Isolation of Cellulolytic Bacteria from Soil and Identification by 16S rRNA Gene Sequencing

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

Submitted by
Faria Mahjabeen
Student ID: 12126004
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Microbiology Program
Department of Mathematics and Natural Sciences
BRAC University
Dhaka, Bangladesh

Declaration

I, hereby certify that the thesis work entitled **"Isolation of cellulolytic bacteria from soil and identification by 16S rRNA gene sequencing"** submitted to the Department of Mathematics and Natural Science, BRAC University in partial fulfillment of the requirement for the degree of Bachelor of Science in Microbiology is a record of work carried out by me under joint supervision and able guidance of my supervisors Dr. Mahboob Hossain **,** Co-ordinator of Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University and Trosporsha Tasnim Khan, Lecturer, Microbiology Program, Department of Mathematics and Natural Sciences, BRAC University. It is further declared that the research work presented here is original and the contents of this report in full or parts have not been submitted to any other university and institution for any degree or diploma.

Candidate

_____

(Faria Mahjabeen)

Certified by:

_____

Trosporsha Tasnim Khan

Supervisor,

Lecturer

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University

Dhaka, Bangladesh

_____

Dr. Mahboob Hossain

Co-supervisor,

Co-ordinator

Microbiology Program

Department of Mathematics and Natural Sciences

BRAC University

Dhaka, Bangladesh

Dedicated To

My Beloved Parents, Brother, Grand Mother And

All My Loved Ones

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Table of Contents

List of Figures

List of Tables

List of Abbreviations

3D	Three- dimensional
CBD	Cellulose Binding Domain
PDB	Protein Data Bank
GHs	Glycosyl Hydrolases
CEF	Cellulose Elementary fibril
CesA	Cellulose Synthase
CD	Catalytic Domain
CBM	Cellulose Binding modules
CAC	Catalytically Active Complex
CMC	Carboxymethyl cellulose
PCR	Polymerase Chain reaction
NA	Nutrient Agar
CMCase	Carboxymethyl cellulase
DNS	Dinitrosalicylic Acid
BSA	Bovine Serum Albumin
CipA	Cellulosome Integrating Protein
SLH	S Layer Homologous
SdbA	Scaffolding Dockerin Binding
OlpA	Outer Layer Protein

Abstract

Cellulases have an escalating demand in many industries as they hold extreme potency for converting the most abundant lignocellulosic material into renewable and sustainable energy, chemicals, fuels and materials. Hence, studies pursue to unfold a novel cellulase that can overcome existing challenges in biorefinery, reduce biofuel production cost, as well as have tremendous applications in industrial processes. Therefore, soil from a dairy farm was screened for potent cellulase producers on CMC agar and the best isolate so far had an extracellular crude enzyme activity of 0.167U/ml and specific activity of 0.333U/mg. The cell morphology, cultural characteristics and biochemical tests revealed the isolate to be facultative anaerobe, gram positive, non motile spore forming rods and presumptively identified it to belong to the genus *Bacillus*. Later on, molecular analysis was carried out by initially amplifying the 16S rRNA gene of the isolate using universal primers 27F and 1492R vie polymerase chain reaction method. The 16S rRNA gene was further sequenced using Sanger sequencing method which revealed the length of the nucleotide sequence to be 1381bp. The sequence was compared to the NCBI nucleotide database. Afterwards a phylogenetic tree was constructed by maximum likelihood method. The organism was found to be *Bacillus subtilis* as indicated by its presence in the same node with *Bacillus subtilis* subsp. *spizizenii.* The isolate obtained shows good cellulase activity but requires further characterization to determine its potentiality as an industrial producer of cellulase.

Chapter 1

1. Introduction

1.1 Background

Throughout the photosynthesis process, the plant produces a large quantity of plant biomass. The cellulose comprises the most it. It is the primary product in the terrestrial environment. Every year it is estimated to be approximately 40 billion tons (Hatami *et al.,* 2008). Thus, cellulose which happens to be the major polymeric component of plant material is the most abundant polysaccharide produced in the biosphere. Around 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 mainly in cellulose, lesser in hemicelluloses and least of all lignin (Shaikh *et al,.* 2013). It is renewable and a great potential resource for bioconversion to value added bio products (Shenkani & Sundara, 2015). This biological conversion of the cellulosic biomass to fuels and chemicals offer a high yield of products vital to economic success and potential for very low cost (Yang *et al.,* 2011).

Cellulase is responsible for the bioconversion of cellulosic and lignocellulosic residues. It hydrolyses β-1,4 glucosidic bonds in cellulose. They are members of glycoside hydrolase families of enzymes, which hydrolyze oligosaccharide and polysaccharide. The extensive intermolecular bonding pattern of cellulose creates a phenomenal crystalline substrate particularly resistant to microbial degradation (Bayer *et al.,* 1998). Hence, cellulolytic activity is a multi complex enzyme system and complete enzymatic hydrolysis requires a synergistic action of 3 enzymes: endo-glucanse, exo-glucanse and glucosidase. These enzymes act sequentially in the synergistic system and efficiently degrade cellulose converting it into an utilizable energy source. Thus, cellulases play a vital role in biomass utilization.

Although most efficient cellulase activities are observed in fungi but there is increasing interest in cellulase production by bacteria since they have higher growth rate as compared to fungi and has good potential to be used in cellulase production (Shaikh *et al.,* 2013). The specific cellulolytic activity shown by the bacterial species is found to be depending on the source of occurrence. Some bacterial species for example: *Cellulomonas* species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* have cellulolytic property. Cellulase enzymes secreted by such microbes are classes of Glycoside Hydrolases (GHs). Depending on the initial biomass source and environmental factors the combination of enzyme and microbe may vary among different

biomass degrading ecosystem. Few evidences from previous studies done in Bangladesh reveals that at present due to the urbanization and rapid growth of population, municipal solid waste is raising in amount causing a threat for the environment. It mostly consists of cellulosic organic content which could be easily used as crude materials to remove municipal solid wastes from the environment using potent organisms from the studies. This biological conversion of solid wastes to bio resources could be used to prevent environmental pollution and public health hazards in urban areas which makes cellulase research imperative in Bangladesh (Shohag et al., 2013). Also, cellulases have a high demand in the textile indutry for sustainable washing as a finishing treatment in the denim garments in order to make them comfortable to wear. The need to import cellulases by means of expensive transportation can be prevented if the enzyme is produced in Bangladesh in a satisfactory amount and hence, the economy can be expanded. New enzyme sources can be developed using emerging biotechnological tools to provide desirable enzyme features, including higher specific activities with more balanced synergism, better thermal stability, better resistance to environmental inhibitors and improved combination of various enzymes' activities that maximize sugar yields at low cost (Yang *et al.,* 2011). There were attempts being made to increase the production of cellulase from bacteria by mutation, protoplast fission, optimization of medium composition and environmental factors in the past (Shenkani & Sundara, 2015).

1.2 Literature review

1.2.1 Enzyme and historical resume

Life happens to be an intricate meshwork which involves a vast majority of chemical reactions taking place in perfect order. Some of these reactions synthesize large molecules while other reactions cleave large molecules and all of them either utilize energy or liberate energy. All the living cells carry on their life processes in low temperature and atmospheric pressure in spite of the fact that these conditions trigger to slow down the reactions. These reactions proceed in extremely high rate in living cells due to the presence of the particular catalysts synthesized or produced inside the body of organism. These biological catalysts were previously known as "fermenters". In order to designate those catalysts, Friedrich Wilhelm Kuhne coined the name "enzyme" in 1878. They were further titled as "manifestations of nature's impatience" as they quicken most of the chemical reactions occurring inside the body.

It started with the work of Dubrunfaut in 1830 who prepared malt extract from germinating barley seeds which possessed the power of converting starch into sugar. In 1833, Payen and Perfoz prepared amylase from malt extract by precipitation from alcohol while Horace De Sausure prepared a substance performing like amylase to convert starch into sugar. Next, Theodor Schwann succeeded in extracting pepsin to digest meat and trypsin, a peptidase in digestive fluids. Hence, the diastases (early names of enzymes) extended to animals. In, 1837 Jones Jacob Berzelius discovered the catalytic nature of these biological diastases. In 1857, Pasteur demonstrated the alcoholic fermentation which explains that the catalytic action could be induced by living cell such as organized ferments (bacteria and yeast) or by non living substances such as unorganized ferments (diastases). Kuhne proposed enzyme to be unorganized ferments while Eduard and Hans Buchner carried out the alcoholic fermentation with yeast extract claiming cell free fermentation in 1897. In 1926, James B. Sumner isolated and purified urease and confirmed the proteinaceous nature of the enzymes. Since then around 250 enzymes have been obtained in pure crystalline form such as trypsin, catalase, Rnase etc. In 1983, the cellulosome concept was discovered in *Clostridium thermocellum*, in which the cellulases were organized into a high molecular weight cellulolytic complex. In the upcoming years, various cellulase genes from this bacterium were cloned and sequenced and their modular structure was recognized (Bayer *et al.,* 1998).

1.2.2 Enzyme classification

With a gradual increase in the knowledge of enzymology, multiple criteria have evolved to name and classify enzymes. Enzymes are divided into six major classes with several sub classes. It includes oxidoreductases which are involved in oxidation and reduction, transferases that transfer functional groups (e.g. amino or phosphate group), hydrolases transfer water that is they catalyze the hydrolysis of a substrate, lyases add (or remove) the element of water, ammonia or CO_2 to (or from) double bonds, isomerases catalyze rearrangements of atoms within a molecule and ligases join a molecule. Carbohydrate hydrolyzing enzymes fall under hydrolases which includes glycosidases such as: cellulase, amylase, sucrase, lactase and maltase (Pratt & Voet, 1999). Glycoside hydrolases (GHs) are enzymes that catalyze the hydrolysis of the glycosidic bond between two carbohydrate residues or a carbohydrate unit linked to a non-carbohydrate aglycon unit.

1.2.3 Families and folds of Glycosyl hydrolases

Glycosyl hydrolases has been classified into more than 45 families on the basis of the similarities on the amino acid sequence with an expectation that it may facilitate the derivation of useful information from the structure and function of these enzymes. Behind this classification was the idea of finding proteins with sufficient similarities in the folds within a family to allow homology modeling. Due to the evolutionary divergence to acquire new specificities, enzymes with different substrate specificities are sometimes found in the same family such as families 1, 13 and 16. On the other hand, enzymes that hydrolyze the same substrate are sometimes found in different families for example: cellulases are found in 13 families. So, the classification based on the sequence and the structural, differs significantly from that of the International Union of Biochemistry (IUB) nomenclature of enzymes based mostly on substrate specificity.

Since the 3D structure of protein is more highly conserved than their sequences. Several sequence based families are found to have related folds. For instance, structural similarity was suggested for family 11 xylanases and family 12 cellulases (Torronen *et al.,* 1993) whereas similarity in the arrangement of catalytic residues and a fold is found in the family 7 with the β-1,3-glucanases and β-1,3-1,4-glucanases of family 16 (Divne & Jones, 1994). All of these family groupings represent the strict conservation of the catalytic machinery and mechanism during evolution.

Table 1.1 reports those various glycosyl hydrolases families for which at least one 3D structure has been determined along with the mechanism of glycosidic bond hydrolysis (Davies & Henrissat, 1995).

Table 1.1 Structure and mechanism in different families of glycosyl hydrolases.

