-amylases, carboxypeptidase, and β- glucanase that hydrolyze the seed reserves. Oksanen, *et al.* (1985) in one of his experiment observed that endoglucanase II as well as exoglucanase II of the *Trichoderma*cellulase system were in charge of a most extreme reduction in the level of polymerization and worth thickness. Galante *et al.* (1998) conducted a research, where he evaluated the performance of Cytolase 219 (mixture of cellulase, pectinase, and xylanase) by utilizing three kinds of white grapes in wine making. The results showed that there would be a 10–35% increase in the extraction of the principal wine, a 70–80% increase in the filtration rate must, 50–120 minutes decrease in squeezing time, 30–70% decrease in viscosity, 20–40% sparing of energy during cooling of fermenter, and a significant improvement in wine stability were found (Kuhad *et al.,* 2011).

1.2.9.7 Agricultural Industries

Different enzyme arrangements comprising of various mixes of cellulases, hemicellulases and pectinases have potential applications in agriculture for upgrading development of products and controlling plant infections. Plant or parasitic protoplasts created utilizing microbial hydrolases can be utilized to deliver mixture strains with desirable properties.

Enzymes for example cellulases and related compounds which have been produced from specific fungi are capable of degrading the cell wall of plant pathogens in controlling the plant disease such as the fungal β-glucanases. In addition, numerous cellulolytic fungi including *Trichoderma sp., Geocladium sp.,Chaetomium sp., and Penicillium sp*. are known to play a key

role in agriculture by encouraging upgraded seed germination, rapid plant growth and blooming, improved root system and expanded product yields. In spite of the fact that these fungi have both direct (presumably through growth promoting diffusible factors) and indirect (by controlling the plant illness and pathogens) impacts on plants. The utilizing of traditionally straw that added an important strategy to improve soil quality and reduce dependence on mineral fertilizers. There have been many studies attempted in order to rapid straw decomposition by microbial routes. Since, promising results have been with the application of cellulolytic fungi such as *Aspergillus, Chaetomium, and Trichoderm, and actinomycetes*. Fontaine et al. (2004) demonstrated that exogenous cellulase supplementation quickened deterioration of cellulose in soil. Subsequently, utilizing exogenous cellulase might be a potential intends to quicken straw decomposition and expansion soil fertility (Kuhad *et al.,* 2011).

1.2.10 Production of Cellulase in Bangladesh

In six major districts of Banladesh namely; Dhaka,Chittagog, Rajshahi, Khulna , Barishal and Sylhet there is about 7,690 tons of municipal solid wastes(MSW) per day. Due to the over growth of population and urbanization, it is expected that the MSW might be increased up to 47,000 tons by the year of 2025. Most of the constituents of the MSW are organic and it's about 75-85%, in fact, approximately 80% of this organic content is cellulosic which can be used as crude materials for cellulase production. At present, MSW in Bangladesh are for the most part gathered and dumped in swamps. Therefore, disposed haphazardly that caused environmental pollution and public health hazards. Methane gas which is emitted from the decaying MSW and considered as the second dominating greenhouse gas have 20times more impact on climate change when compare to the carbon dioxide (CO_2). Therefore, it has now become crucial to utilize the cellulosic biomass of MSW. Bacteria which can utilize cellulosic MSW as the raw material are able to produce the cellulase enzyme. With this view, most of the cellulase producing bacteria were isolated and screened from MSW and cow dung which is available all over the country (Shohag *et al.,* 2013).

Chapter 2

2.0 Materials and Methods

2.1 Working place:

This experiment was conducted in the Microbiology laboratory of the Department of Mathematics and Natural Sciences, BRAC University.

2.2 Solutions and reagents:

All the necessary solutions and reagents were available in the laboratory and in the media room of the department. Therefore, they were used without further purification as these were of reagent grade. The list of the chemicals is given in the appendix-I.

2.3 Media

Nutrient agar (NA) was used for the initial isolation of bacteria and short term preservation. While, Carboxymethylcellulose (CMC) agar was used for primary screening (sub culturing) of pure cultures of bacterial isolates from NA plate.

2.4 Soil sample collection

In order to carry out the experiment, soil sample was collected from Bagan Bari Dairy Farm, Kalatia post office, Aksail village, Keranigonj, Bangladesh. The samples were collected in sterile container and stored at 4ºC until use.

2.5 Isolation of cellulolytic bacteria:

Initially the sample was spread on NA plate. For this 1g of collected soil samples were taken and added to 10 mL of sterile distilled water. After that, serial dilution was carried out in sterile saline. Then, 0.1ml (100 µl) of soil suspension of appropriate dilution was spread on NA agar plates and incubated at 37◦C for 24 hours. The colonies were preserved at 4°C for further identification and screening for cellulase production.

2.6 Primary screening of cellulolytic bacteria:

Pure cultures of bacterial isolates were transferred onto CMC agar plates by needle inoculation and incubated for 48 hours. The CMC agar 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 % $(NH4)_2SO_4$ and 0.2% gelatin. Then CMC agar plates were flooded with 1% Gram's iodine for 3-5 mins at room temperature to see the cellulolytic activity of isolated strain. Appearance of clear zone around the bacterial colonies indicated cellulose hydrolysis. However, the bacterial colonies having the largest clear zone were selected for identification and cellulase production in submerged system.

$$\text{Clear zone ratio} = \frac{\text{Clear zone diameter}}{\text{Colony diameter}}$$

2.7 Secondary screening and production of cellulase enzyme:

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

2.7.1 Inoculum development

Pure cultures of selected bacterial isolates were individually maintained on CMC supplemented minimal agar plates at 4˚C, until used. The isolates that showed a maximum zone of hydrolysis were inoculated in broth medium (5ml each) containing 0.03 % $MgSO_4$, 0.2 % K_2HPO_4, 1 % glucose, 0.25% $(NH4)_2SO_4$ and 1 % peptone at pH 7 for 24 hours of fermentation period, at 37 ◦C. After 24h of fermentation period these vegetative cells were utilized as inoculum source.

2.7.2 Cellulase enzyme production (submerged fermentation process):

Newly isolated bacteria were screened for cellulase enzyme production in submerged fermentation process. Fermentation medium (50 ml) was prepared using 1% CMC (as cellulose substrate), 0.2 % K_2HPO_4, 0.03 % MgSO4, 1 % peptone, 0.25 % $(NH4)_2SO4$, 1% gelatin and autoclaved at 121°C for 15min. After sterilization, the medium was allowed to cool at room temperature. The medium was inoculated with 2.5 ml of selected bacterial isolates and incubated at 37◦C on rotary shaker at 150 rpm for 48hours.

2.7.3 Collection of crude enzyme:

After termination of the fermentation period the fermented broth was centrifuged at 6,000 rpm for 10 min at 4˚C to remove the unwanted material. The clear supernatant thus obtained after centrifugation served as crude enzyme source.

2.7.4 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. Crude enzyme was added to 1.0 ml of 1 % CMC (used as substrate) in 0.05 M phosphate buffer (pH 4.8) and incubated in water bath at 50˚C for 30 min. After incubation, reaction was stopped by the addition of 1.5ml of DNS reagent , boiled at 100˚C in water bath for 10 min and were cooled under running tap water. Sugars liberated were determined by measuring absorbance at 540 nm. Cellulase production was estimated by using glucose calibration curve. Using the blank as reference, absorbance was taken and a graph was plotted for values of glucose concentration against corresponding absorbance. 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 (Islam *et al.,* 2014):

$$\text{Enzyme (cellulase) activity} = \frac{\text{Product concentration X 1000 X Dilution factor}}{\text{Molecular weight of glucose X Incubation time}}$$

2.8 Culture preservation

20% glycerol broth was used for preservation purpose. One loop-full of bacterial inoculum was transferred to 5 ml nutrient broth medium and incubated at 37°C for 24 hours. Then, 800 µl of bacterial suspension was mixed with 200 µl autoclaved glycerol. In 1.5ml centrifuge tubes and sealed with parafilm tapes. These were stored in -20◦C refrigerator.

2.9 Determination of extracellular protein concentration and specific activity

Different dilutions of BSA solutions from 0.2 -10 were prepared by mixing stock BSA solution (1 mg/ ml) and water in the test tube. The final volume in each of the test tubes was 5 ml and the BSA reagent was 0.05 to 1 mg/ ml. From these different dilutions, pipette out 0.2 ml protein solution to different test tubes and 2 ml of alkaline copper sulphate reagent (analytical reagent) was added and mixed well. This solution was incubated at room temperature for 10 mins and then 0.2 ml of reagent Folin Ciocalteau solution (reagent solutions) was added to each tube and incubates for 30 min. The colorimeter was set to zero with blank and the optical density (measure the absorbance) at 660 nm was taken. The absorbance against protein concentration was plotted to get a standard calibration curve.

Specific activity was calculated using the formula:

$$\text{Specific activity (Umg}^{-1}\text{)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Extracellular protein concentration (mg/ml)}}$$

2.10 Presumptive Identification of cellulase producing bacteria:

The best isolate was preliminary identified by means of morphological, cultural and biochemical characterization.

2.10.1 Gram's staining:

For morphological characterization colonies were stained by Gram's staining technique. A smear of cell sample was air dried and heat fixed by carefully passing the slide through a Bunsen burner three times. Then, the slide was flooded with the primary stain (crystal violet) for 1 minute and rinsed with running tap water to remove unbound crystal violet. The slide was then flooded with Gram's iodine for 30 seconds, 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 45 seconds, rinsed with running tap water and allowed to air dry. The dry slide was covered with immersion oil and viewed under a microscope.

2.10.2 Spore Staining

The smear of bacterial sample to be tested was air dired and heat fixed. The slide was placed over a water bath and malachite green was added continuously so that the dye did not dry out. The slide was heated for 2 to 3 minutes. After heating, the slide was cooled and rinsed thoroughly with tap water. Then the smear was stained with safranin for 30 seconds, washed with tap water and blot dried with bibulous paper. Finally, bacterial observation was made under the oil immersion lens (1000X) for the presence of endospores.

2.10.3 Cultural characterization:

The bacterial isolate was streaked on NA plate and incubated at 37◦C for 24 hours. After incubation colony characteristics were noted by observing the colony diameter, colour, shape, size, elevation, border, nature of colony and pigmentation.

2.10.4 Biochemical characterization:

The isolate was characterized through the following biochemical tests:

Table 2.1: LIST OF BIOCHEMICAL TEST DONE

1.Catalase test	**2. Oxidase test**
3. Indole test	4. Methyl red test
5. Vogas-proskuras test.	6. Cimmon citrate test
7. Casein hydrolysis test	8. Nitrate reduction test
9. Starch hydrolysis test	10. MIU test (motility indole urease)
11. Triple sugar iron (TSI test)	12. Urease test
13. Growth at 45◦C	14. Growth at 65◦C
15..Growth in 7% NaCl 17.Carbohydrate Utilization Test ✓ Fructose test ✓ Sucrose test ✓ Maltose test ✓ Glycerol ✓ D-xylose	16.Gelatin hydrolysis test 18. Lipid hydrolysis 19. MSA test 20 .Blood agar test 21.Lecithinase test

2.10.4.1 Catalase Test

A sterile microscopic slide was placed on a petri deish and a small amount of organism picked using a sterile inoculating loop. Then 1 drop of 3% H_2O_2 was placed on the organism on the microscopic slide by using a dropper. Finally, it was observed for the presence or absence of bubble formation for interpreting the results.