Family	Enzyme	Organism	EC number	PDB* code	Mechanism
1	β-glucosidase	*Trifolium repens*	3.2.1.21	–	retaining
2	β-galactosidase	*Escherichia coli*	3.2.1.23	1BGL	retaining
5	endoglucanase A	*Clostridium cellulolyticum*	3.2.1.4	–	retaining
6	cellobiohydrolase II	*Trichoderma reesei*	3.2.1.91	3CBH	inverting
	endoglucanase	*Thermononospora fusca*	3.2.1.4	1TML	inverting
7	cellobiohydrolase I	*Trichoderma reesei*	3.2.1.91	1CEL	retaining
	endoglucanase I	*Humicola insolens*	3.2.1.4	–	retaining
9	endoglucanase D	*Clostridium thermocellum*	3.2.1.4	–	inverting
10	xylanase A	*Streptomyces lividans*	3.2.1.8	1XAS	retaining
11	xylanase	*Bacillus circulans*	3.2.1.8	1BCX	retaining
13	α-amylase	*Aspergillus oryzae*	3.2.1.1	6TAA	retaining
14	β-amylase	*Glycine max*	3.2.1.2	1BTC	inverting
15	glucoamylase	*Aspergillus awamori*	3.2.1.3	3GLY	inverting
16	β-1,3-1,4-glucanase	*Bacillus sp.*	3.2.1.73	1BYH	retaining
17	β-1,3-1,4-glucanase	*Hordeum vulgare*	3.2.1.73	1GHR	unknown†
18	chitinase	*Serratia marcescens*	3.2.1.14	1CTN	retaining
19	chitinase	*Hordeum vulgare*	3.2.1.14	1BAA	inverting
20	chitobiase	*Serratia marcescens*	3.2.1.52	–	retaining
22	lysozyme	Hen egg white	3.2.1.17	1HEL	retaining
23	lysozyme	Goose	3.2.1.17	153L	unknown
24	lysozyme	Bacteriophage T4	3.2.1.17	1LYD	unknown
33	sialidase	*Salmonella typhimurium*	3.2.1.18	2SIL	retaining
34	neuraminidase	Influenza virus B	3.2.1.18	1NSB	retaining
45	endoglucanase V	*Humicola insolens*	3.2.1.4	1ENG	inverting
48	cellobiohydrolase CelS (Cel48A)	*Thermobifida fusca*	3.2.1.176	_	inverting
	endo-β-1,4-glucanase	*Thermobifida fusca*	3.2.1.4	_	inverting
	chitinase	*Serratia marcescens*	3.2.1.14	_	inverting

Many glycosyl hydrolase have a modular structure that consists of a catalytic domain and one or more non catalytic domains. Some of the non catalytic domains contribute for substrate binding while most of them has unknown functions. Since, this dissertation focuses on the cellulase enzyme and there are 13 families of cellulases (family 1,2,5,6,7,9,16,17 and 45; out of which family 6 and 7 contains two types of cellulases in each) according to the table, the main folds in the catalytic domains of the enzymes of those families are shown below (Davies & Henrissat, 1995):

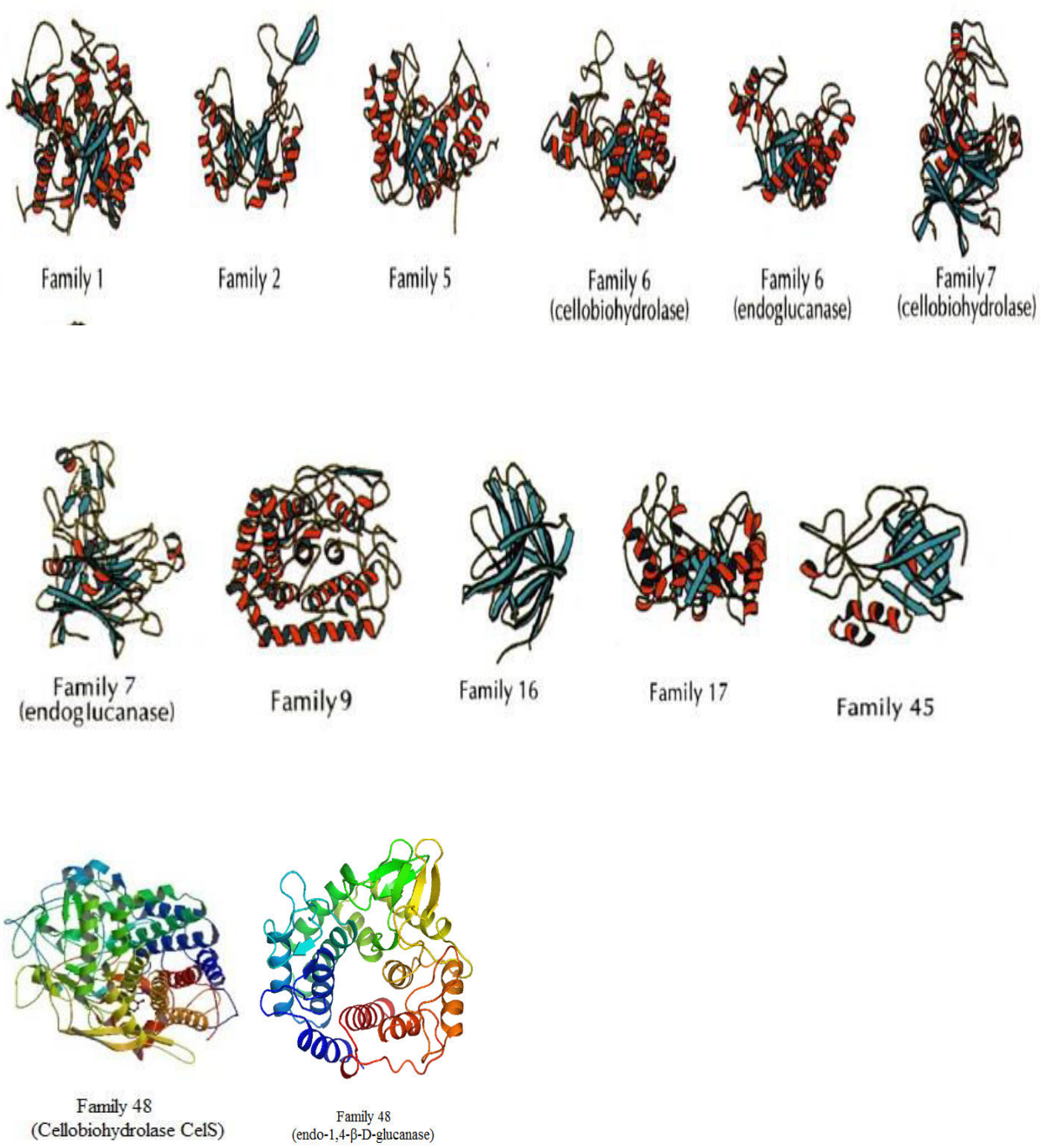


Figure 1.1 Main folds of the catalytic domains of the enzymes belonging to the respective families

Substrates for these enzymes are shown below:

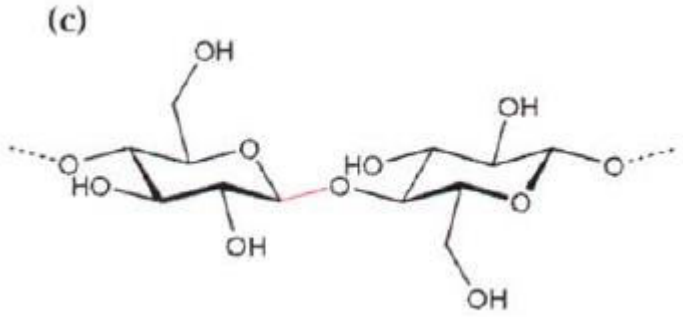


Figure 1.2: Cellulose for endoglucanases and cellobiohydrolases of families 5,6,7,9,45 and 48.

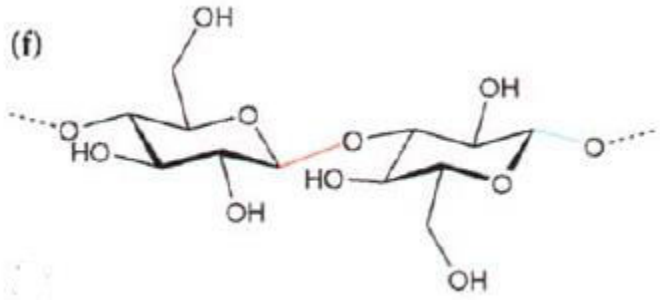


Figure 1.3: Mixed β-1,3-1,4-glucan with the bond hydrolyzed by β-1,3-glucanases (family 16) in red and that cleaved by β-1,3-1,4-glucanases (family 17) in blue.

1.2.4 Structure of cellulase

Proteins with hydrolytic activity such as cellulase comprises of a catalytic domain (CD) and one or more cellulose binding domain (CBD) joined by unstructured linker sequences. The catalytic domain covers more than 70% of protein sequence. The sequence analysis of this domain is significantly variable in different cellulases in fact having three dimensional arrangements (pocket or crater, cleft or groove and tunnel). It is N-glycosylated and cleave glycosidic bond with the acid hydrolysis mechanism using a proton donor and a base such as glutamic and aspartic acid. CBDS assist in the hydrolysis by keeping the substrate closer to CD increasing the rate of catalysis during starting and processivity. It is O-glycosylated and occurs as single,

double or even triple domain in a protein. They can be found in either C or N terminal and occasionally in the centre. Upon removal of the cellulose binding modules (CBM) from the enzyme or from the scaffolding in cellulosomes dramatically decrease its enzymatic activity. The linker peptide is a sequence of amino acids connecting the CBD and CD. This linker consists of 6-59 amino acids and functions as a flexible hinge allowing independent function of each domain. The sequence varies among cellulases. Treonine and serine residue of the linker is highly O-glycosylated to prevent proteolysis. In case of its absence, the CBD and CD obstruct each other and the affinity reduces (Castañeda & Mallol, 2013).

1.2.5 Cellulase systems

Cellulase systems of microorganisms can be regarded as complexed and non complexed. They consist of either secreted or cell associated enzymes categorized according to their mode of action and structural properties. Three major types of cellulase activities recognized are:

- **Endoglucanases**: they cut at random internal amorphous sites in the cellulose polysaccharide chain generating oligosaccharides and new chain ends.
- **Exoglucanases**: they act on the reducing and non reducing ends of the cellulose chains liberating cellobiose or cellooligosaccharides as major products.
- **β-Glucosidases**: they break down soluble cellodextrins and cellobiose to glucose.

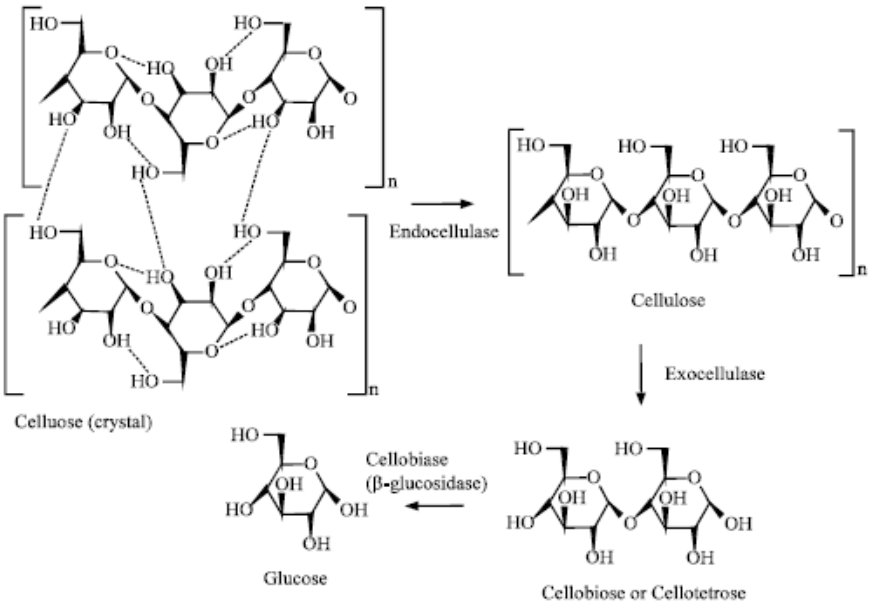


Figure 1.4: Mode of action of cellulase enzyme

Non complexed cellulase system: Aerobic fungi and bacteria mostly have free and secreted cellulase system. Typical example can be cellulase system of *T. reesi*. Fungi produce two exoglucanases CBHI and CBHII, eight endoglucanses EGI-EGVIII and seven β-glucosidases BGI-BGVII. Aerobic bacteria also produce all components of cellulytic system including exo and endo glucanases.

Complexed cellulase system (cellulosomes): It is native to anaerobic bacteria and contains stable enzyme complexes. In *C.thermocellum*, the cellulosome consists of a non catalytic cipA protein having different catalytic modules responsible for exo and endo glucanase activity. Cellulosomes vary among organism (Sukumaran *et al.,* 2005).

1.2.6 Cellulose – the substrate

Cellulose is the most common organic polymer in the total annual biomass production by photosynthesis process especially in the tropics and considered as an abundant and renewable raw material for production of different products (Klemm *et al.,* 2002). An efficient way for the utilization of this sustainable resource is the microbial hydrolysis lignocellulosic waste and fermentation of the resultant reducing sugar for the production of desired metabolites or bio fuels. Lignocellulose happens to be the major structural component of all cell plants and a

renewable organic material containing three vital parts: cellulose (40-50%), hemicellulose (20-40%) and lignin (20-30%) (Castañeda & Mallol, 2013).

Cellulose is a crytalline polymer which is an unusual feature to acquire. The crystalline structure of cellulose was discovered in the 19th century and first established by Carl von Nageli in 1858 varified by X ray crystallography. Cellulose chains are connected tightly by inter and intra chain hydrogen bonds and the adjacent sheets which overlie one another are held together by weak Van Der Waals forces. In nature, cellulose fibers are embedded in a matrix of other biopolymers such as hemicellulose and lignin (Marchessault & Sundararajan, 1993 and Lynd *et al,*. 1999). An important feature of this crystalline array is that relative impermeability towards large molecules like enzymes as well as small molecule like water. There are crystalline (ordered) and amorphous (less ordered) region in the polymeric structure along several types of surface irregularities (Cowling, 1975 and Fan *et al,*. 1980). The heterogeneous structure makes the fiber to swell when partially hydrated resulting in the micro pores and cavities become sufficiently large to allow penetration of larger molecules like enzymes (Sukumaran *et al.,* 2005).

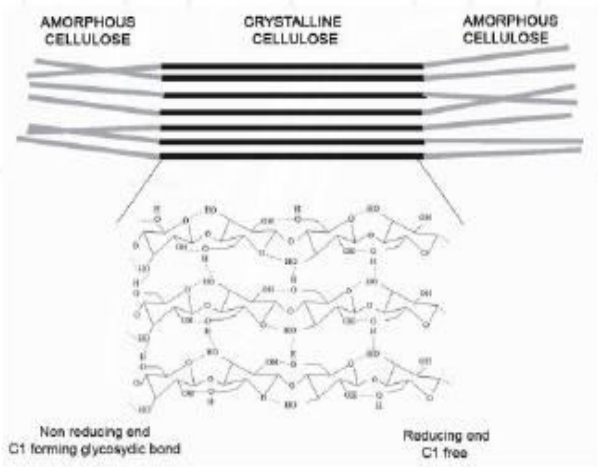


Figure 1.5: Crystalline and amorphous structure of cellulose. The crystalline structure is conserved by hydrogen bonds and Van Der Waals forces, in amorphous structure exists twists and torsions that alter the ordered arrangement. Reducing and non-reducing are shown.

At the molecular level, cellulose is a linear polymer of glucose composed of anhydoglucose units coupled to each other by β-1, 4- glycosidic bonds. The number of glucose molecules in the cellulose molecules varies and degree of polymerization ranges from 250 to well over 10.000 depending on the source and treatment method (Klemm *et al.,* 2005). The nature of cellulosic substrate and its physical state is important for enzymatic hydrolysis. Al though. Lignocellulosic materials are to recalcitrant to microbial actions, suitable biomass pretreatment is required to disrupt the lignin structure and increase the accessibility of enzyme. Thus, the rate of biodegradation can be increased along with high yields using low amount of enzyme (Lynd *et al.,* 2002 and Yang *et al.,* 2011).

1.2.7 Cellulose degradation mediated by non cellulosomal enzyme

Aerobic cellulolytic bacteria and fungi degrade cellulose with the help of sets of soluble cellulases. Once the cellulase recognizes a free chain end, it threads the chain into the tunnel to form catalytically active complex (CAC). Cellulose degradation in water is energetically unfavourable. Therefore, the tunnels or clefts of the CDs contain hydrophobic and polar residues that form favorable contacts with the chain. Once the cellulase forms a CAC with a cellodextrin chain, depending on the directionality of the enzyme the degradation of the cellulose begins through a retaining or inverting mechanism. After the reaction the product is expelled so that another CAC can be formed by threading another cellobiose unit into the CD. Two amino acid residues are responsible for the hydrolysis of glycosidic bond: an acid as proton donor and a base. Depending on the position of these catalytic residues the hydrolysis occurs via either overall retention or inversion of anomeric carbon (Castañeda & Mallol, 2013).

- The retaining glycoside hydrolase mechanism produces a compound after hydrolysis that has the same configuration of the anomeric compound as the substrate had before hydrolysis causing a net retention of the configuration.

- The inverting glycoside hydrolase mechanism causes inversion of the configuration at the anomeric carbon by a single nucleophilic displacement where the hydrolysis of a glycosidic bond creates a product with the configuration and vice versa (Castañeda & Mallol, 2013).

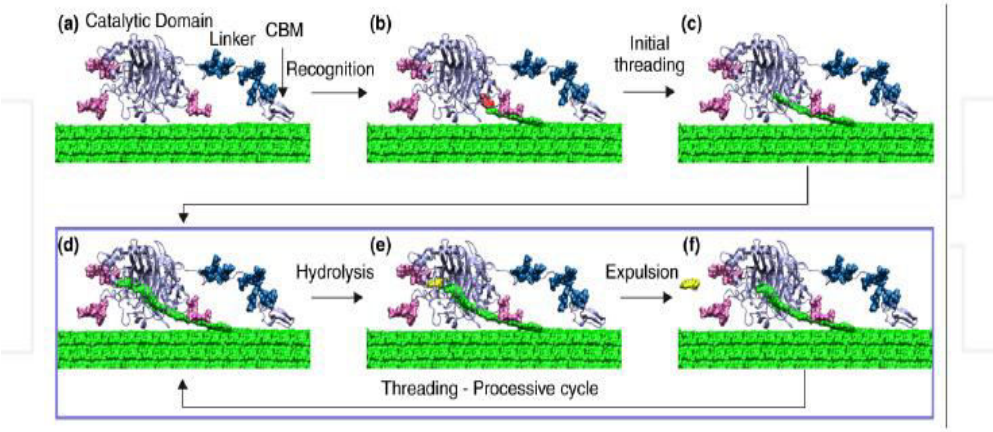


Figure 1.6: Non cellulosomal enzyme processivity: a) Cel7A binding to cellulose, b) recognition of a reducing end of a cellulose chain, c) initial threading of the cellulose chain into the catalytic tunnel, d) threading and formation of a catalytically active complex, e) hydrolysis in a processive cycle and f) product expulsion and threading of another cellobiose

1.2.8 Cellulose degradation mediated by cellulosome

The cellulosome structure is made up of two components: a) scaffolding proteins having no enzymatic activity with enzyme binding sites called cohesins; b) enzymes with dockerins proteins interacting with cohesins in the scaffolding protein. The number of cohesins on the scaffolding protein and CBM varies upon the bacterial species. They bind the cellulosome tightly to the substrate and concentrate the enzyme to a particular site of the substrate. The cohesin-dockerin interaction interconnects the different scaffolding component where the specificities among the complexes direct the overall supramolecular architecture of the participating components (Bayer *et al.*, 2004).

An hypothetical organization of *C.thermocellum* cellulosome reveals nine type I cohesins of a scaffolding protein, cellulosome integrating protein (CipA), interact with type I dockerins on various catalytic subunits. A CBD of CipA enters into cellulose and disorder its crystalline structure to supply the catalytic subunits with easily hydrolysable substrates i.e. amorphous region in the cellulose. CipA is attached to the cell by scaffolding dockerin binding (SdbA) containing S layer homologous (SLH) domains and a type II cohesin. Interaction between

cohesins and dockerins is mediated by Ca^{2+} ion. Outer layer protein (OlpA) contains a type I cohesin and three SLHs anchor a protein containing a type I dockerin (Ohmiya *et al.,* 1997).

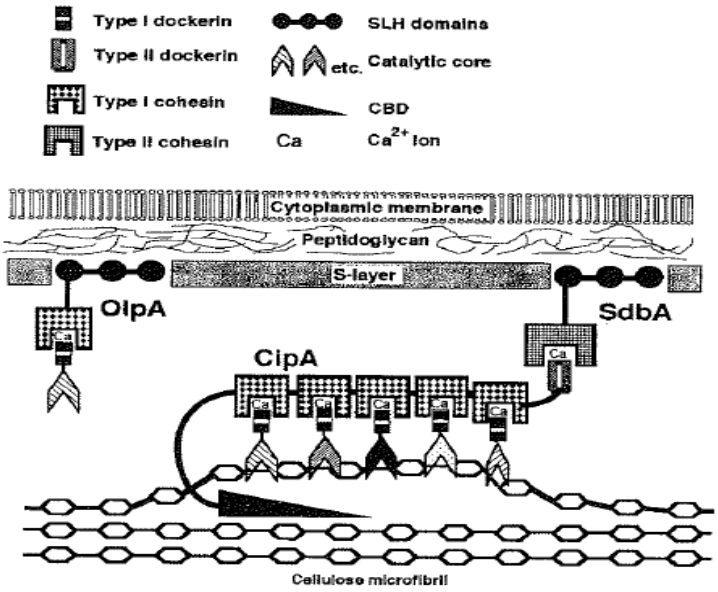


Figure 1.7: The cellulosome of *C.thermocellum*

To sum up, the enzymatic cellulosome system is greater in terms of potentiality than non cellulosomal degradative system due to its structural organization, efficient binding to the substrate and the variety of hydrolytic enzymes acting synergistically (Ohmiya *et al.,* 1997).

1.2.9 Major microorganisms employed in cellulase production

Cellullolytic microorganisms are carbohydrate degraders and unable to utilize proteins and lipids as energy source (Lynd *et al.,* 2002). Although cellulolytic microbes especially *Cellulomonas* and *Cytophaga* along with fungi can utilize other carbohydrates in addition to cellulose (Poulsen & petersen, 1988; Rajoka & Malik, 1997) whereas anaerobic cellulolytic microbes tend to utilize cellulose only (Ng & Zeikus, 1982; Thurston, 1993). The ability of secreting large amount of extracellular protein associated with large amount of cellulase production is a characteristic of some particular strains of fungi and bacteria. For example, one extensively studied fungi *Trichoderma reesei* can convert native also cellulose to glucose. Most commonly studied fungi are *Trichoderma, Humicola, Penicillium, Aspergillus* and among bacteria are *Bacilli, Pseudomonas, Cellulomonas* and Actinomycetes are *Streptomyces* and *Actinomucor.*

Several fungi can metabolize cellulose as energy source while other secretes complex cellulase system that has applications in enzymatic hydrolysis of cellulose. Besides *Trichoderma, Humicola, Penicillium* and *Aspergillus* are able to yield high level of extracellular cellulases. Aerobic bacteria such as *Cellulomonas, Cytophaga* and *Cellovibrio* can degrade cellulose in pure culture (Sukumaran *et al.,* 2005). However, the microbes commercially exploited for cellulase preparations are mostly limited to ones mentioned below:

Table 1.2: List of microorganism used for cellulase production (Yang *et al.,* 2011)

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

Filamentous fungi (aerobic)		
Acremonium cellulolyticus	NC/HC	M
Acrophialophora nainiana	HC/HC	M
Aspergillus acculeatus	NC/HC	M
Aspergillus fumigatus	NC/HC	M/T
Aspergillus niger	NC/HC	M
Aspergillus oryzae	NC/HC	M
Fusarium solani	NC/HC	M
Humicola grisea var. *thermoidea*	NC/HC	T
Irpex lacteus	NC/HC/LN	M
Penicillium funmiculosum	NC/HC	M
Penicillium atrovenetum	NC/HC	T
Penicillium citrinum	NC/HC	M
Phanerochaete chrysosporium	NC/HC/LN	M
Schizophyllum commune	NC/HC	M
Sclerotium rolfsii	NC/HC	M
Sporotrichum cellulophilum	NC/HC	T
Talaromyces emersonii	NC/HC	T
Thielavia terrestris	NC/HC	T
Trichoderma koningii	NC/HC	M
Trichoderma. reesei	NC/HC	M
Trichoderma viride	NC/HC	M

Anaerobic fungi		
Anaeromyces elegans	NC/HC	M
Anaeromyces mucronatus	NC/HC	M
Caecomyces CR4	NC/HC	M
Neocallimastic frontalis	Cellulosome	M
Neocallimastic hurleyensis	Cellulosome	M
Neocallimastic patriciarum	Cellulosome	M
Orpinomyces joyonii	Cellulosome	M
Orpinomyces PC-2	Cellulosome	M
Piromyces communis	Cellulosome	M
Piromyces equi	Cellulosome	M
Piromyces E2	Cellulosome	M

AT: Alkali tolerant; HC: Hemicellulase; LN: Ligninase; M: Mesophilic; NC: Noncomplexed cellulase; T: Thermophilic.