2.10.4.2 Oxidase Test

This test was carried out by taking a filter paper soaked with a substrate Kovacs oxidase reagent. The paper was moistened with distilled water. The colony to be tested was picked with a loop and smeared in the filter paper. The result was observed by the presence or absence of color changes.

2.10.4.3 Indole test

The tryptophan broth was inoculated with bacterial culture and incubated at 37°C for 24 hours in ambient air. Then 0.5 ml of Kovac‘s reagent was added to the broth culture. Finally, it was observed for color changes to determine whether the result is positive (cheery red ring) or negative (yellow).

2.10.4.4 Methyl red test (MR TEST)

Bacterial culture are inoculated in 3.5ml aliquot of the broth in a clean test tube and incubated overnight at a temperature of 37°C for 24 hours. The day after, 5 drops of the pH indicator methyl red was added and the medium was observed for the immediate development of color.

2.10.4.5 Voges-Proskaure (VP) Test

After 24 hours incubation of pure bacterial culture in VP broth a 3.5 ml of broth was dispensed to a clean test tube clean test tube. Then 0.6ml (12 drops) of Barrit’s reagent A and 0.2ml (4drops) of Barrit’s reagent B was added and gently shake the medium to atmospheric oxygen. The tube was then allowed to remain undistributed for 10-15mins. Finally, the medium observed for color changes to determine whether the result is positive (pink-red) or negative (yellow).

2.10.4.6 Cimmon citrate test

When microorganisms utilize citrate, they remove the acid from the medium, which raises the pH and turns the pH indicator from green to blue. A single colony was picked from the pertri dish and streaked on the slant of Simmons citrate agar using a needle. . The screw caps loosen for oxygen entry and incubated at 37°C for 48 hours. Finally, the medium observed for color changes to determine whether the result is positive (prussian) or negative (forest green).

2.10.4.7 Casein hydrolysis test

These results in a clear zone and soluble peptides can then be absorbed on skim milk agar. After Skim milk agar preparation, the pure bacterial culture was inoculated on the plate either as a straight line or a zig-zag and then incubated at 37°C for 24hours. The plate was observed for the appearance of clear zone.

2.10.4.8 Nitrate reduction test

Nitrate broth was added to test tubes and durham tubes were placed in an inverted manner. Fresh bacterial cultural was inoculated and incubated at 37°C for 24 to 48 hours. 1 drop of each Sulfanilic acid and alpha napthylamine was addedafter the incubation period. If no color change was observed nitrite's absence was proven. No zinc powder was added as it is hazardous.

2.10.4.9 Starch hydrolysis test

This biochemical test was carried out by preparing a starch agar media and autoclaved at 37 degrees Celsius for 24 hours. The next step was to inoculate the bacterial sample, this being done by streaking the straight line. After that it was incubated for 24 hours at 37°C. The last step was to add iodine. If a clear zone was formed then the result would turn out positive. If not then it would be negative.

2.10.4.10 Motility Indole Urease (MIU) test

This test was carried out by adding 6ml urea-glucose solution in a sterile test tube and then isolated bacterial colony was inoculated by stabbing into the test tube. Later on, an incubation period of 24 hours at 37°C was carried out. A positive motility test is indicated by a diffuse cloud of growth away from the line of inoculation. A red ring on addition of Kovac's reagent would indicate indole positive while no ring for negative test. The entire medium would turn reddish urea hydrolysis observed.

2.10.4.11 Triple sugar iron (TSI test)

To conduct the test, a straight inoculating needle was used to pick an isolated colony and inoculated the TSI slant by first stabbing the butt down to the bottom, and then streaking the surface of the slant. The results were observed after 24 hours of incubation at 37°C

2.10.4.12 Growth at 45◦C

Nutrient agar was prepared, it was streak plated on the agar media and incubated for 24 hours at 45°C and observed for the presence or absence of growth.

2.10.4.13 Growth at 65◦C

Nutrient agar was prepared, it was streak plated on the agar media and incubated for 24 hours at 65°C and observed for the presence or absence of growth.

2.10.4.14 Growth in 7% NaCl

7% NaCl was incorporated in NA and after being autoclaved at standard conditions; it was inoculated and incubated for 24 hours at 37 °C.

2.10.4.15 Carbohydrate Utilization Test

The lactose, glycerol, maltose, D-xylose and the sucrose broths were prepared by autoclaved at 15 psi for 121°C for 15 minutes. In all of these tests, the bacteria were inoculated to the medium using a transfer loop a, tubes were not shaken in order to avoid bubble of air formation inside the tubes and incubated at 37 °C for 24 hours . The media were observed for color change and gas production.

2.10.4.16 Gelatin Hydrolysis Test

In this test, heavy inoculums of an 18-24 hour old bacterial culture was stabbed into tubes containing nutrient gelatin (3ml) and incubated at 37 °C for 48 hours for gelatin liquefaction. To confirm that gelatin liquefaction activity, the tubes were immersed in an ice bath for 15-30 minutes and the result was observed.

2.10.4.17 Manitol Salt agar (MSA) test

This test was carried out by streaking a plate of manitol salt agar with appropriate bacterial culture obtained from isolated colonies and later on after incubation the colonies were observed.

2.10.4.18 Blood Agar Test

For this test, the blood agar (suing ship blood) was prepared following by the laboratory protocol and then sterilized by autoclaving at 121oC for 15 minutes. The plates were inoculated

with bacterial culture and incubated in a carbon dioxide enriched atmosphere at 35-37 o C overnight. The growth characteristics of colonies were checked for result.

2.10.4.19 Lecithinase Test

After preparing the egg yolk media it was placed under anaerobic conditions for 18-24 hours. Then immediately following inoculation, the medium was placed in an inverted position (agar side up), in an anaerobic atmosphere and incubated at 37ºC for 48 hours. After incubation, the plate was observed for the appearance of lecithinase.

2.11 Characterization of the crude cellulase of newly isolated cellulolytic strain:

2.11.1 Effect of pH on activity of crude cellulase

The optimum pH for the crude enzyme was determined by incubating crude enzyme with substrate (1% CMC) prepared in appropriate buffers; 0.05 M citrate buffer (pH 3.0 to 6.0), 0.05 M sodium phosphate buffer (pH 6.0 to 8.0), 0.05 M Tris-HCl (pH 8.0 to 9.0) and 0.05 M glycine-NaOH (pH 9.0 to 10.0). Crude enzyme (5fold dilution) mixture was added to those buffers and incubated for 30 min at 50˚C. Cellulase activity was assayed by DNS method as previously described above. The optical density (OD) was observed absorbance at 540nm in a spectrophotometer. The control was prepared by adding 1% CMC (substrate) in the 0.05 M of 1ml citrate buffer (pH 5.0) before mixing the distilled water (dH_2O) and O.D was observed by DNS method.

2.11.2 Effect of temperature on activity of crude cellulases

The effect of temperature on activity of cellulase was determined by incubating crude enzyme (5fold dilution)) with 1 %CMC in 0.05 M phosphate buffer (pH 6.5) at temperatures including 25°C,35°C,,45°C,55°C,65°C,75°C and 85°C. Cellulase activity was assayed by DNS method as described above. The optical density (OD) was measured at 540 nm in spectrophotometer and the analysis was made in later part of the results and discussion. The control was prepared by adding 1% CMC (substrate) in the 0.05 M citrate buffer (1ml) (pH 5.0) before mixing the distilled water (dH_2O) and DNS.

2.12 Filter paper and Cotton degradation:

The best isolate was selected and then inoculated in nutrient broth at 37ºC for 24 hours. For carrying out this experiment, a sterile mineral buffered solution of pH 7.5 was prepared and individually supplemented with filter paper strips (0.15g) of 1 cm x 2 cm and cotton (0.12g) as a sole source of carbon. After that, the medium was supplemented with two drops of 10mM (0.01 M) glucose to possibly induce cellulase production (Maki *et al*., 2011). The buffered solution with filter paper and cotton supplement was inoculated. Finally, the culture was incubated for maximum 6 days at 50 °C in shaking incubator at 120rpm and observed daily for visual conformation of degradation (Shaikh *et al.,* 2013).

Chapter 3

3.0 Results

3.1 Isolation and primary screening for cellulase producing bacteria:

The study was conducted using the soil sample collected from Bagan Bari Dairy Farm, Kalatia post office, Aksail village, Keranigonj, Bangladesh. Total 68 isolates were obtained after spreading 10^{-5} dilution plate. All of these 68 isolates were selected for the calculated ratios of zone of hydrolysis to the colony diameter on CMC agar. Among them 31 colonies were able to produce cellulase while 32 colonies were unable to produce cellulase. Besides, there were 5 colonies that showed Clear zone throughout the media. The percentage of these types of bacteria is given in the following pie chart (figure 3.1)

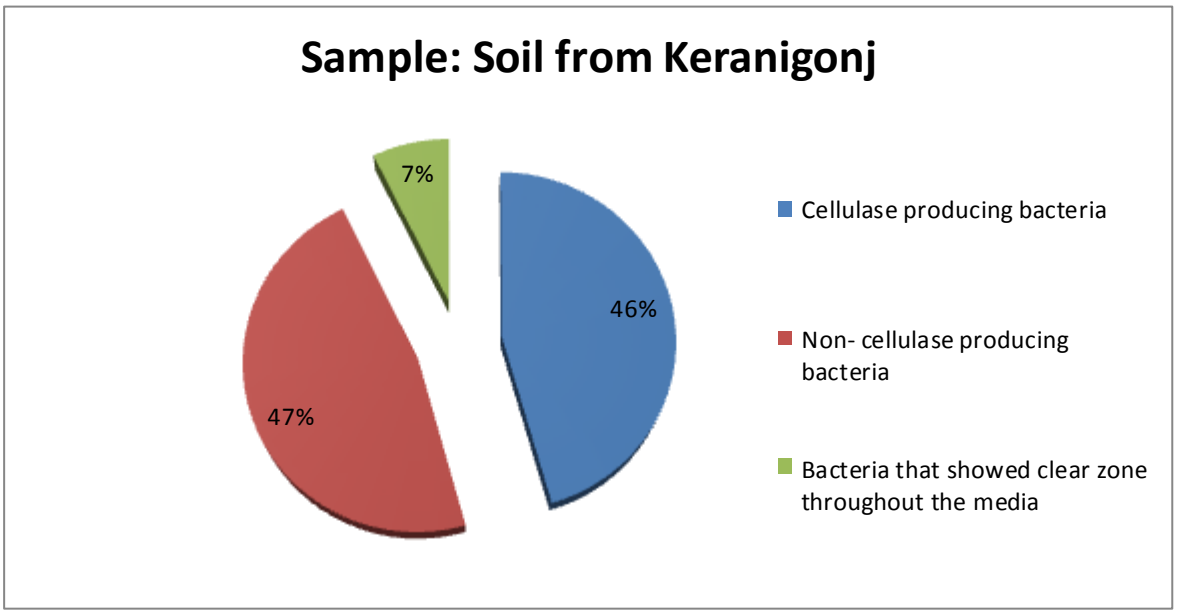


Figure 3.1: Percentage of the types of bacteria in the soil

The percentage of cellulase producers was found to be 46% while non-cellulase producers were 47%. And 7% of the total isolates showed clear zone throughout the media. This result indicates the soil to be good source of cellulolytic bacteria.