1.2.10 Applications of cellulases

Bioconversion of biomass using cellulase enzyme paved a way of research in the industrial applications of this enzyme in animal feed, food, textiles, detergents and paper industry. The uprising need for an alternative of renewable energy and fuels as well as shortage of fossil fuel have raised an interest over bioconversion of lignocellulosic material using cellulase enzyme. In other fields, however, the intensive use of this enzyme has made its use imperative (Sukumaran *et al.,* 2005)

Microbial cellulases find application in the following industries:

Table 1.3: Applications of cellulases (Kuha *et al.,* 2011)

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

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

1.2.11 Aim of the study

Rapid depletion of the fossil fuel has created adverse effects on the environment by global warming and air pollution along with creating shortage of energy. Hence, lignocellulosic biomolecules are used as an alternative source of energy (Sharma *et al.,* 2004). Although, cellulases have a wide range of industrial applications in Bangladesh, the obstacles of developing cost effective processes for converting biomass to fuels and chemicals are yet to be fully realized. Hence, there's an urgent need of isolation of novel cellulase to discover and develop, that can overcome existing challenges in biorefinery, reduce biofuel production cost, as well as have tremendous applications in industrial processes (Rashid *et al.,* 2015; Shil *et al.,* 2014). The aim of this project includes:

- Isolation of cellulolytic bacteria from soil sample.
- Determination of the abundance of the cellulolytic bacteria in the collected sample.
- Screening for the best isolate through
  - Observation of clear zone of cellulose hydrolysis on CMC agar
  - Determination of cellulase activity using DNSA method
  - Determination of specific activity
- Identification of the best isolate by
  - Cultural, morphological and biochemical characteristics.
  - Sequencing of 16S rRNA gene and construction of phylogenetic tree.

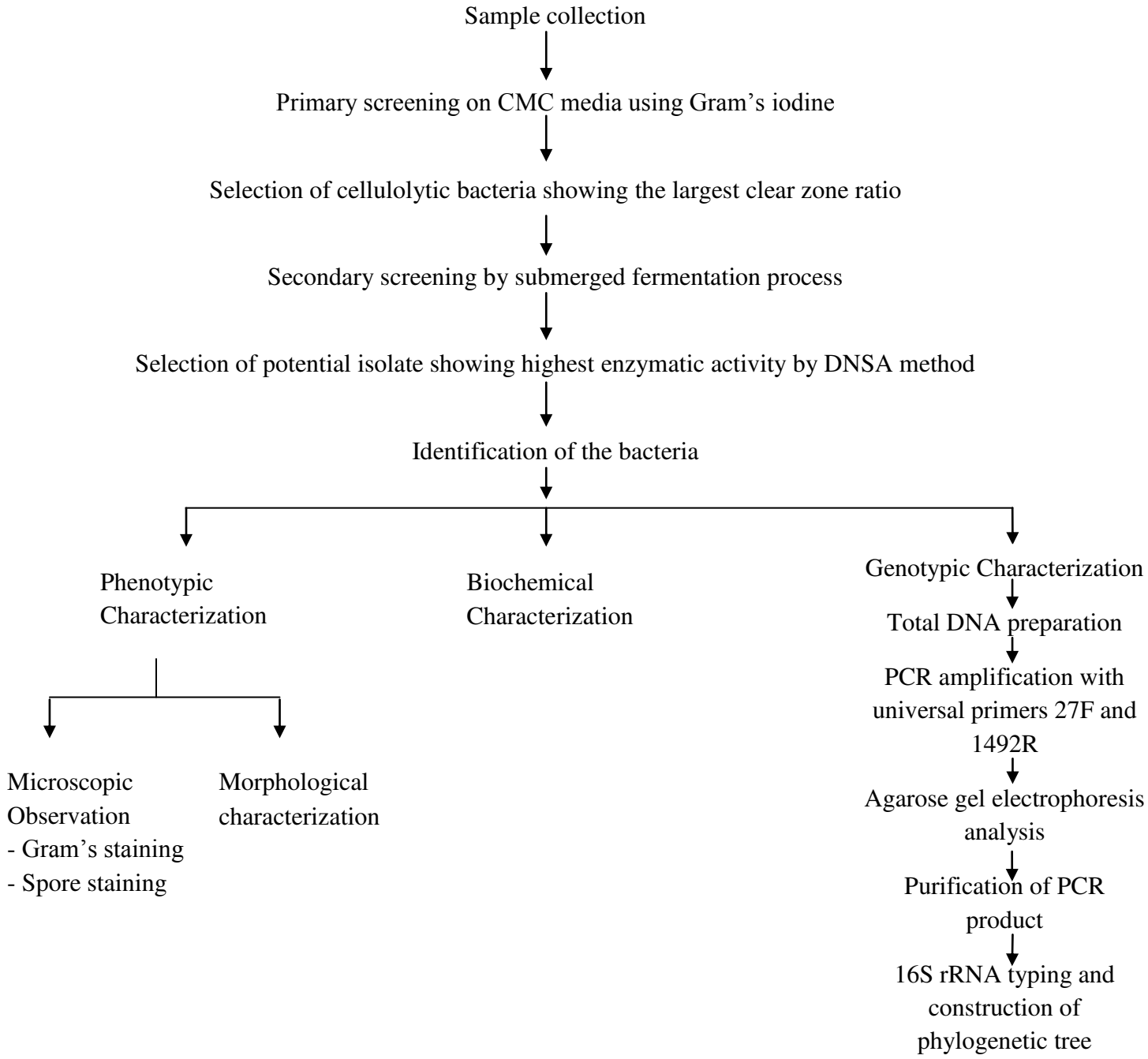
Chapter 2

2. Materials and methods

2.1 Study place

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

2.2 Flow Diagram of the Study Design



2.3 Handling of laboratory apparatus and glassware

All glasswares were washed with mild detergents, rinsed at least three times with distilled water and allowed to air dry. Petri dishes were heat sterilized at 180◦ for an hour in the sterilizer (Oven, Model: MH6548SR, LG, China) before use. Micropipette tips, glass pipettes and micro centrifuge tubes were sterilized by autoclaving at 121◦C for 15 min at 15 psi (SAARC).

2.4 Sample collection

Soil sample was collected from Bagan Bari Dairy Farm, Keranigonj. The samples were collected in sterile container and stored at 4◦C until use.

2.5 Serial dilution

Test tubes containing 9 ml of saline water were autoclaved before use. Ten fold serial dilutions of the soil sample were prepared in autoclaved saline water. Initially, 1 g of soil was mixed with 9 ml of saline water in a test tube. This makes a dilution of 10^{-1} which is further diluted in subsequent steps to obtain a final dilution of 10^{-6}.

2.6 Primary screening of cellulose degrading bacteria

0.1 ml sample from the test tubes labeled 10^{-4}, 10^{-5} and 10^{-6} were spread plated on NA plates. The NA plates were then incubated at 37◦C for 24 hours. The plates showing discrete colonies were selected for further study. The colonies were transferred using needle to carboxymethyl cellulose (CMC) media containing 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 1 % agar, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and 0.2 % gelatin (Irfan *et al.,* 2012). After incubation at 37◦C for 48 hours, the CMC agar plates were flooded with gram's iodine and allowed to stand at room temperature for around 10 minutes. The ratio of the clear zone diameter to colony diameter of CMC hydrolysis were measured and recorded (Shaikh *et al.,* 2013). The bacterial colonies having largest ratio were selected for cellulase production in submerged system.

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

2.7 Maintenance of pure culture

The colonies showing significant clear zone were streaked on to the Nutrient Agar medium, incubated at 37◦C for 24 hours and then stored at 4◦C until use (Immanuel *et al.,* 2006).

2.8 Secondary screening and production of cellulase enzyme

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

2.8.1 Inoculum development

One loopful of bacterial colonies from the pure cultures of the selected bacterial isolates were inoculated in inoculum broth containing 0.03% $MgSO_4$, 0.2% K_2HPO_4, 1% glucose, 0.25% $(NH_4)_2SO_4$ and 1 % peptone and incubated at 37◦C for 24hrs of fermentation period. After 24hr these vegetative cells were used as inoculum source for the production medium (Irfan *et al.,* 2012).

2.8.2 Submerged fermentation process

50 ml of CMC broth containing 1.0 % peptone, 1.0 % carboxymethylcellulose (CMC), 0.2 % K_2HPO_4, 0.03 % $MgSO_4.7H_2O$, 0.25 % $(NH_4)_2SO_4$ and 0.2 % gelatin was prepared in 250 ml Erlenmeyer flasks for each of the selected isolated strains and autoclaved at 121◦C for 15 minutes. The media were inoculated with 2.5ml of selected bacterial isolate from the inoculum media and incubated at 37◦C for 48hrs at 150 rpm (Shaikh *et al.,* 2013).

2.8.3 Preparation of crude enzyme

Following fermentation, the fermented broth was centrifuged at 16000rpm for 10 minutes in order to remove unwanted materials. After centrifugation the clear supernatent was collected to serve as crude enzyme source and utilized for determination of enzymatic activity (Shaikh *et al.,* 2013).

2.9 Cellulase enzyme assay

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

2.9.1 Preparation of glucose standard curve

Initially, 0.2- 1.2mg/ml concentrations of glucose solutions were prepared. 1 ml from each of the dilutions were transferred in the test tubes labeled of respective concentration along with 1 extra test tube in which 1 ml of distilled water was transferred to be served as control. 1 ml of 0.05M citrate buffer (pH 4.8) was added in all the test tubes followed by 3 ml of Dinitrosalicylic acid (DNS) reagent. All the test tubes were then placed in boiling water (100◦C) for 10 minutes in water bath (Water bath, korea). Afterwards the test tubes were taken out from the water bath and allowed to cool to room temperature. The absorbances of each of the sample were then measured at 540 nm in a spectrophotometer. A standard curve was prepared plotting the standard glucose concentration (mg/ml) (x-axis) against the respective absorbance (y-axis) in order to achieve a best fit straight line (Ghose, 257-268).

2.9.2 Reducing sugar estimation by Dinitrosalicylic acid (DNSA) method

The amounts of reducing sugar liberated in the hydrolysis were measured by DNSA method. The enzyme unit (EU) was determined as the amount of cellulase required to release 1 µmole of reducing sugar per ml per min under the following standard assay conditions (Shaikh *et al.,* 2013):

The cellulase activities of each of the bacterial strains were assayed in triplicate. The reaction mixtures contained 1ml 0.05M citrate buffer (pH 4.8), 1 ml substrate solution (1% CMC dissolved in citrate buffer) and 1 ml crude enzyme solution. The blanks contained 1 ml distilled water instead of the enzyme. The reaction was carried out at 50◦C for 30 minutes. After incubation, 1.5 ml of DNS reagent was added to all the test tubes in order to stop the reaction, waited for 10 minutes and then boiled at 100◦C for 10 minutes in water bath. The absorbance was measured at 540 nm. Glucose liberation was estimated using glucose calibration curve (Shoham *et al.,* 1999) and enzymatic activity was determined by the following equation (Islam *et al.,* 2014):

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

2.10 Determination of extracellular protein concentration

Protein concentration of the crude enzyme samples were determined by the Folin lowry method using Bovine Serum Albumin (BSA) as standard and expressed as mgml^{-1} (Lowry *et al.,* 1952).