The experiments were carried out three times and the average of the ratio is shown in Table 3.1.

Table 3.1: Zone of hydrolysis of 68 different isolates

Isolate Numbers	Average clear zone ratio of zone	Isolate Numbers	Average clear zone ratio of zone
1	1.32	58	No hydrolysis
2	2.00	21	No hydrolysis
3	2.20	22	No hydrolysis
4	No hydrolysis	23	No hydrolysis
5	3.27	24	1.43
6	1.07	25	No hydrolysis
7	1.41	26	3.78
8	No hydrolysis	27	1.84
9	No hydrolysis	28	1.33
10	No hydrolysis	29	1.91
11	No hydrolysis	30	Clear zone throughout the media
12	No hydrolysis	31	1.80
14	No hydrolysis	32	2.96
15	3.80	33	3.20
16	No hydrolysis	34	Clear zone throughout the media
17	No hydrolysis	35	Clear zone throughout the media
18	No hydrolysis	36	No hydrolysis
19	2.01	37	2.50
40	1.00	38	2.30
41	No hydrolysis	59	No hydrolysis
42	No hydrolysis	60	No hydrolysis
43	1.57	61	No hydrolysis
44	2.00	62	1.95
45	Clear zone throughout the media	63	2.50
46	No hydrolysis	64	Clear zone throughout the media
47	4.87	65	1.94
48	2.29	66	3.85
49	1.74	67	3.00
50	No hydrolysis	68	1.13
51	No hydrolysis		
52	No hydrolysis		
53	No hydrolysis		
54	No hydrolysis		
55	No hydrolysis		
56	No hydrolysis		
57	No hydrolysis		

The 31 isolates were capable of producing cellulase and their clear zone ratio is presented in bar diagram (Figure 3.1.1). The best of them were then selected to carry out secondary screening.

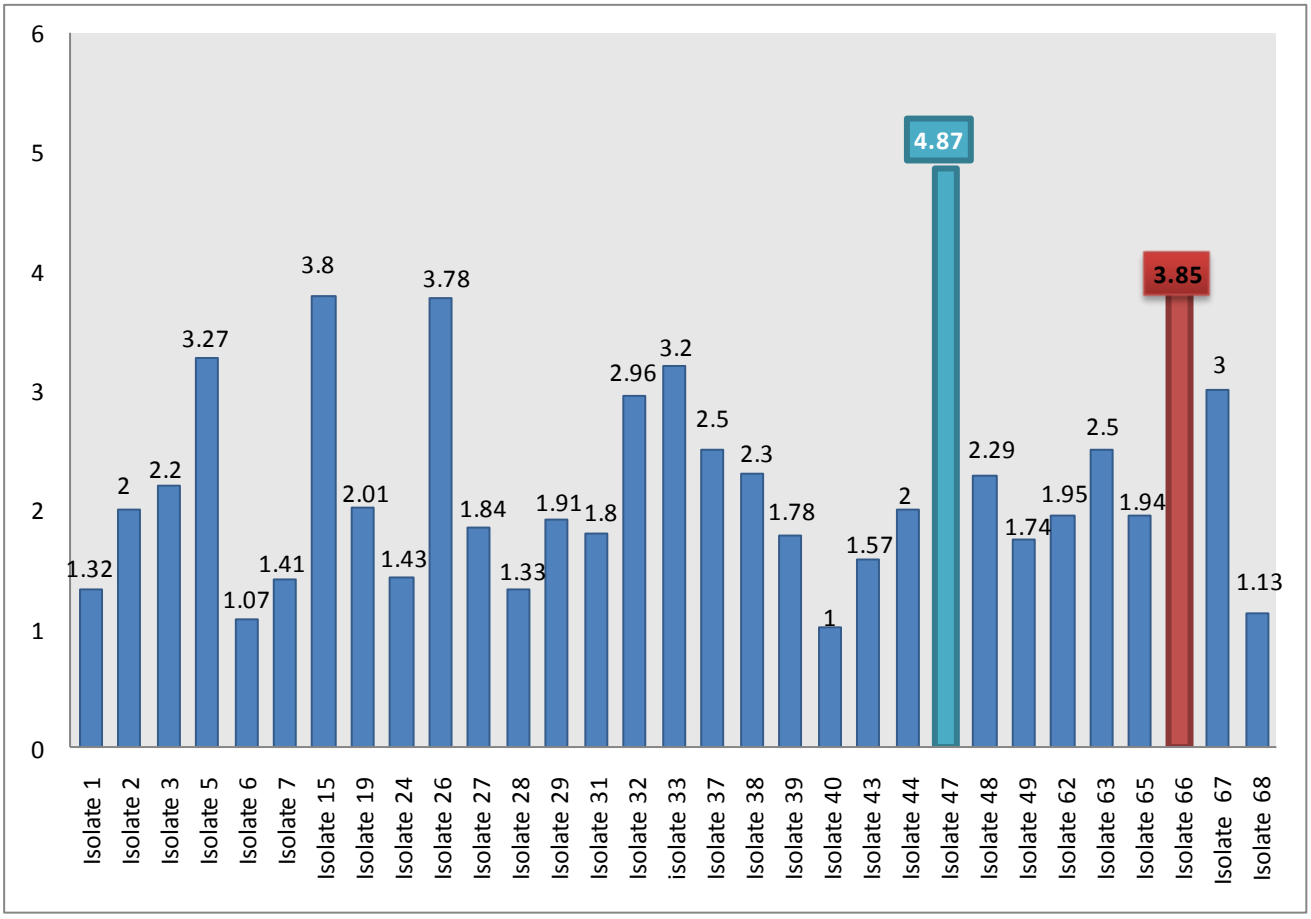


Figure 3.1.1: Ratio of clear zone diameter to colony diameter of total 31 isolates.

Among them two isolates with maximum clear zone ratios on CMC agar were selected- isolate 47 and 66 shown is figure 3.1.1. Isolates 47 and 66 were better cellulase producer and were then analyzed through secondary screening.

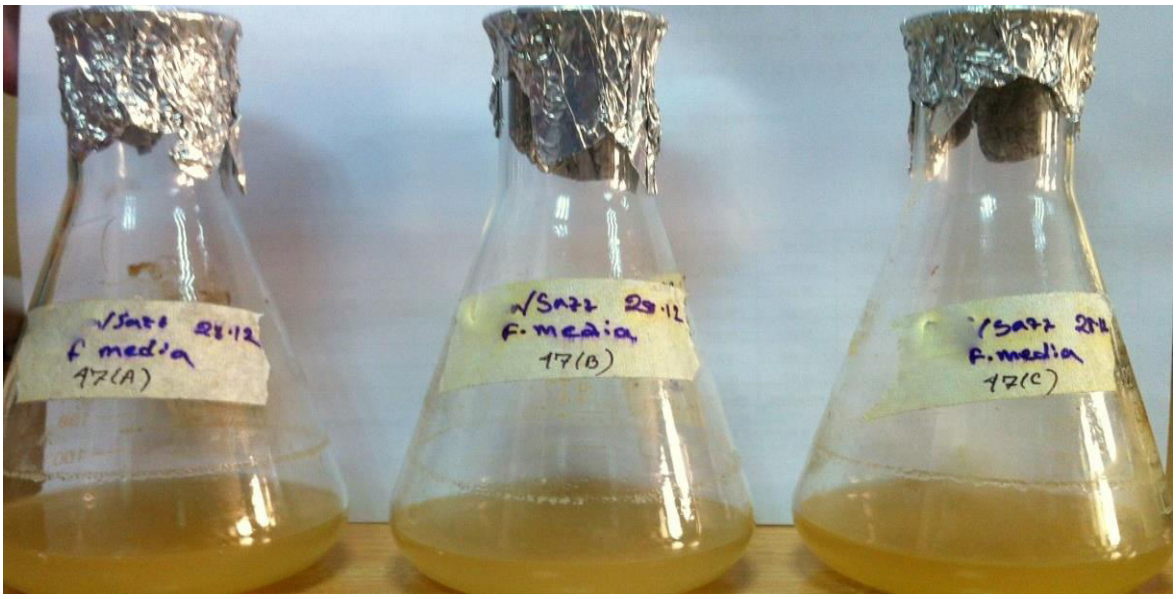
a **b**

Figure 3.1.2: Isolate 47 (a) and isolate 66 (b) showing clear zone of cellulose hydrolysis on CMC following addition of Grams's Iodine.

3.2 Secondary screening and production of cellulase enzyme

On the basis of primary screening the potential isolates 47 and 66 were then evaluated for their enzyme productivity in submerge fermentation process as shown in the (figure 3.2.1). For the enzyme activity study, the crude enzyme samples were assayed by cellulase enzyme assay method (DNS).

a



b

Figure 3.2.1: Crude enzyme after submerged fermentation process from potential isolates 47(a) and 66 (b).

3.2.1 Enzyme activity assay

The enzyme unit (EU) of crude enzyme was determined by using DNSA method. Glucose liberated by the cellulase enzyme was estimated by glucose calibration curve as shown in the figure (3.2.2).

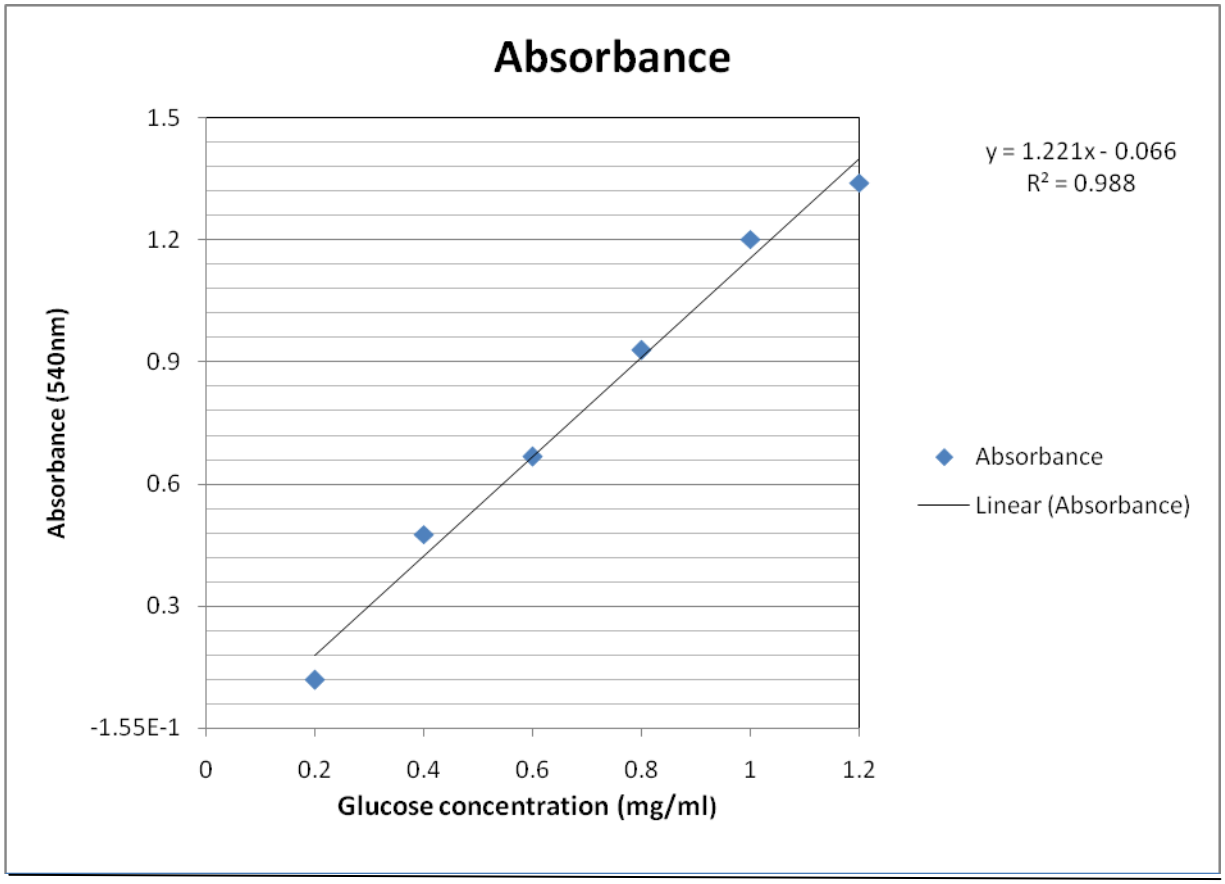


Figure 3.2.2: A glucose standard curve for the determination of the quantity of reducing sugar produced from enzyme assays. Absorbance read at 540 nm.

After analyzing the above data, cellulase activity of both isolates were determined in figure (3.2.3).

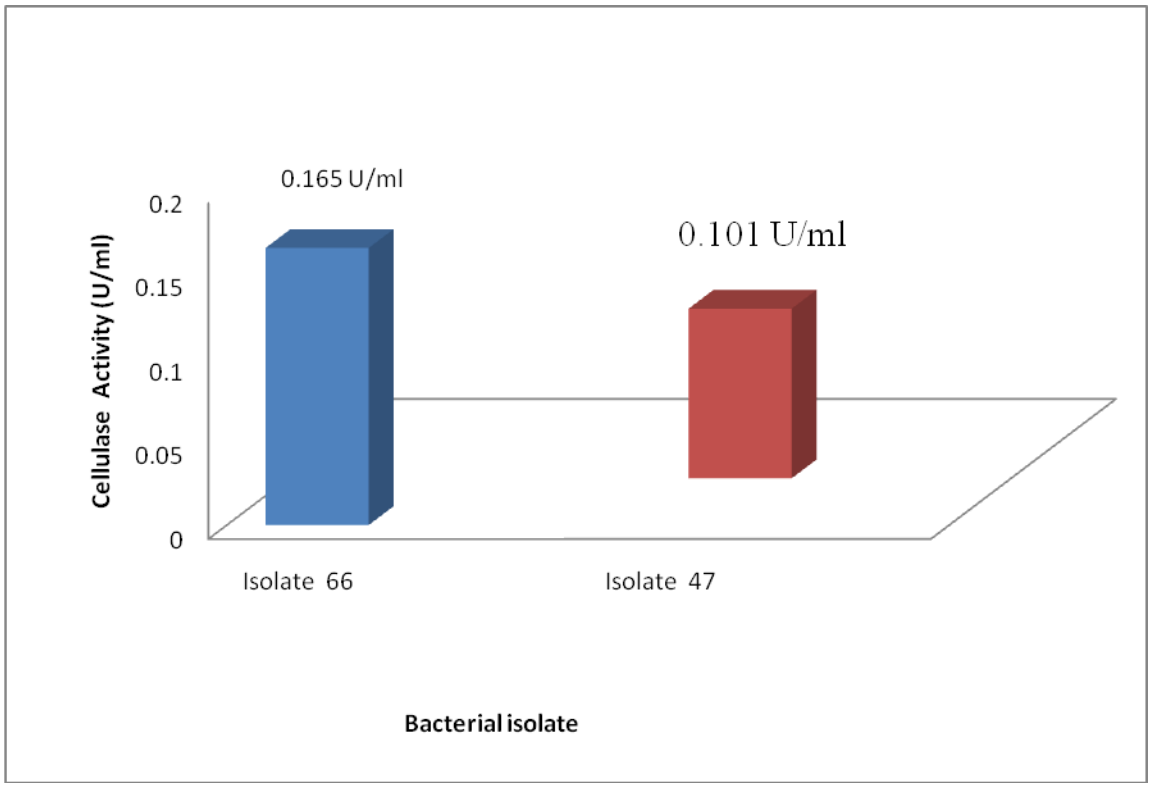


Figure 3.2.3: Cellulase activity of isolated bacteria 47 and 66 in submerged fermentation at 37◦C.

The enzyme activity of isolates 47 and 66 were 0.101 U/ml and 0.165 U/ml respectively at 37◦C**.** Data analysis clearly indicated that the highest enzyme activity was obtained with isolate 66 and thus selected for further characterization and identification.

3.2.2 Determine the concentration of extracellular proteins by Lowry's method

The protein concentrations at different range from 0 to 1.0 were taken and measured their optical density (O.D). As shown in the figure (3.2.4), the O.D increased as the protein concentration increased and the highest peak of O.D was found at the protein concentration of 1.0.

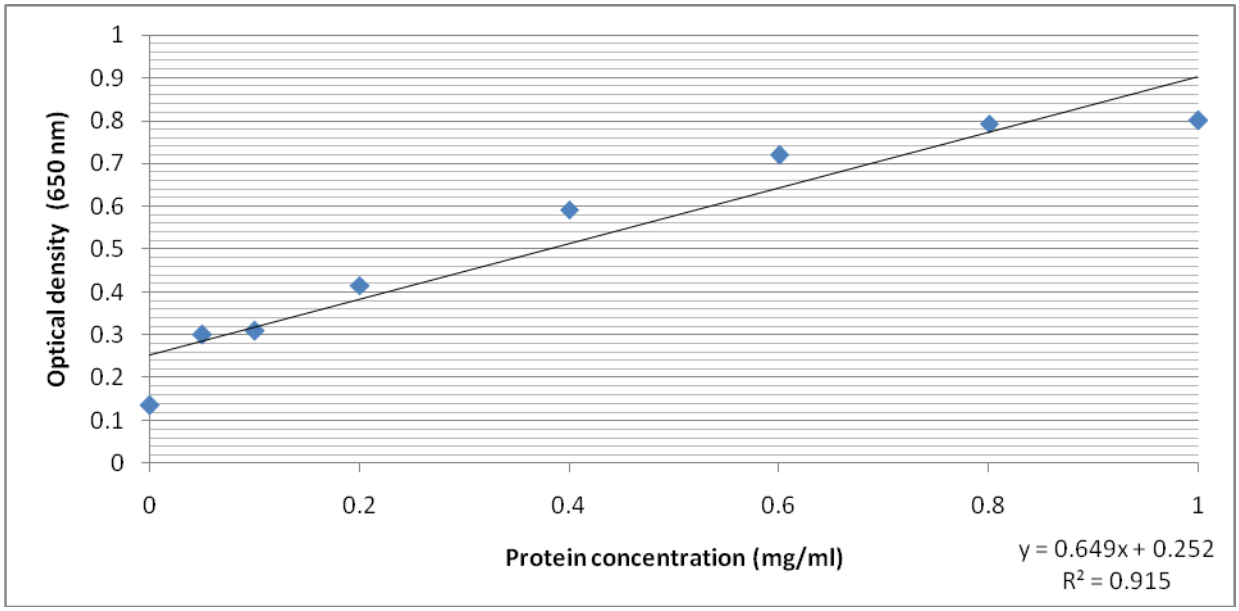


Figure 3.2.4: Standard curve for estimation of extracellular protein concentration by Lowry's method

3.2.3 Specific Enzyme activity

After measuring the specific activity of both isolate 47 and 66, it clearly showed that the isolate 66 has specific activity of about 0.333 U/ml that was higher than that of isolate 47 as shown in figure (3.2.6).

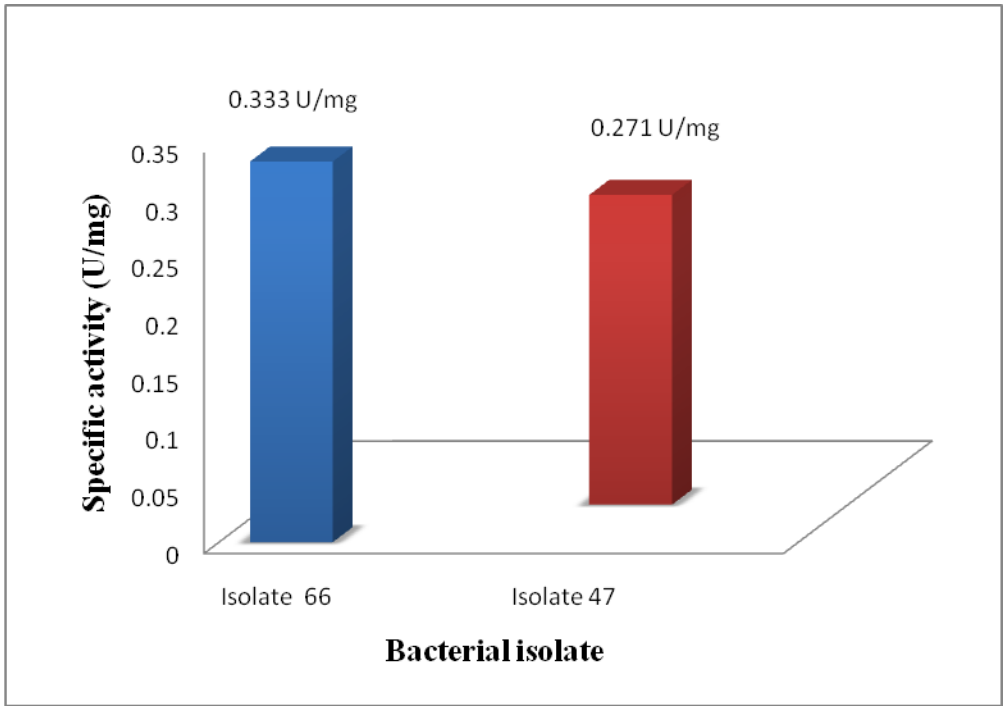


Figure 3.2.5: Specific enzyme activity of isolate 47 and 66.