Different dilutions of BSA solutions were prepared by mixing stock BSA solution (mg/ml) and distilled water in the test tubes. The final volume in each of the test tube was 5 ml. The BSA range was from 0.05 to 1mg/ml. From this different dilutions, 0.2 ml of protein solutions and the crude enzymes from both the isolates were transferred to different test tubes and 2 ml alkaline copper sulphate reagent (analytical reagent) was added. The solutions were then mixed properly. After that, the solutions were incubated at room temperature for 10 minutes followed by adding 0.2ml of reagent Folin Ciocalteau solution (reagent solutions) to each tube and incubated for 30 minutes. Then the absorbances were measured at 650 nm using spectrophotometer (UVmini-1240, Shimadzu Corporation). The absorbances were plotted (y-axis) against protein concentrations (x-axis) to get a standard calibration curve with a best fit line. The absorbances of the unknown samples were measured and the concentrations were determined using the standard curve.

2.10.1 Determination of specific activity

Specific activity of the crude enzyme was determined using the following formula:

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

The potential isolate with the highest enzyme activity as well as specific activity was chosen for carrying out the further characterization and identification steps.

2.11 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 autoclaved centrifuge tubes. It was then sealed with parafilm tapes. These were stored in -20◦C refrigerator.

2.12 Identification of the bacteria

2.12.1 Morphological characterization of the bacteria

Using sterile technique, a NA plate was streaked to obtain isolated discrete colonies. The plates were then incubated at 37◦C for 24 hours. After incubation, the bacterial colonies were evaluated for size, pigmentation, form, margin, elevation and texture (Cappuccino & Sherman, 2005).

2.12.2 Microscopic Observation of the bacteria

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

2.12.2.1 Gram stain

Using sterile technique, a drop of saline was placed on the slide and a small amount of the bacteria were then transferred to the drop of saline with a sterile cooled inoculating loop. A smear was then prepared by mixing and spreading the bacteria by means of a circular motion of the loop. The smear was then allowed to air dry followed by heat fixation. The smear was flooded with crystal violet and let stand for 1 minute. Then, the smear was gently washed with tap water. It was then flooded again with the gram's iodine mordant and let stand for 1 minute followed by gentle wash with tap water. After that, the smear was decolorized with 95% ethyl alcohol and gently washed with tap water. Finally, it was counterstained with safranin for 45 seconds and gently washed with tap water. The slide was then blot dried with bibulous paper and examined under oil immersion (Cappuccino & Sherman, 2005).

2.12.2.2 Spore stain

Nutrient agar was prepared with 5 mg/L of $MnSO_4.H_2O$ in order to incubate the bacteria for 48 hours in a nutrient deficient condition to encourage sporulation (Logan & Vos, 2009). After incubation, using sterile technique, smear was prepared in the usual manner on clean glass slide. It was allowed to air dry and heat fixed in the usual manner. The smear was flooded with malachite green while placed over a water bath and allowed to steam for 2 to 3 minutes. The stain was prevented from drying out by constant application of the dye. The slides were removed, cooled and washed under running tap water. The smear was then counterstained with safranin for 30seconds and washed with tap water. The slide was then blot dried with bibulous paper and examined under oil immersion (Cappuccino & Sherman, 2005).

2.12.3 Biochemical characterization of the bacteria

Several biochemical tests were carried out in order to have a presumptive identification of the potential bacteria chosen before. Most of the methods were done according to the microbiology laboratory manual (Cappuccino & Sherman, 2005). The biochemical tests performed were; Carbohydrate fermentation (Sucrose, fructose, glycerol, maltose and D-xylose), Triple sugar iron agar test, IMViC test (Indole production test, Methyl red test, Voges- Proskauer test, Citrate utilization test), Urease test, Nitrate reduction test, Catalase test, Oxidase test, Casein hydrolysis test, Motility test, Gelatin hydrolysis test, Mannitol Salt Agar, Starch hydrolysis, Blood agar, growth at 45◦C, 65◦C and 7% NaCl media and anaerobic growth.

2.12.3.1 Carbohydrate Utilization test

Phenol red sucrose, fructose, glycerol, maltose and D-xylose broths were prepared by autoclaving at 15 psi 121◦C for 15 minutes (Autoclave, SAARC) in separate test tubes. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the broths by means of loop inoculation. All the tubes were incubated for 24 hours at 37◦C (Cappuccino & Sherman, 2005).

2.12.3.2 Triple Sugar Iron Agar test

Triple sugar iron slants were prepared in the test tubes and autoclaved at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab and streak inoculation method. The tubes were incubated for 24 hours at 37◦C (Cappuccino & Sherman, 2005).

2.12.3.3 Indole Production test

Tryptophan broth of 5 ml in each test tube was prepared by autoclaving at 15 psi, 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes and the tubes were incubated for 48 hours at 37◦C. In order to test for indole production, 5 drops of kovac's reagent was added directly into the tubes (MacWilliams, 2009).

2.12.3.4 Methyl red test

MR-VP broth of 7 ml in each test tubes were prepared by autoclaving at 15 psi 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was

inoculated into the tubes and the tubes were incubated for 24 hours at 37◦C. After 24 hours 3.5 ml from the culture tubes were transferred to clean test tubes for Voges- Proskauer test and the remaining broth were re-incubated for additional 24 hour. After 48 hour incubation 5 drops of methyl red indicator was added directly into the remaining aliquot of the culture tubes to observe the immediate development of a red color. (Cappuccino & Sherman, 2005)

2.12.3.5 Voges Proskauer test

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

2.12.3.6 Citrate utilization test

Simmons citrate agar slants of 2 ml in each vials were prepared by autoclaving at 15 psi, 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the vials by means of a streak inoculation method with an inoculating needle and the vials were incubated for 48 hours at 37◦C (Cappuccino & Sherman, 2005).

2.12.3.7 MIU (Motility- Indole- Urease) test

MIU media was prepared by autoclaving at 15 psi, 121◦C. the media was cooled to about 50-55◦C and 100ml of urease reagent was added aseptically to 900 ml base medium. After that, 6ml solution was transferred to each sterile test tube and allowed to form a semi solid medium. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a stab inoculation method with an inoculating needle and the tubes were then incubated for 24 hours at 37◦C (Acharya, 2015).

2.12.3.8 Nitrate reduction test

Nitrate broth of 6 ml in each test tubes were prepared by autoclaving at 15 psi, 121◦C. Using sterile technique, small amount of the experimental bacteria from 24 hour pure culture was inoculated into the tubes by means of a loop inoculation method with an inoculating loop and the tubes were incubated for 24 to 48 hours at 37◦C. After incubation, 5 drops of reagent A and 5

drops of reagent B was added to each broth. If there was no red color development, a small amount of zinc was added to each broth (Cappuccino & Sherman, 2005).

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

2.12.3.9 Catalase test

A microscopic slide was placed inside a petri dish. Using a sterile inoculating loop, a small amount of bacteria from 24 hour pure culture was placed onto the microscopic slide. 1 drop of 3% H_2O_2 was placed onto the organism on the microscopic slide using a dropper and observed for immediate bubble formation (Reiner, 2010).

2.12.3.10 Oxidase test

A small piece of filter paper was soaked in Gaby and Hadley oxidase test reagent and let dry. Using an inoculating loop, a well isolated colony from pure 24 hour culture was picked and rubbed onto filter paper and observed for color change (Shields & Cathcart, 2010).

2.12.3.11 Gelatin hydrolysis test

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

2.12.3.12 Mannitol Salt Agar test

Using sterile technique, a plate of MSA agar was streaked by picking a loopful colony of 24 hour old pure culture to obtain isolated colonies. The plates were then incubated at 37◦C for 24 hours (Shields & Tsang, 2013).

2.12.3.13 Starch hydrolysis test

Using sterile technique, a starch agar plate was streaked by picking a loopful colony of 24 hour old pure culture with an inoculating loop. The plates were then incubated at 37◦C for 48 hours and the hydrolysis was observed using gram's iodine (Cappuccino & Sherman, 2005).

2.12.3.14 Casein hydrolysis test

Distilled water and agar solution was taken in separate conical flasks and both were autoclaved at 121◦C, 15 psi. Skim milk powder (28 g/L) was then added to the autoclaved distilled water aseptically and boiled for 1 minute to dissolve completely. After that, the milk solution was mixed with agar solution. The media was dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, a milk agar plate was inoculated by 24 hour old culture by means of streak plate method. The plates were then incubated at 37◦C for 24 hours (Sturm, 2013).

2.12.3.15 Blood agar test

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

2.12.3.16 Growth at 45◦C, 65◦C, 7% NaCl and anaerobic condition

Nutrient agar and nutrient agar supplemented with 7% NaCl were prepared in separate conical flasks and both were autoclaved at 121◦C, 15 psi. The media were then dispensed into sterile plates while liquid and left for a while to solidify. Using sterile technique, nutrient agar plates were streaked by picking a loopful colony of 24 hour old pure culture with an inoculating loop by means of streak plate method. The plates were then incubated at 45◦C and 65◦C for 24- 48 hours. The NA plates supplemented with 7% NaCl and the NA plates placed in anaerobic jar were incubated at 37◦C for 24 hours (Cappuccino & Sherman, 2005).

2.12.4 Genotypic characterization

In order to achieve the complete identification of the bacteria via molecular analysis the following procedures were carried out:

2.12.4.1 Total DNA preparation

The wizard® Genomic DNA Purification Kit was used in order to obtain genomic DNA from the potential experimental bacterial isolate.

One loopful of bacteria was aseptically transferred to Luria Bertani broth and allowed to grow overnight. 1 ml from the overnight culture was added to 1.5 ml micro centrifuge tube. Then it was centrifuged (Centrifuge; Eppendorf, Germany) at 13,500 RPM for 2 minute in order to separate the cells. The bacterial pellet was resuspended and DNA was extracted according to the protocol provided with the wizard® Genomic DNA Purification Kit. The DNA was stored at 2 to 8◦C until use.

2.12.4.2 Polymerase chain reaction (PCR)

The 16S rRNA gene from the isolated DNA was amplified by Polymerase Chain Reaction (PCR) using bacteria universal primers (27F – AGAGTTTGATCCTGGCTCAG and 1492R-GGTTACCTTGTTACGACTT). A reaction mixture was prepared containing 5µl of 10X reaction buffer that contains 1.5mM $MgCl_2$, 1µl of 10mM dNTPs and 39.75 µl of nuclease free water. Then each of the DNA primers was added in an amount so that the final concentration of DNA primers become 0.2mM in the final reaction mixture. Afterwards, 2µl of DNA template was added along with 0.25 µl of Taq polymerase. The final reaction volume should be 50 µl. All the steps were performed on ice.

Table 2.1: Reaction set up for PCR carried out in 50µl reaction volume

Ingredients	Volume Added
10x master mix	6 µl
Forward primer(10 mM)	1 µl
Reverse primer (10 mM)	1 µl
Template DNA	2 µl
Taq Polymerase	0.25 µl

Nuclease free water	39.75 µl

The PCR reactions were performed in a thermal cycler (Applied Biosystems, USA). The PCR program initiated with initial denaturation of template DNA at 94◦C for 2 minute, followed by 30 cycles of the following steps.

Table 2.2: PCR reaction condition

PCR condition	Temp (◦C)	Time	
Initial Denaturation	94	2 min	
Denaturation	94	30 sec	30 Cycles
Annealing	52	30 sec	
Extension	72	2 min	
Final Extension	72	5 min	

A single final extension was done at 72 ◦C for 5 minutes (Atanda *et al.,* 2014).

2.12.4.3 Detection of amplicon by electrophoresis analysis

After PCR reaction, amplification was checked by horizontal electrophoresis in 1.0% agarose slab gel in Tris –borate EDTA (TBE) buffer. Agarose was dissolved in 1x Tris borate EDTA buffer to give a final concentration of 1.0% agarose and was heated to dissolve in a microwave oven for about 30 seconds and then allowed to cool down to about 50◦C. Then it was poured on the tray previously set with the comb and allowed to solidify. 6µl aliquot of the PCR product was mixed 2µl of loading dye and was loaded into the individual well of the gel. 1 Kb plus DNA marker (Invitrogen, USA) was used to identify the amplicon size. The gel was then stained in staining solution (10µl EtBr in 150ml TBE) for 15-30min. and destained in distilled water for

10min. The EtBr stained DNA bands were observed on a UV transilluminator at 365 nm (UV Transilluminator, Wealtec).