3.3 Identification of cellulase producing bacteria:

The potent isolate was preliminary identified by means of morphological, cultural and biochemical characterization.

3.3.1 Morphological characterization:

The isolate 66 morphological features were determined by Gram stain test and spore stain test.

3.3.1.1 Microscopic observation:

a. Gram staining:

The isolate 66 retained the purple color of crystal violet stain implying that it was Gram positive rods as shown in figure (3.3.1).

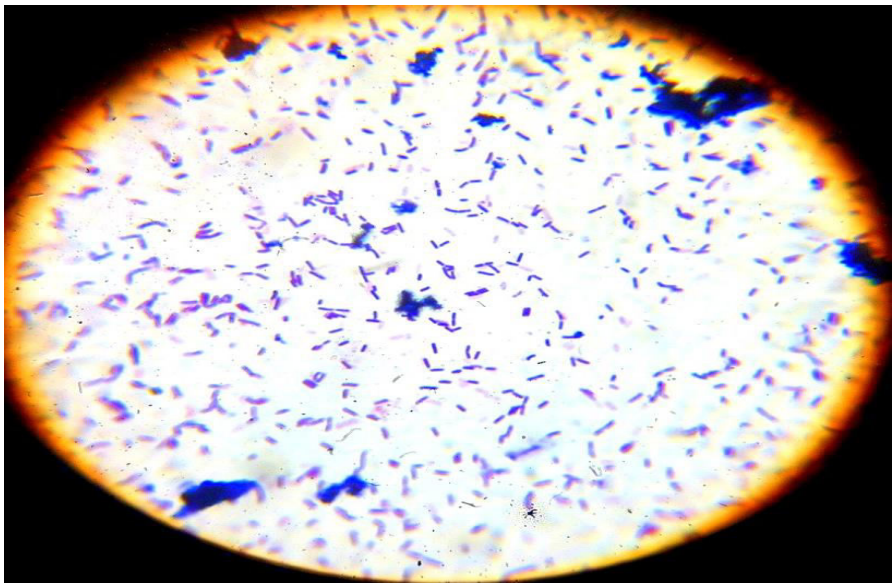


Figure 3.3.1: The cell morphology was observed under bright field microscope (1000X, Krüss, Germany).

The isolate 66 showed cylindrical (rod) shape, single bacillus like arrangement, purple color which indicates gram positive.

b. Spore staining: For the isolate 66, spores were observed in immersion oil field at 1000 times magnification under microscope as shown the in figure (3.3.2).

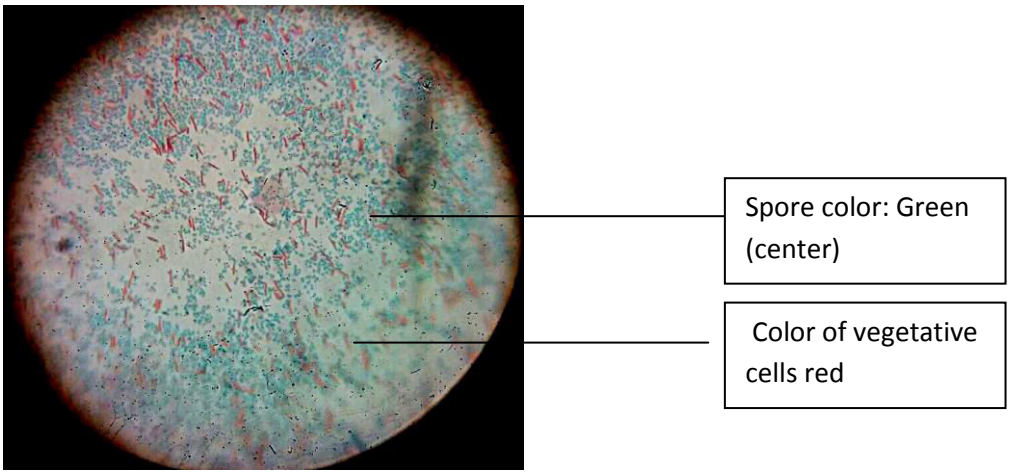


Figure 3.3.2: The cell morphology was observed under bright field microscope.

3.3.1.2 Cultural characteristics

Isolate 66 was further characterized. It was streaked on NA plates and the colony characteristics were observed (figure 3.3.3) and recorded as shown in table (3.3.1).

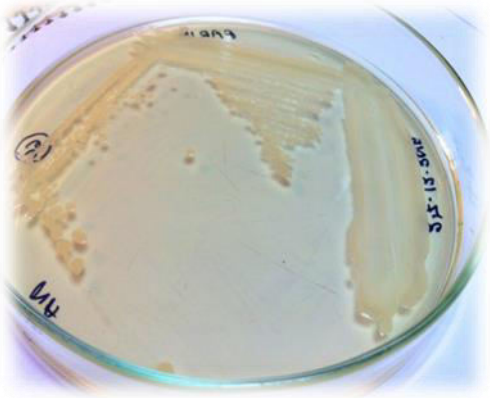


Figure: 3.3.3: Colonies on Nutrient agar medium after 24 hours incubation

Table 3.3.1: Colony characteristics of isolate 66

Size	**Moderate**
Pigmentation	**No pigmentation (Opaque and white to creamy)**
Form	**Round**
Margin	**Undulate**
Elevation	**Convex**
Texture	**Mucoid**
Edges	**Entire**
Surface	**Dull-thick and Opaque**

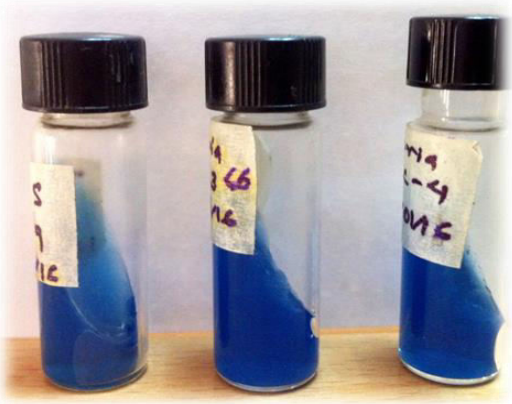
3.3.2 Biochemical characterization:

For characterization of the selected isolate 66, all the biochemical tests were done in triplicate to avoid any false positive or false negative results.

A number of biochemical tests were carried out in order to have presumptive identification of the bacteria. Out of these, sucrose, fructose , glycerol, triple sugar iron agar test, indole test, voges-proskauer test, citrate utilization test, nitrate reduction test, catalase test, gelatin hydrolysis test, mannitol salt agar test, starch hydrolysis test, casein hydrolysis test, blood agar test, growth at 45◦C, growth in 7% NaCl and growth in anaerobic test gave positive results while maltose and D-xylose utilization test, methyl res test, motility test, urease test, oxidase test and growth at 65◦C gave negative results. All the biochemical tests were done in triplicate to avoid any false positive or false negative results. The results of the tests along with their interpretations are given in the following table 3.3.2.

Table 3.3.2: Biochemical test results and their interpretation

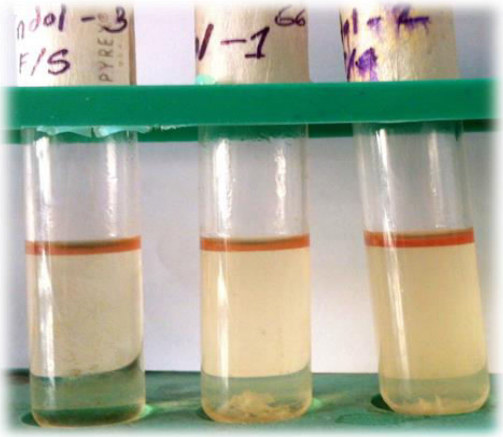
Biochemical Tests	Results
Sucrose Utilization test	**+**
Fructose Utilization test	**+**
Glycerol Utilization test	**+**
Maltose Utilization test	**-**
D-Xylose Utilization test	**-**
Triple Sugar Iron agar test	Alkaline slant (red) and acid butt (yellow) without gas and Hydrogen sulfide production
Indole production test	**+**
Methyl red test	**-**
Voges-Proskauer test	**+**
Citrate Utilization test	**+**
Motility- Indole- Urease (MIU) test	Motility **'-'**, Indole **'+'**, Urease **'-'.**
Nitrate reduction test	**+**
Catalase test	**+ (weak)**
Oxidase test	**-**
Gelatin hydrolysis test	**+**
Mannitol salt agar test	**+**
Starch hydrolysis test	**+**
Casein hydrolysis test	**+**
Blood agar test	**+ (Beta hemolytic)**
Growth at 45◦C	**+**
Growth at 65◦C	**-**
Growth in 7% NaCl	**+ (weak)**
Growth in anaerobic condition	**+**



Citrate Utilization test

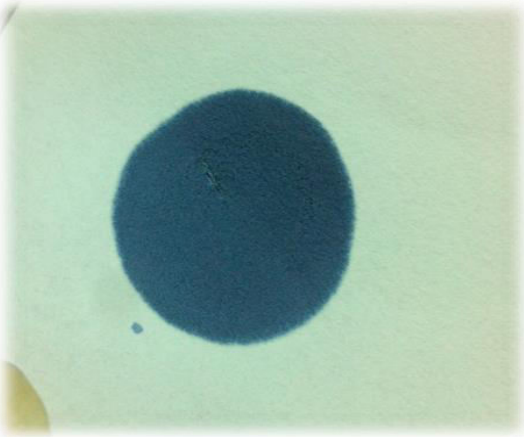
Nitrate reduction test

Starch hydrolysis test



Indole production test

Methyl red test



Oxidase test

Figure 3.3.4: Biochemical tests performed with the isolate 66

As, it is hard to completely identify the species only on the basis of performances of biochemical tests given by isolate 66 , so it is also considered as presumptive identification, the bacteria were tentatively identified to be *Bacillus*.

3.4 Characterization of crude enzyme from cellulolytic bacterial isolate

3.4.1 Effect of pH on activity of cellulase

The effects of pH on cellulase activity were examined by using CMC in 0.05M buffer solution with pH ranging from 3.0 – 10.0. Data illustrated in Figure (3.3.1) clearly indicated that the highest enzyme activity of isolate 66 was found to be 0.494U/ml at pH 5.0. Increasing or decreasing pH beyond this resulted in decline in enzyme activity shown in the figure (3.3.1).

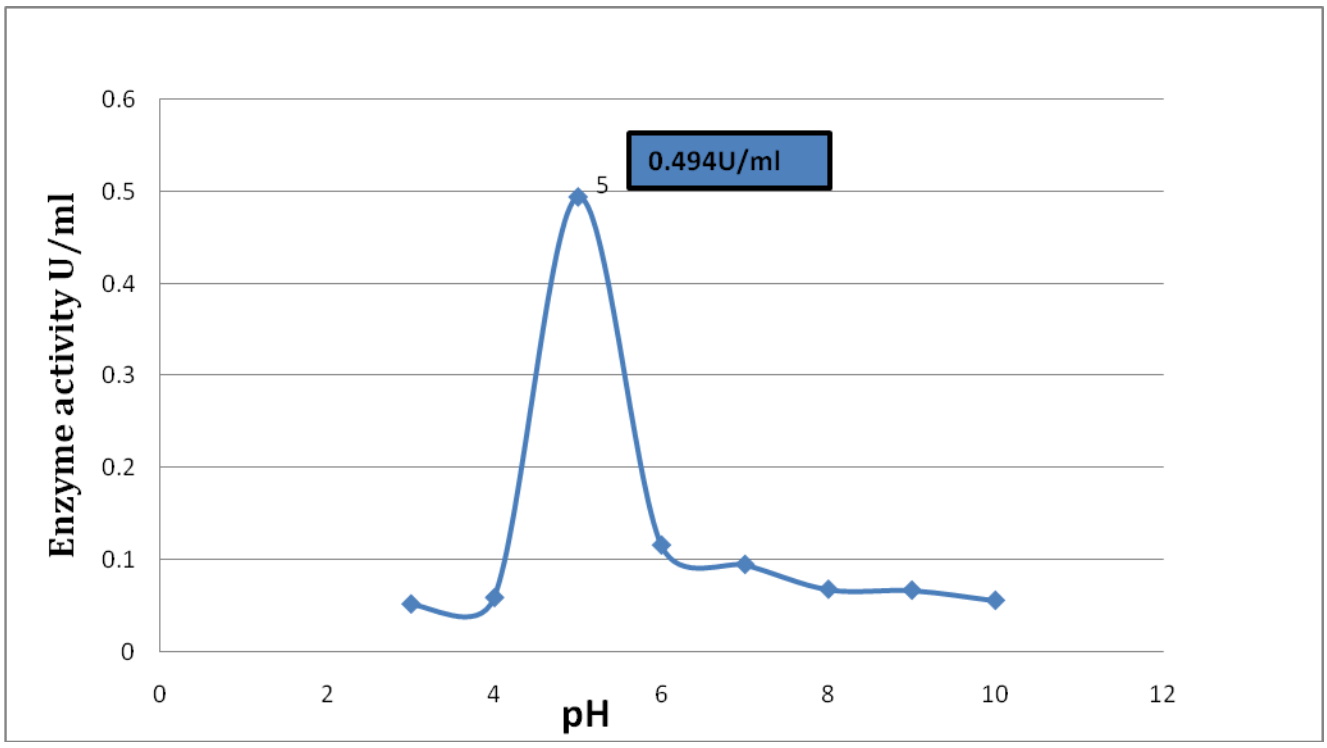


Figure 3.3.1: Effect of pH on the activity of cellulase collected from isolate 66.

3.4.2 Effect of temperature on activity of cellulase

Effects of temperature from 25°C to 85°C were studied. Enzyme activity was found in the range of 0.063 U/ml to 0.133U/ml.Data illustrated in Figure 3.3.2, clearly indicated that the highest enzyme activity of isolate 66 was found to be 0.133U/ml at 65 °C. As the temperature increased from 35˚C enzyme activity increased but activity started to decline as temperature increased above 65˚C.Whereas, the lowest enzyme activity was found to be 0.063U/ml at 85˚C shown in the figure (3.3.2) below.

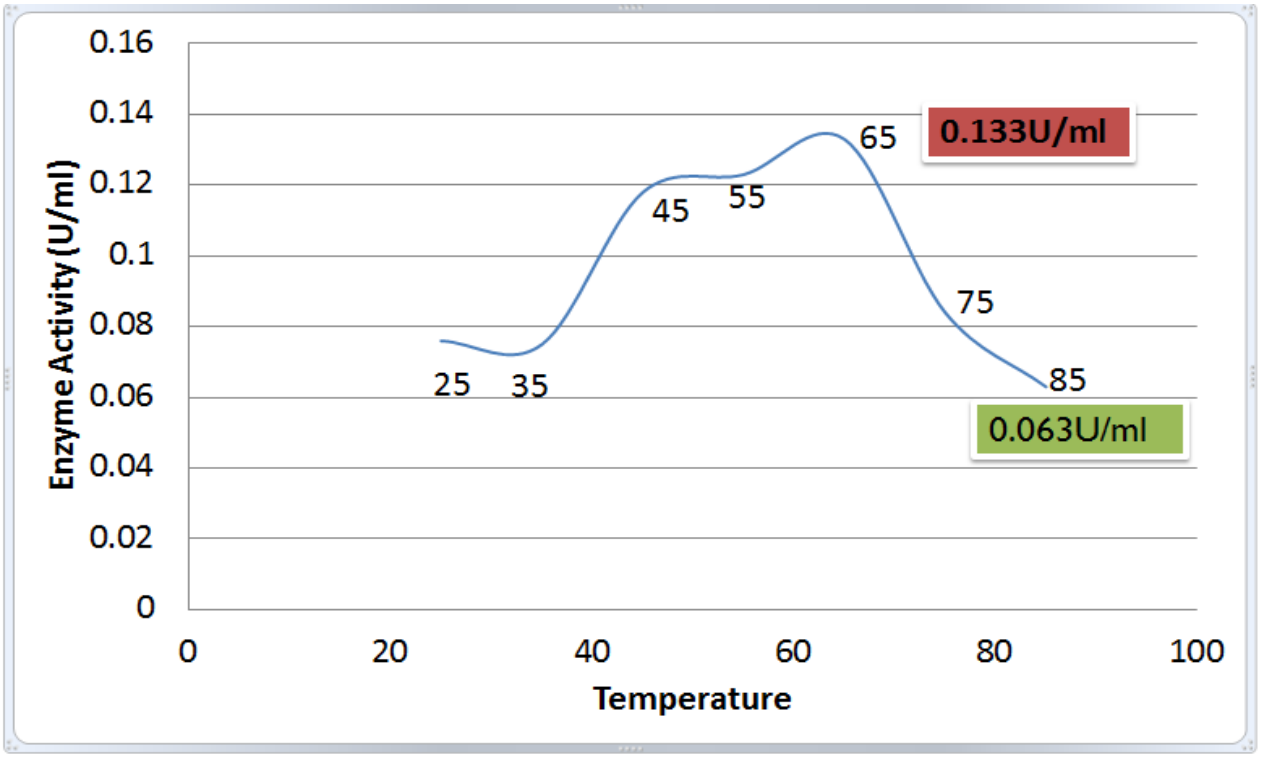


Figure 3.3.2: Effect of temperature on the activity of cellulases from isolate 66.

3.5 Degradation of Filter paper and Cotton:

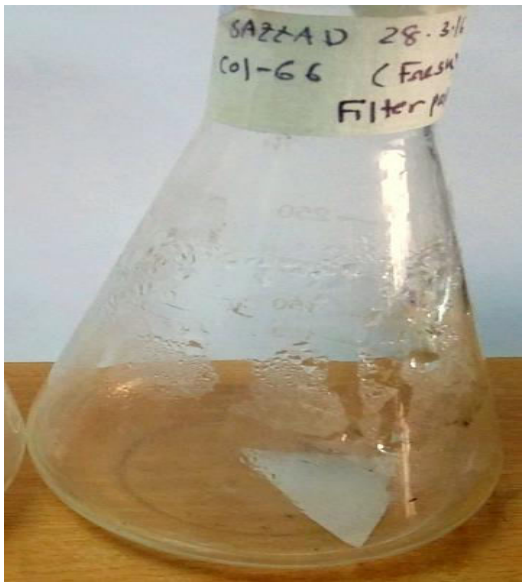


Figure 3.4.1(a): Isolate 66 before incubation

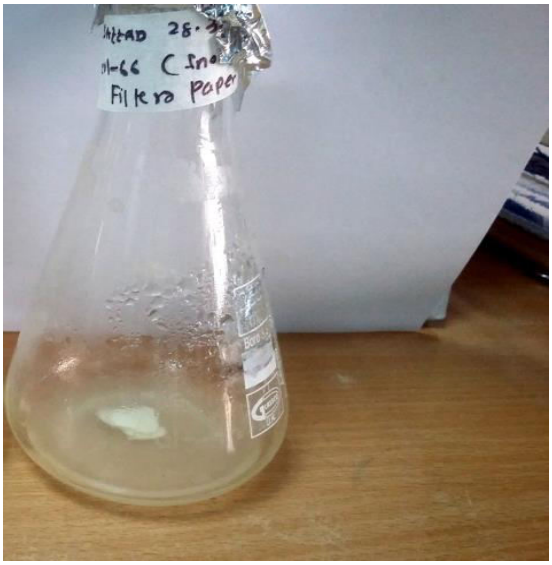


Figure 3.4.1 (b): Isolate 66after incubation

(Filter paper: 1 cm x 2 cm)

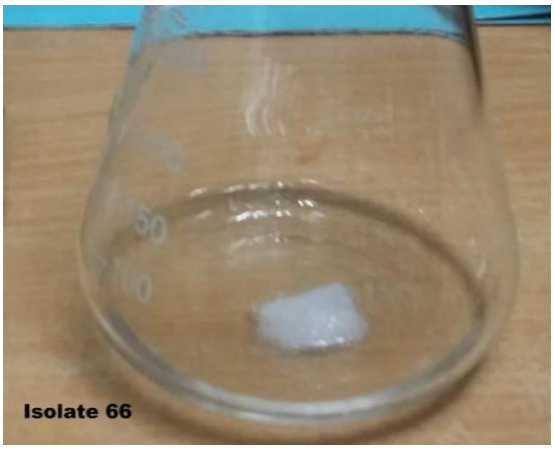


Figure 3.4.2(a): Isolate 66 before incubation

(Cotton: 0.12g)

Figure 3.4.2 (b): Isolate 66 after icubation

The figures (3. 4.1 and 3.4.2) show the degradation of filter paper and cotton by the isolate 66. The figure 3.4.1 (a) shows the condition of filter paper strips (1 cm x 2 cm) before incubation. The isolate 66 was able to degrade the filter paper as shown in figure 3.4.1 (b), in a shaking incubator at 50 °C and 120 rpm for a time period of 6 days. Whereas, the condition of cotton before and after incubation as shown in figure (3.4.2: a and b) indicates that the isolate 66 was unable to degrade the cotton.