2.12.4.4 Purification of PCR products

Amplified PCR products were purified by using the purification kit Wizard® SV Gel and PCR Clean-Up System.

2.12.4.5 Measurement of DNA concentration and purity

DNA concentration was also measured using NanoDrop 2000 spectrophotometer (Thermo scientific). 2 µl of nuclease free water in which DNA was eluted was used as blank. Another 2 µl of sample was loaded and DNA concentration was shown at ng/ µl unit. The OD260 /OD280 ratio was also shown with the software indicating the purity of the sample. Pure DNA preparations have OD260/OD280 values of 1.8.

2.13 Sequence analysis of 16s rRNA gene

These purified PCR products were sequenced by Sanger sequencing method using ABI Genetic Analyzer (Model: 3700) and was performed by 1st BASE Laboratories, Malaysia.

2.13.1 Sequence trimming and submission

The forward and reverse sequences of the DNA were screened for removal of non essential vector sequences using the program BLASTn by the software Seqman (Lasergene). The corrected partial sequence was then submitted to NCBI, Nucleotide databank for alignment with the closest species (Singh *et al.,* 2015).

2.13.2 Downloading homologous sequences

The corrected partial sequence in FASTA format was subjected to BLASTn to retrieve homologous sequences belonging to divergent species available at the NCBI database based on higher percentage identity and E value ($<10^{-5}$). A total of 10 divergent species and their respective sequences were downloaded and saved in FASTA format (Singh *et al.,* 2015).

2.13.3 Multiple sequence alignment

The sequences of 10 divergent species along with the query sequence in FASTA format were subjected to multiple sequence alignment using MEGA6 software (Tamura et al., 2013) to identify the evolutionary conserved regions of 16S ribosomal RNA gene among species (Singh *et al.,* 2015).

2.13.4 Phylogenetic inference

The MEGA6 software (Tamura *et al.,* 2013) was used for phylogenetic tree construction for determining the closest species. The phylogenetic tree of the sequences was inferred using maximum likelihood ratio (with 500 bootstrap replication) using the nucleotide substitution type and Tamura-Nei model, with uniform rates for rates among sites and with complete deletion of the gaps/ missing data (Singh *et al.,* 2015).

Chapter 3

3. Results

3.1 Primary screening

Total of 68 colonies from the dilution 10^{-5} plate were obtained and their ability to produce cellulase was determined by primary screening on Carboxy Methyl Cellulose (CMC) agar to select the potential isolates showing best zone of hydrolysis. The average of the ratio is given in the following table:

Table 3.1.1: The ratio of the clear zone diameter to the colony diameter of 68 isolates

Isolate number	Clear zone ratio (Average)	Isolate number	Clear zone ratio (Average)
1	1.32	**16**	No hydrolysis
2	2.00	**17**	No hydrolysis
3	2.20	**18**	No hydrolysis
4	No hydrolysis	**19**	2.01
5	3.27	**20**	No hydrolysis
6	1.07	**21**	No hydrolysis
7	1.41	**22**	No hydrolysis
8	No hydrolysis	**23**	No hydrolysis
9	No hydrolysis	**24**	1.43
10	No hydrolysis	**25**	No hydrolysis
11	No hydrolysis	**26**	3.78
12	No hydrolysis	**27**	1.84
13	No hydrolysis	**28**	1.33
14	No hydrolysis	**29**	1.91
15	3.80	**30**	Clear zone throughout the media

Table 3.1.1: The ratio of the clear zone diameter to the colony diameter of 68 isolates (continued)

Isolate number	Clear zone ratio (Average)	Isolate number	Clear zone ratio (Average)
31	1.80	**50**	No hydrolysis
32	2.96	**51**	No hydrolysis
33	3.20	**52**	No hydrolysis
34	Clear zone throughout the media	**53**	No hydrolysis
35	Clear zone throughout the media	**54**	No hydrolysis
36	No hydrolysis	**55**	No hydrolysis
37	2.50	**56**	No hydrolysis
38	2.30	**57**	No hydrolysis
39	1.78	**58**	No hydrolysis
40	1.00	**59**	No hydrolysis
41	No hydrolysis	**60**	No hydrolysis
42	No hydrolysis	**61**	No hydrolysis
43	1.57	**62**	1.95
44	2.00	**63**	2.50
45	Clear zone throughout the media	**64**	Clear zone throughout the media
46	No hydrolysis	**65**	1.94
47	4.87	**66**	3.85
48	2.29	**67**	3.00
49	1.74	**68**	1.13

From the table 3.1.1, both cellulase producing (31 colonies) bacteria and those unable to produce cellulase (32 colonies) were found along with bacteria that showed clear zone throughout the media (5 colonies). All the bacteria were able to grow on the CMC agar. The percentage of the types of bacteria is given in the following pie chart.

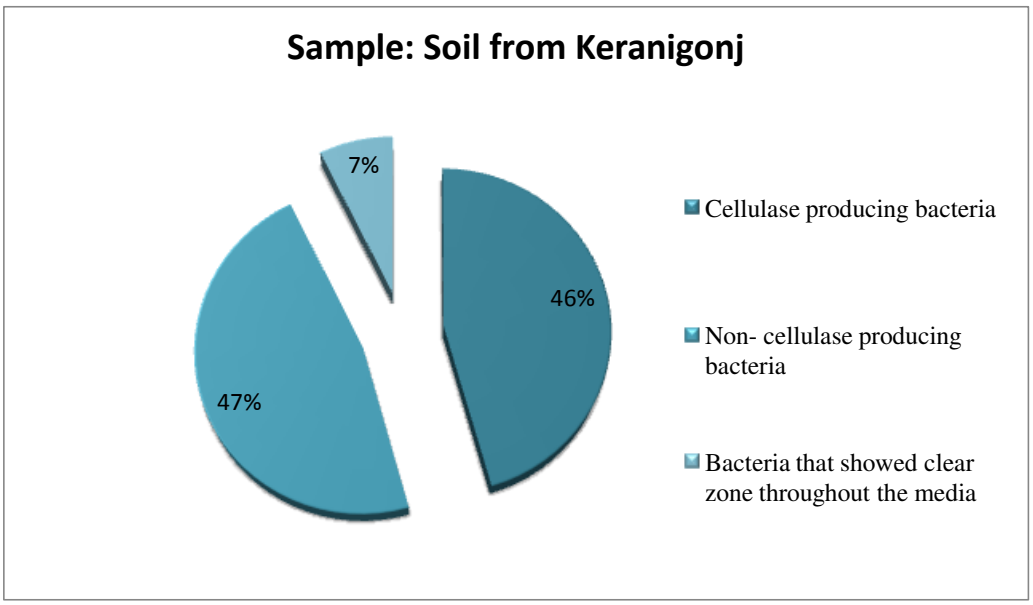


Figure 3.1.1: The percentage of the types of bacteria in the soil sample

The percentage of cellulase producers and non cellulase producers were almost equal. This revealed the sample source to be a good source of cellulase producing bacteria. However, there might be better natural sources that can be further studied.

The ratios of clear zone diameter to colony diameter of the 31 isolates giving zone of hydrolysis are shown in bar chart in figure 3.1.2:

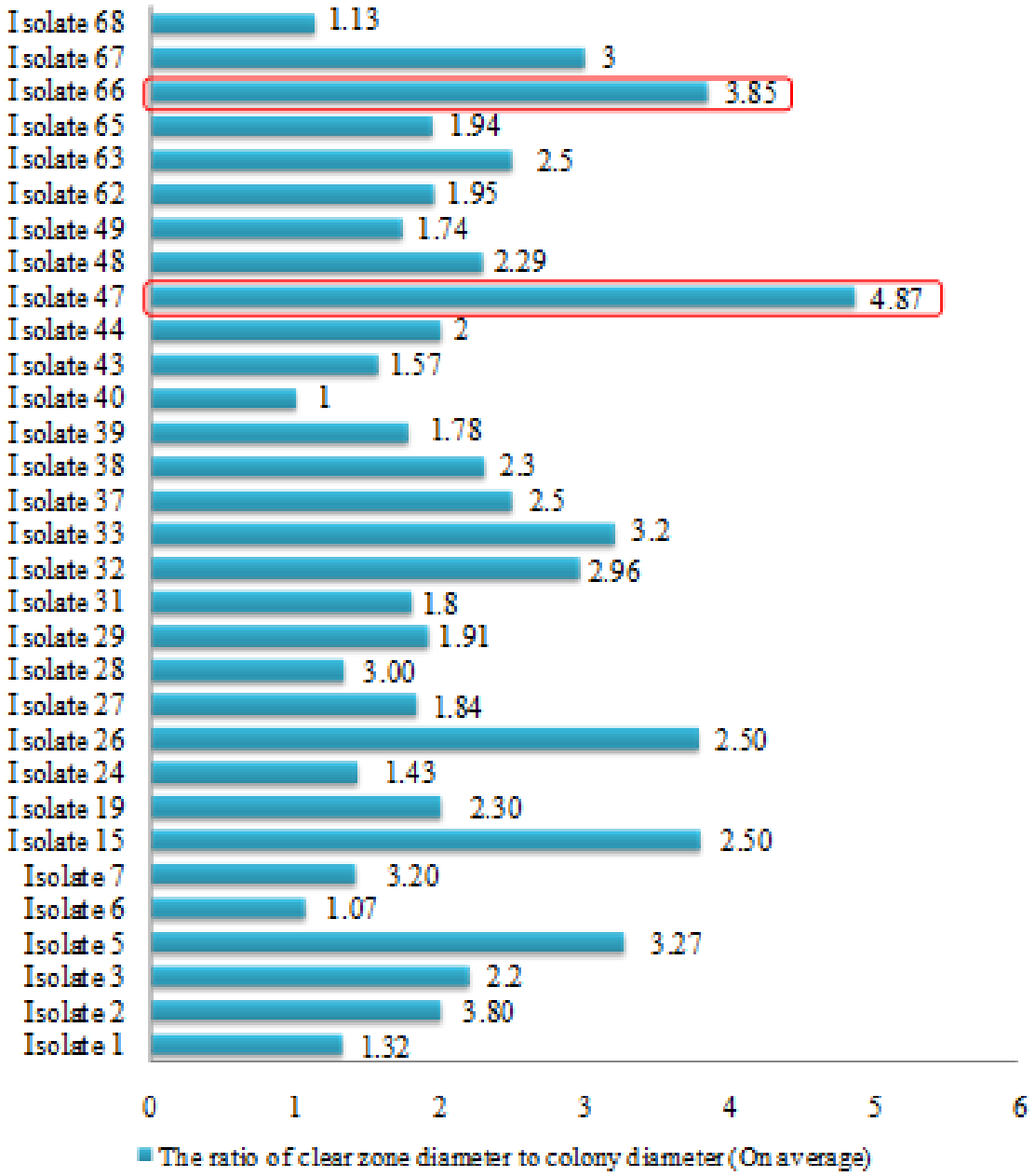


Figure 3.1.2: The ratio of clear zone diameter to colony diameter of the selected isolates

From the above 31 isolates, only two isolates (isolate 47 and 66) showing the best hydrolysis ratio were selected for further secondary screening.

Figure 3.1.3: Zone of hydrolysis of isolate 47 (left) and 66 (right)

3.2 Secondary screening

These two bacterial isolates (47 and 66) were screened for cellulase production in submerged fermentation process using CMC broth. A glucose standard curve was prepared for the estimation of reducing sugar (figure 3.2.1).