Chapter 4

4.0 Discussion

It is well known that plants are the most available source of renewable carbon and energy on the earth. Annually, the production of cellulose is about 4×10^9 tons from plants and consists of highly stable polymer of β-1,4-linked glycosyl residues along with other polysaccharides (Coughlan, 1990, Yin *et al.,*2010). At present it has become crucial to develop an effective method to hydrolyze cellulose in various industries. Bio-based products and bioenergy from less costly renewable lignocellulosic and cellulosic materials would convey advantages to the local economy, environment and national energy security (Zhang, 2008). The cost of an enzyme is one of the main factors determining the economics of a process. In this respect, the major goals for future cellulase research would be to reduce cellulase production cost and improve the performance of cellulases to make them more effective (Sukumaran *et al.,* 2005).

The main objective of this present study is to isolate, identify and screen bacteria from soil samples that have high cellulase activity. The present study conducted here, to obtain a cellulase producer that would allow cellulase production at low cost; that can be utilized in the industries, a process that would reduce environmental pollution through biodegradation and cellulose decomposition as well.

Cellulases are the inducible bioactive compounds produced by microorganisms like bacteria and fungi, during their growth on cellulosic matters. Cellulase is used in enzymatic hydrolysis of cellulosic substances due to the increasing knowledge of its mode of action. Although a large number of microorganisms are capable of degrading cellulose (collected from cow dung), among them only a few are able to produce significant quantities of cell-free bioactive compounds (Bai *et al.,* 2012). Fungi are the main cellulase-producing microorganisms, though a few bacteria have also been reported as potential cellulase producer and have a higher growth rate than fungi. In this present study 68 cellulotytic bacteria were isolated from soil sample collected from Bagan Bari Dairy Farm, Keranigonj, Bangladesh.

For the characterization of the selected isolate, the basic routine laboratory works like morphological, cultural and different biochemical characteristics tests , DNS method (for assaying cellulase enzyme and reducing sugar) and characterization of crude enzyme were performed. The cellulolytic activity shown by the isolated bacterial species was reported to depend on the source of occurrence in various natural environments because of the various amounts of cellulose that occurs in these environments and natural wastes present (Shankar and Isaiarasu, 2011). A rapid primary screening of 68 isolates were carried out for their cellulase activity by using media containing 1 % CMC as a sole source of carbon and after 48 hr of incubation, the plates were flooded with 1% gram's iodine to check cellulase hydrolysis (Ariffin *et al.,* 2006, Sadhu and Maiti, 2013, Irfan *et al.,* 2012). Among these, 31 colonies had the capability of hydrolyzing cellulose. In addition, Das *et al*. (2010) isolated eight bacterial strains from cow dung samples and found most of these were *Bacillu ssp*. and in another study Bai *et al.* (2012) isolated around 21 bacterial samples from the cow dung of which 9 strains were found to be cellulase producer and surprisingly they were *Bacillus subtilis* (Irfan *et al.,* 2012, Bai *et al.,* 2012). In this study quite a good number of cellulase producers were found. Cellulase can also be isolated from cattle waste, woody biomass, cow manure and compost. Cellulase producing bacteria were found commonly in all environments which are enable them to degrade the cellulose and are prevalent in waste materials (Bai *et al.,* 2012, Sharma *et al.,* 1985, Sleat *el al.,* 1984, Palop *et al.,* 1989, Lee *et al.,* 1975). The clear zone ratio of two of the best isolates found in this study were 3.85 (isolate 66) and 4.87 (isolate 47) which were much higher than the mean ratio found from the isolates of farming soil 2.1 and forest soil 1.6 (Hatami *et al.,* 2008).

The crude enzyme produced by the Isolates 47 and 66 were assayed for enzyme activity by DNS method. Regarding this, other previous enzyme activity assays that were done by using the crude enzyme indicated the similar ability, shown by the *Bacillus* genus to secrete extracellular proteins (Schallmey *et al*., 2004; Lin *et al.,* 2012).Since cellulases are active at about a pH range of 5.0- 6.5 for *Bacillus* strains (Mawadza *et al.,* 2000), the enzyme assay was carried out at 50◦C and pH 7, considering them to be the optimum temperature and pH. The enzyme activity of isolate 47 was 0.101 U/ml and isolate 66 was 0.165 U/ml respectively at 50◦C**.** However, these results were approximately in correlation with the finding of many other workers. For instance, *Bacillus sp*. SMIA-2 expressed a promising level (0.83 U/ml) of cellulase

activity (Ladeira *et al.,* 2015) and two strains of *Bacillus thuringiensis* showed enzyme activity of0.072 U/ml and 0.059 U/ml (Lin et al., 2012). All the data compared above illustrated that the enzyme activity for both isolates (47 and 66) was higher than enzyme activities obtained with *Bacillus thurengiensis*. It can be said that these isolates were good cellulase producers.

The determination of total extracellular protein concentration was carried out by Lowry method (Lowry *et al*., 1951). Isolate 66 had a higher extracellular protein concentration (0.495mg/ml) and specific activity (0.333U/mg) compared to isolate 47 (0.373mg/ml; 0.271U/mg). In addition studies resembling similar result of protein concentration with this study reported 0.460 mg/ml for *Bacillus subtilis* KO strain (Shabeb *et al.,* 2010) and 1.01 mg/ml for *Bacillus spp*. isolated from sugarcane bagasse and corn steep liquor (Ladeira*et al.,* 2014).
Comparing the results based on enzymatic activity and specific activity of the two isolates, the one with the better performance (isolate 66) was chosen. The selected potent bacterial isolate 66 was characterized for its morphological, cultural and biochemical characteristics as described by Bergey's manual of systematic bacteriology (Schleifer, 2009, Logan and Vos, 2009). The colonies of isolate 66 on NA agar plate (Figure:3.3.4) were moderate in size and irregular shaped, opaque and white in color, with undulate margins, convex and texture was mucoid. According to Bergey's Manual of systematic bacteriology, *Bacillus* spp produce colonies that may give the appearance of a mixed culture. Cells rod-shaped, straight or slightly curved, occurring singly and in pairs, some in chains, and occasionally as long filaments, endo spores former and gram positive. The colonies are basically irregular in shape and of moderate (2–5 mm) diameter, they become opaque, with surfaces that are dull and which may become wrinkled; color is whitish, and may become creamy or brown; textures range from moist and butyrous or mucoid, through membranous with an underlying mucoid matrix, with or without mucoid beading at the surface, to rough and crusty as they dry (Schleifer, 2009, Logan and Vos, 2009). In this study, microscopic examination of this isolate 66 revealed that it was gram positive (retained the purple color of crystal violet), spore forming rod (cylindrical) and non-motile). Most of the biochemical tests were done following the method from Cappuccino and Sherman (2005). Considering the outcome of spore staining, it could be observed that the isolate 66 is capable of producing spores when the environment turns adverse out of nutrient depletion. To encourage the ability of the bacteria to sporulate a small amount of $MnSO_4.H_2O$ was added. The

position of the spore inside the cell cannot be fully visualized using light microscope. Although, few repeated attempts revealed the position of the spore vaguely to be central mostly. According to Bergey's manual, the position of the spores of *Bacillus subtilis*could is central as well as ellipsoidal, paracentral and subterminal along with non-swelling sporangia (Schleifer, 2009, Logan and Vos, 2009). The cultural and microscopic characteristics demonstrated by Bergey's Manual of systematic bacteriology, for *Bacillus* was quite similar to the characteristics expressed by the isolate 66. Hence, the isolate could be tentatively predicted as *Bacillus* spp.

Besides, the isolate was able to grow on blood agar (Figure 3.3.19) and showed positive growth at 45◦C and 7% NaCl (Figure 3.3.23) which indicated as weakly positive. The isolate 66 was also able to grow anaerobically (Figure 3.3.24) at 37◦C but unable to grow at 65◦C (Figure 3.3.22). As many Bacillus species, however, grow anaerobically using nitrate or nitrite as an electron acceptor (Logan and Vos, 2009). The biochemical characteristics of the isolate 66 was found positive for acid from sucrose, fructose , glycerol, triple sugar iron agar test, indole test, voges-proskauer test, citrate utilization test, nitrate reduction test, catalase test, gelatin hydrolysis test, mannitol salt agar test, starch hydrolysis test, casein hydrolysis test. While, negative results were for acid from maltose, D-xylose, methyl red test, motility test, urease test and oxidase test. The results obtained from the isolate 66 was then interpreted from Bergey's Manual of Systemic Bacteriology, revealed that it was mostly similar with the characteristics of *Bacillus* spp. given that positive for catalase, variable for oxidase, hydrolysis of casein, gelatin and starch, utilization of citrate, nitrate reduction, Voges–Proskauer, production of a small acid without gas from glucose, xylose, sucrose, fructose, glycerol and maltose, motility and cellobiose. On the contrary, *Bacillus subtilis*also showed negative results for anaerobic growth and hydrolysis in Bergey's manual. However, four of the tests in this study including motility (-ve), anaerobic (+ve), acid from xylose and maltose (-ve) were totally opposite of that mentioned in Bergey's Manual. . The symbols- '+' and '-' in Bergey's Manual indicated that the results were positive for >85% of the indicated bacteria and only 0-15% positive for various other closely related species and sub species of *Bacillus* spp (Logan and Vos, 2009). From the previous studies, biochemical characteristics were positive for indole, MR, VP, citrate, catalase, oxidase, gelatin, nitrate and negative for urease which was presumptively identified as *Bacillus spp*. (Shenkani and Sundara, 2015). Another report was found giving biochemical test results to be positive for indole, catalase, oxidase MR-VP, citrate, nitrate, gelatine, starch and casein. Among sugar fermentation

positive results were obtained with glucose, lactose, maltose, xylose, mannitol, fructose and negative for sucrose which was then identified as *Bacillus* spp. (Shaikh *et al.*, 2013). Thus, the results clearly vary between strains as well as species. However, it could probably be considered as the bacteria belong from genus *Bacillus*. Also, there are always possibilities of bacteria from genus such as *Bacillus* to bring up evolutionary changes due to environmental stress quite easily during subsequent sub culturing, incubation period and refrigeration. Furthermore, in order to receive complete identification of the bacteria, there are a lot of biochemical tests yet to be performed. Due to the limited resource, all the biochemical tests could not performed. Hence, the result could only tentatively predict that the isolate belong to the genus *Bacillus*. Further identification can be carried out in molecular level for the confirmation of the species.

To determine the optimum pH, the enzyme activity assay was carried out in buffer mixtures at various pH values (3-10) at the predetermined temperature (65°C) using water bath. 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). In this present study, the highest enzyme activity of isolate 66 was found to be 0.4454 U/ml at pH 5.0 (Figure: 3.3.1) suggesting that the enzyme is an acid cellulase, which was close to the optimal pH value 4.8 of most bacterial cellulases (Shu *et al.,* 2013) and a pH of 5.0 to 6.5 of *Bacillus* strains (Mawadza *et al.,* 2000). Acid cellulases act at a pH range of 3.8 to 5.8 (Mosjov, 2012). In addition, present findings were significant from *M. circinelloides*which has a pH range of 4-7 (Saha *et al.,* 2004) and pH 4.5-7 for *B. circulans* (Kim *et al.,* 1995). Increasing or decreasing pH beyond this resulted in decline in enzyme activity (shown in figure 3.3.1). For example, a second highest enzyme activity of isolates 66 was seen at pH 6 which is about 0.066 U/ml while, the lowest enzyme activity was found to be 0.002 U/ml at pH 3.0. According to some previous studies, cellulases are active at the pH range of 6.0 to 7.0 from *A. Niger* (Akiba*et al.,* 1995) and 5.0 to 7.0 from *Lysobacter* sp. (Ogura *et al.,* 2006). The optimum pH for the enzyme was found to be range between 6.5 for *Bacillus subtilis* (verma *et al.,* 2012*).* This range of pH is important for this enzyme, which can be used, in alkaline environments such as in processing of paper pulp.

Temperature plays an important role in enzyme production and activity. In this study, the highest enzyme activity of isolate 66 was found to be 0.133 U/ml at 65 °C. The results illustrated by Figure (3.3.2), clearly showed that cellulase production, expressed as enzyme activity, gradually increased as the temperature values increased from 45 ˚C to 65˚C and reached its maximum at 65 ˚C. Similar findings were also reported by Saha *et al.* (2004) and Mawadza *et al.* (2000) where the highest activity of cellulase was found at 55˚C from *Bacillus strains CH43* and at 65 ˚C from M. *circinelloides*. Other studies showed that Cellulases from some species of *Bacillus subtilis subsp subtilis* A-53, *B. subtilis*YJ1 and *Bacillus* strains RH68 have optimum temperature of 50˚C , 60˚C , 70˚C (RH68) (Yin *et al.,* 2010, Irfan *et al.,* 2012) . Besides, there are some other *Bacillus* strains have the optimum temperature of 70°C (Yin *et al.,* 2010 , Liang *et al.,* 2009, Assareh *et al.,* 2012). Thermostability and pH stability of enzymes are of great importance since these properties determine their applications in specific industries. For example, cellulases in detergents should be able to work within the temperature range of 50 to 70^0C typically used in washing (Ladeira *et al.,* 2015).

As isolate 66 showed highest cellulase activity, so it was tested for filter paper and cotton degradation. After continuous observation of 6 days incubation in a shaking incubator at 50 °C and 120 rpm, it could be concluded that isolate 66 was able to degrade the filter paper (Figure 3.4.1 a & b) but was less efficient for cotton treatment. However, degradation of cellulosic materials is a complex process requiring participation by a number of microbial enzymes in some cases, pretreatment by other microbial population or chemicals may help or induce degradation. In order to find these microorganisms, it is essential to search for those habitats that contain these substrates (Huang and Monk, 2004; Shaikh *et al.,* 2013). Besides, this enzyme needs to be tested for its ability to hydrolyze a lot of other substrates like straw, rice husks, sugarcane bagasse etc. Further testing with various metal ions and inhibitors is also necessary to identify its full potential.

After analyzing all the results above, it has revealed that the cow dung mixed soil is a good source for isolation of cellulase producers since it is a rich source of cellulose. The selected potent bacterial isolate 66 presumptively identified through its morphological, cultural and biochemical characteristics, thus, > 85% results indicated that it could be *Bacillus* spp. The isolated wild *Bacillus spp was* able to produce promising level of cellulases under thermophilic conditions from submerged fermentation process. Moreover, the *Bacillu*s strain isolated showed fast growing capability within 24 hours of time period which can be utilized as a good cellulase producer for various industrial applications. The functional tests conducted here to determine the conditions for cellulolytic activity including the optimum temperature for cellulase activity from *Bacillus spp.* was found to be 65°C; this thermostable property of cellulase points towards its suitability as an industrial enzyme. Additionally, enzyme activity was found to be high at the range of pH 4 to pH 7 with an optimum of pH 5 suggesting that the isolate bears acidic cellulases. The isolate also showed the good enzyme activity and specific activity. Furthermore, the isolate can also be utilized as a potent organism for filter paper degradation and high growth rate along with the ability to secrete proteins extracellularly which indicates salient features of the *Bacillu*s species might be implemented in several industrial applications for example in pulp and paper industries . However, more studies are needed before industrial application of this isolates can be evaluated properly. Nevertheless, identification in molecular level for the confirmation of the species, better understanding of physiology of the microorganisms and utilization of the knowledge with engineering for cost effective production of cellulases will be helpful in industrial process development.

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Appendices

Appendix- I

Media compositions

The composition of all media used in the study is given below.

Nutrient Agar

Component	Amount (g/L)
Peptone	5.0
Sodium chloride	5.0
Beef extract	3.0
Agar	15.0
Final pH	7.0

Saline

Component	Amount (g/L)
Sodium Chloride	9.0

Luria Bertani Broth

Component	Amount (g/L)
Tryptone	10.0
Yeast extract	5.0
Sodium chloride	10.0

Carboxymethylcellulose (CMC) Media

Component	Amount (g/L)
Peptone	10.0
Carboxymethylcellulose	10.0
Dipotassium phosphate	2.0
Agar	10.0
Magnesium sulphate heptahydrate	0.3
Ammonium sulphate	2.5
Gelatin	2.0

Inoculum broth

Component	Amount (g/L)
$MgSO_4$	0.3
K_2HPO_4	2.0
Glucose	10.0
$(NH_4)_2SO_4$	2.5
Peptone	10.0

Fermentation broth

Component	Amount (g/L)
Peptone	10.0
CMC	10.0
K_2HPO_4	2.0
$MgSO_4.7H_2O$	0.3
$(NH_4)_2SO_4$	2.5
Gelatin	2.0

Starch Agar

Component	Amount (g/ L)
Beef extract	3.0
Soluble starch	10.0
Agar	12.0

Simmon's Citrate Agar

Component	Amount (g/L)
Magnesium sulphate	0.2
Ammoniun dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bacto agar	15.0
Bacto bromo thymol blue	0.08

Tryptophan Broth

Component	Amount (g/L)
Peptone	10.0
Sodium chloride	5.0

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

Methyl red Voges- Proskauer (MRVP) Media

Component	Amount (g/L)
Peptone	7.0
Dextrose	5.0
Dipotassium hydrogen phosphate	5.0
Final pH	7.0

Triple Sugar Iron Agar

Component	Amount (g/L)
Bio-polytone	20.0
Sodium chloride	5.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Phenol red	0.0125
Agar	13.0
Final pH	7.3

Motility Indole Urease (MIU) Agar

Component	Amount (g/L)
Tryptone	10
Phenol red	0.1
Agar	2.0
Sodium chloride	5.0
pH (at 25°C)	6.8 ± at 25°C

Gelatin Broth

Component	Amount (g/L)
Peptone	5.0
Beef extract	3.0
Gelatin	120.0
Final pH	6.8 ± 0.2 at 25°C

Nitrate Reduction Broth

Component	Amount (g/L)
Beef extract	3.0
Gelatin peptone	5.0
Potassium nitrate	1.0

Mannitol Salt Agar

Component	Amount (g/L)
Proteose peptone	10.0
Beef extract	1.0
Sodium chloride	75.0
D-mannitol	10.0
Phenol red	0.025
Agar	15.0
Final pH	7.4 ± 0.2 at 25°C

Blood Agar Base

Component	Amount (g/L)
Beef heart infusion from (beef extract)	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH	6.8 ± 0.2 at 25°C

Sugar Fermentation Broth

Component	Amount (g/L)
Sugar	5.0
Trypticase	10.0
Sodium chloride	5.0
Phenol red	A very small amount until the broth turns red

Appendix – II

Reagents and Buffers

Gram's iodine (300 ml)

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-Dinitrosalicyclic acid (100 ml)

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. The solution was stored at room temperature in an amber bottle to prevent exposure to light.

BSA stock solution (1 mg/ml)

In order to make 15 ml stock solution of BSA (Bovine Serum Albumin), 0.015 g of powdered molecular biology grade BSA was added to 15 ml distilled water and mixed well. The solution was then stored at 4◦C for further use.

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

Crystal Violet (100 ml)

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.

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 temeperature.

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.

Kovac's Reagent (150 ml)

To a reagent bottle, 150 ml of reagent grade isoamyl alcohol, 10 g of p-dimethylaminobenzaldehyde (DMAB) and 50 ml of HCl (concentrated) were added and mixed. The reagent bottle was then covered with an aluminum foil to prevent exposure of reagent to light and stored at 4°C.

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 destilled 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 µl 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-α-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)^{a} to form a colourless solution and stored at 4°C.

Sodium Phosphate Buffer (0.05 M 50 ml)

For 50 ml of 0.05 M NaH2PO4, 0.35 g was added to 50 ml distilled water. For 50 ml of 0.2 M Na2HPO4, 0.67 g was added to 50 ml distilled water. To make sodium phosphate buffer of pH 6, 1.28 ml of Na2HPO4 was mixed with 3.73 ml NaH2PO4 and the volume was adjusted to 50 ml using distilled water. For pH 7, 2.89 ml Na2HPO4 was added to 2.12 ml NaH2PO4 and the volume was adjusted to 50 ml by adding distilled water. The buffer was autoclaved and stores 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.

Tris – Hydrochloric Acid Buffer (0.05 M 50 ml)

To 45 ml distilled water, 0.3941 g Tris-HCl was added and made upto 50 by adding sodium hydroxide**.** The buffer was autoclaved and stores at 4°C.

Glycine – Sodium Hydroxide Buffer (0.05 M 50 ml)

To 25 ml distilled water, 0.117 g glysine was added. To 25 ml distilled water, 0.03g sodium hydroxide was added. The two solutions were mixed. The final solution was autoclaved at stored at 4°C.

Appendix – III

Instruments

The instruments used in the study are given below.

Instrument	Manufacturer
Weighing Machine	Adam equipment, UK
Incubator	SAARC
Laminar Flow Hood	SAARC
Autoclave Machine	SAARC
Sterilizer	Labtech, Singapore
Shaking Incubator, Model: WIS-20R	Daihan Scientific Companies, Korea
Water Bath	Daihan Scientific Companies, Korea
Spectrophotometer, UV mini - 1240	Shimadzu Corporation, Australia
NanoDrop 2000 Spectrophotometer	Thermo Scientific, USA
Table Top Centrifuge	Digisystem, Taiwan
Microscope	A. Krüssoptronic, Germany
Power Supply Machine: Elite 300 plus	Wealtec Corp, USA
UV Transilluminator, Model: MD-20	Wealtec Corp, USA
-20°C Freezer	Siemens, Germany
Magnetic Stirrer, Model: JSHS-180	JSR, Korea
Vortex Machine	VWR International
Microwave Oven, Model:MH6548SR	LG, China
pH Meter: pHep Tester	Hanna Instruments, Romania
Microcentrifuge Machine: Minispin Plus	Eppendorf, Germany
Micropipette	Eppendorf, Germany
Disposable Micropipette tips	Eppendorf, Ireland
Microcentrifuge tubes	Tarsons Products, Pvt Ltd, Kolkata
ABI Genetic Analyzer, Model: 3700	Applied Biosystems, USA