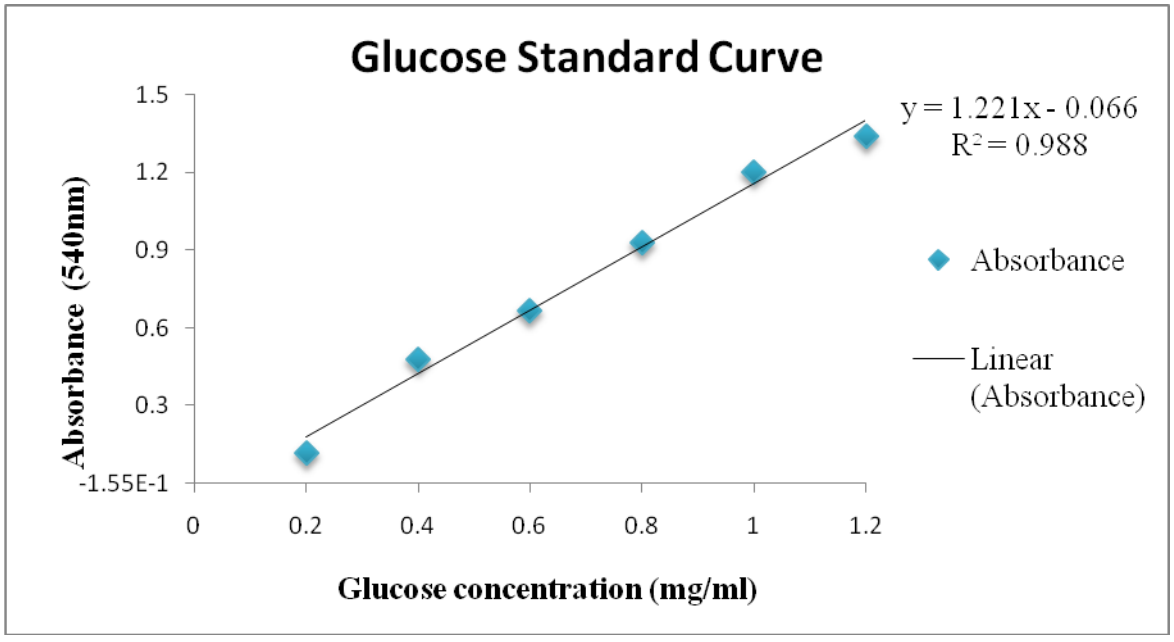


Figure 3.2.1: Glucose calibration curve

The cellulase activities of the isolate 47 and 66 were found to be 0.101 U/ml and 0.165 U/ml respectively. This is shown in figure 3.2.2.

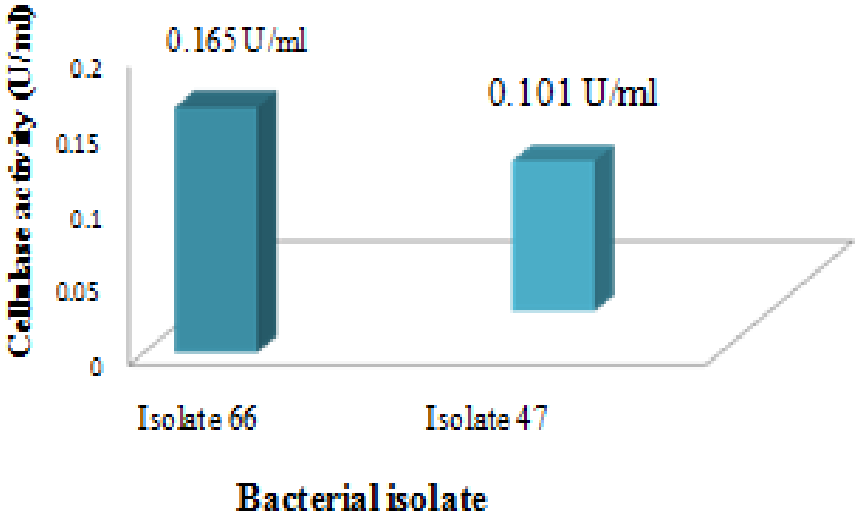


Figure 3.2.2: Cellulase activity of isolated bacterial strains in submerged fermentation at 37◦C

3.3 Estimation of extracellular protein concentration and specific activity

The standard curve of BSA (figure 3.3.1) was used to determine the extracellular protein concentration (mg/ml) and specific activity (U/mg).

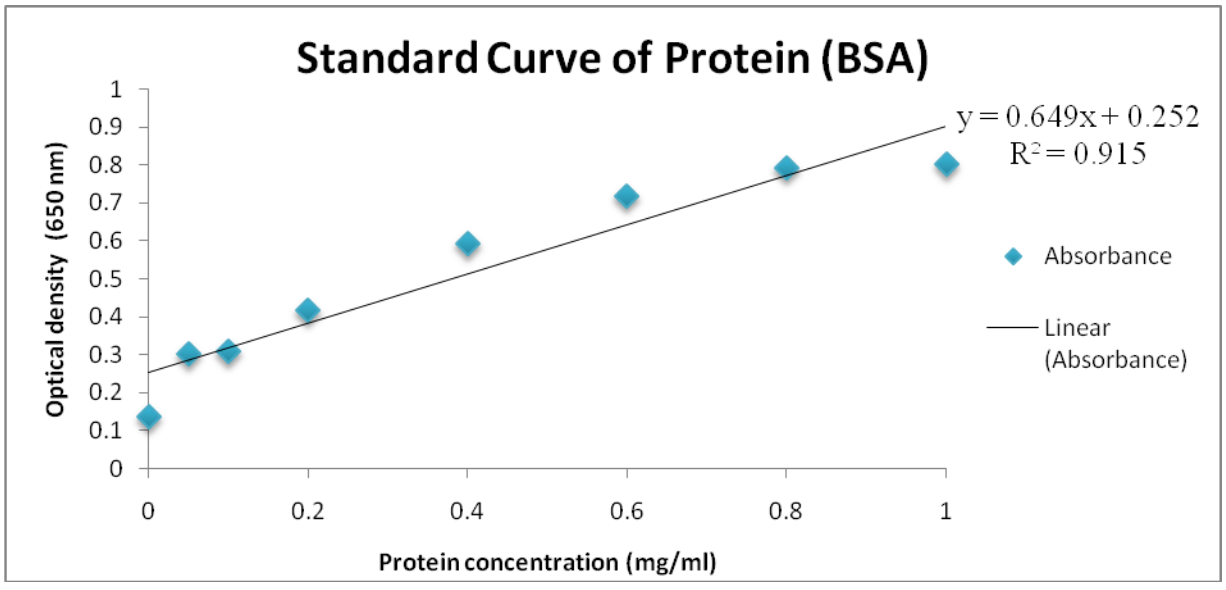


Figure 3.3.1: Standard curve for BSA

The specific activity of isolate 66 was 0.333 U/mg while isolate 47 showed 0.271 U/mg. This is represented in figure 3.3.2.

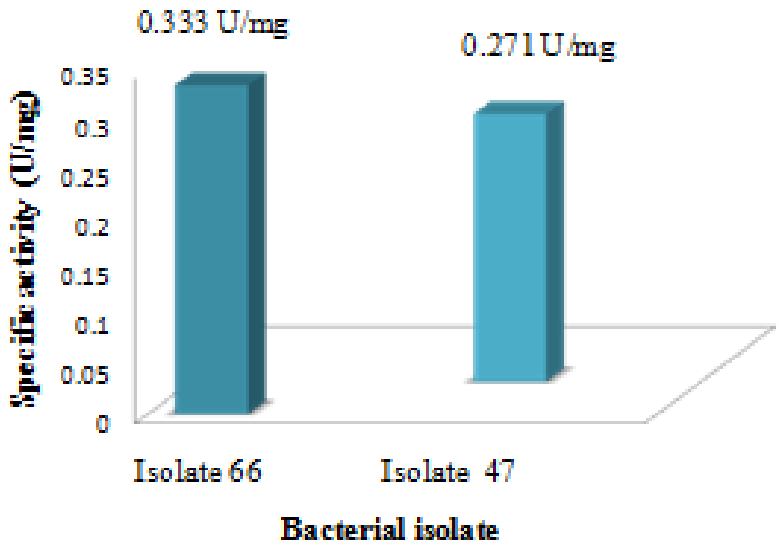


Figure 3.3.2: Specific activity of isolate 66 and isolate 47

Since, isolate 66 showed higher specific activity as well as enzymatic activity, compared to isolate 47, it was selected for further study: presumptive identification by biochemical characterization along with molecular identification followed by bioinformatics analysis.

3.4 Cultural characteristics

The well isolated colonies on the nutrient agar plates were evaluated in the following manner:

Table 3.4.1: Colony morphology of isolate 66

Parameters	Results shown by the isolate 66
Size	Moderate
Pigmentation	No pigmentation (Opaque and white)
Form	Circular (unbroken, peripheral edge)
Margin	Undulate (wavy indentations)

Elevation	Convex (Dome- shaped elevation)
Texture	Mucoid (with an underlying mucoid matrix)

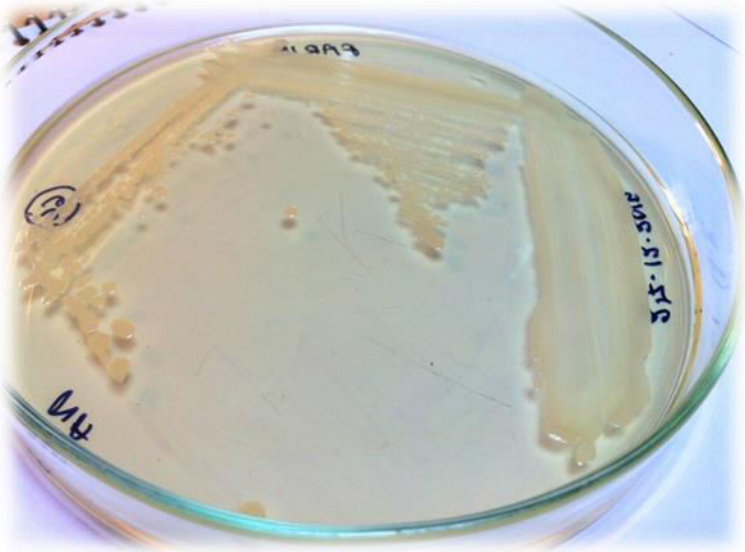


Figure 3.4.2: Discrete isolated colonies of isolate 66 on Nutrient agar plate for the observation of colony morphology

3.5 Microscopic observation

3.5.1 Gram stain

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

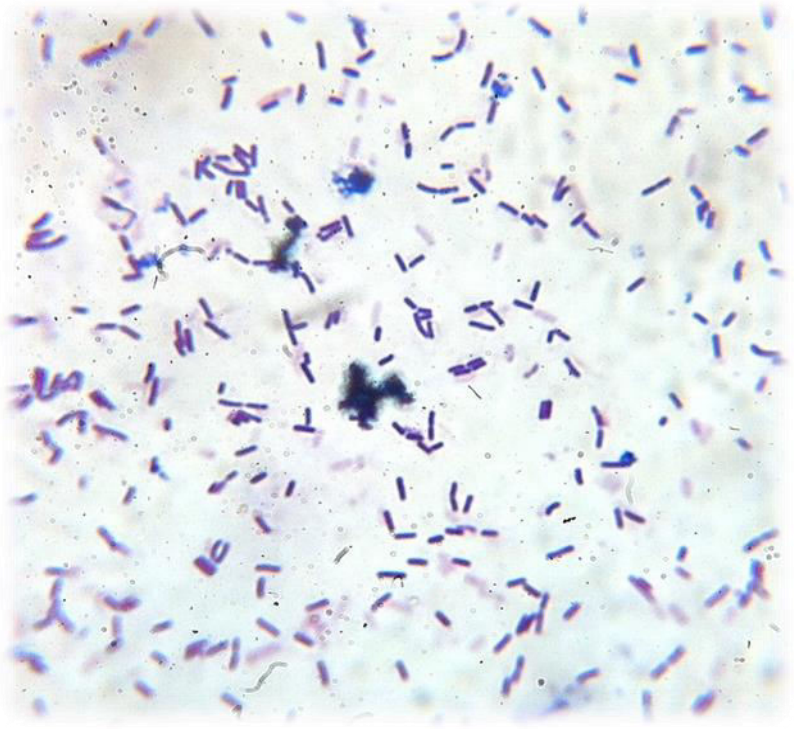
Parameters	Isolate 66
	
Cell shape	Cylindrical (rod)
Cell arrangement	Single bacillus
Cell color	Purple
Gram reaction	Positive

Figure 3.5.1.1: Gram staining

3.5.2 Spore stain

The cells were observed under light microscope (Krüss, Germany). The addition of $MnSO_4.H_2O$ in the nutrient agar media encouraged the sporulation creating nutrient deficient condition for the bacteria. Spores were visible under immersion oil field.

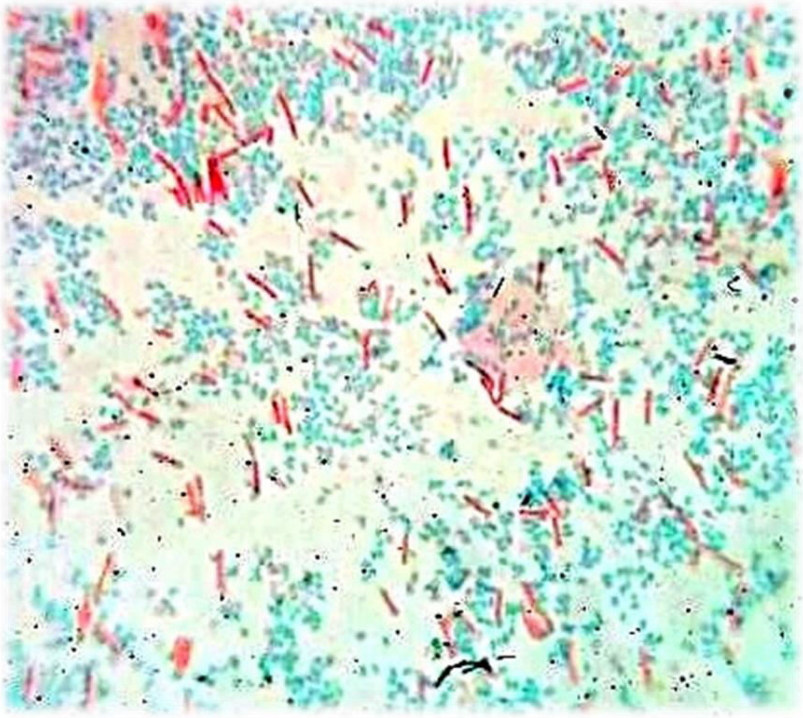
Parameters	Isolate 66
	
Color of spores	Green
Color of vegetative cells	Red
Location of endospore	Central

Figure 3.5.2.1: Spore staining

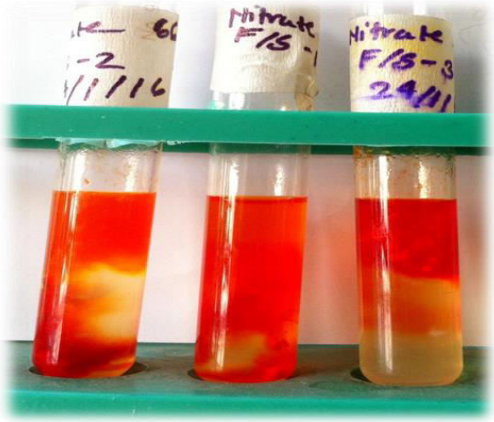
3.6 Biochemical characterization

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 are given in the following table 3.6.1:

Table 3.6.1: Biochemical test results

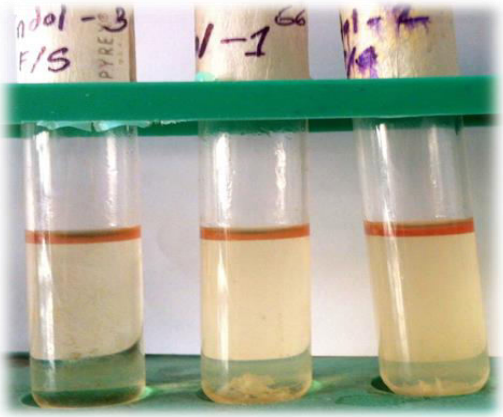
Biochemical Tests	Results
Sucrose Utilization test	+
Fructose Utilization test	+
Glycerol Utilization test	+
Maltose Utilization test	-
D-Xylose Utilization test	-
Triple Sugar Iron agar test	Alkaline slant (red) and acid butt (yellow) without gas and Hydrogen sulfide production
Indole production test	+
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



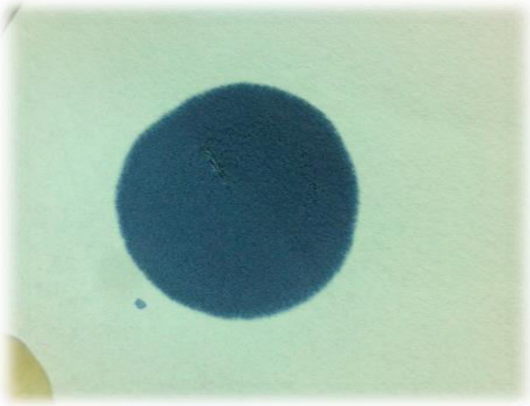
Nitrate reduction test

Starch hydrolysis test



Indole production test

Methyl red test



Oxidase test

Figure 3.6.1: Biochemical tests performed with the isolate 66

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

3.7 Estimation of DNA band size

PCR was carried out to amplify 16S rRNA gene with two universal primers: 27F - AGAGTTTGATCCTGGCTCAG and 1492R- GGTTACCTTGTTACGACTT. PCR product size was found to be around 1600bp by agarose gel electrophoresis.

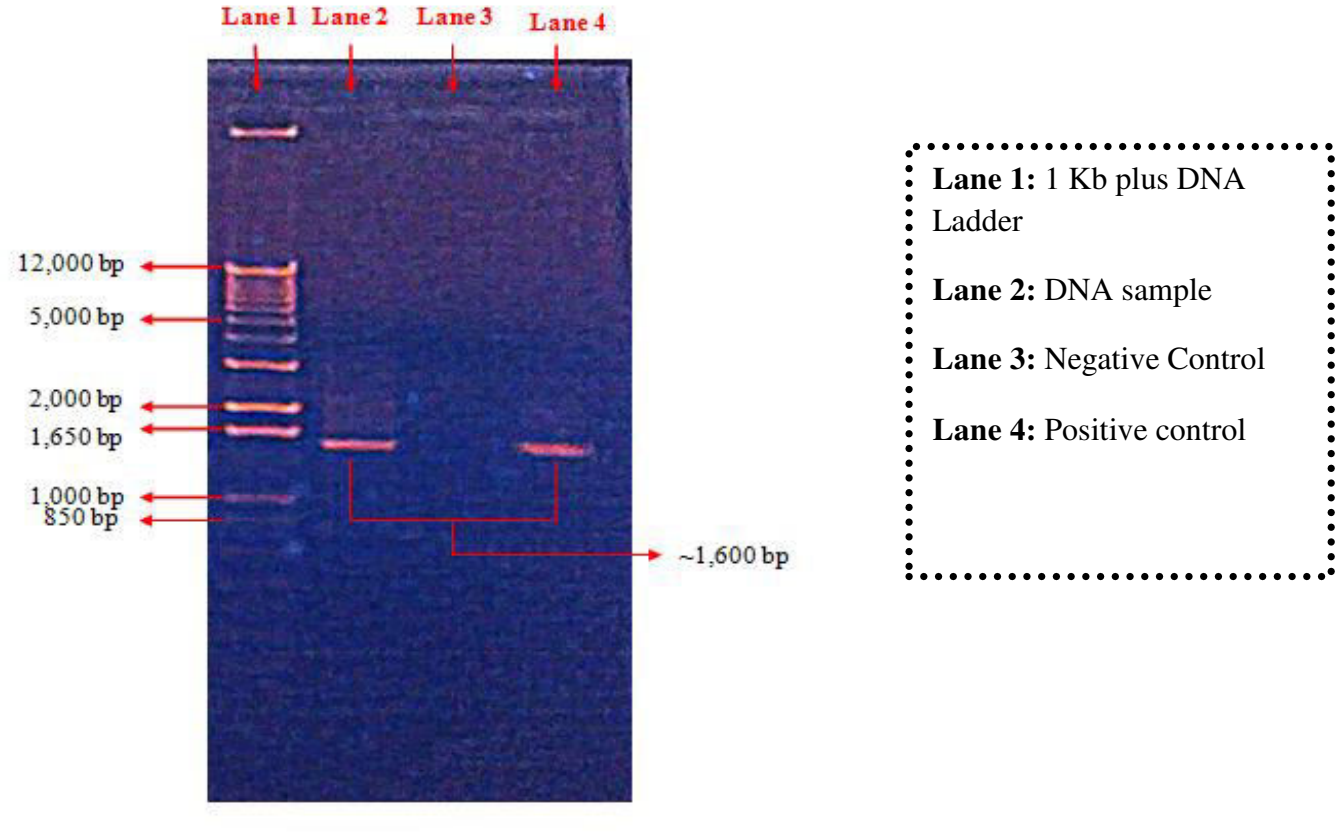


Figure 3.7.1: Agarose gel electrophoresis result of the amplification of 16S rRNA gene of the isolate 66. The DNA band size was estimated to be around 1600 bp according to the 1 Kb plus ladder and a positive control on the other side of the sample.

3.8 Sequence analysis

3.8.1 Estimation of DNA concentration and purity

The PCR product of Isolate 66 was later on analyzed for DNA concentration and purity by Nanodrop 2000 (Thermo scientific, USA) (table 3.8.1).

Table 3.8.1: DNA concentration and purity of the amplicon of isolate 66

DNA concentration	41.0 ng/µl
Purity	1.92

The concentration of DNA and purity was well enough to carry out sequencing process.

3.8.2 Sequence analysis of 16S rRNA gene

The method Sanger sequencing was followed using ABI Genetic analyzer by 1st BASE Laboratories in Malaysia. The trimmed and cleaned query sequence is given in table 3.8.2.1:

Table 3.8.2.1: Trimmed and corrected DNA sequence of 16S rRNA gene of isolate 66 (fasta format)

Query sequence (1381 bp)	>EMBOSS_001 CAGATGGGAGCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCT GCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACC GCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCAT TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGT GATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAA TCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGG ATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGAC GGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTG GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGAT GTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAA GAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGT GGCGAAGGCGACTCTCTGGTCTGTAACTGACACTGAGGAGCGAAAGCGTGGGGAGCGAAC AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTT CCGCCCCTTAGTGCTGTAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAG ACTGAAACTCAAAGGAATTGACAGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC GAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGAGATAGGAC GTCCCCTTCGGGGGCAGAGTGACAGGTGGTGGCATGGTTGTCGTCCCCTCGTGTCGTGAG ATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTG GGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCAT CATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCAGCGAA ACCGCGAGGTTAAGCCAATCCCACAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTC GACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTC CCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCGGTGA G

The query sequence was then submitted to NCBI, nucleotide databank and subjected to BLASTn. The top 20 homologous sequences are given below depending on their higher percentage identity and E value ($<10^{-5}$) from the NCBI database.

Table 3.8.2.2: Top 20 sequences of NCBI list with higher percentage of identity (99%) and 0.0 E value.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Bacillus subtilis subsp. *spizizenii* strain ATCC 6633 16S ribosomal RNA gene, partial sequence	2455	2455	100%	0.0	99%	NR_118486.1
Bacillus subtilis strain JCM 1465 16S ribosomal RNA gene, partial sequence	2455	2455	100%	0.0	99%	NR_113265.1
Bacillus subtilis strain NBRC 13719 16S ribosomal RNA gene, partial sequence	2455	2455	100%	0.0	99%	NR_112629.1
Bacillus subtilis subsp. *subtilis* strain OS-6.2 16S ribosomal RNA gene, partial sequence	2455	2455	100%	0.0	99%	NR_114996.1
Bacillus subtilis strain DSM 10 16S ribosomal RNA gene, partial sequence	2455	2455	100%	0.0	99%	NR_027552.1
Bacillus subtilis subsp. *subtilis* strain OS-44.a 16S ribosomal RNA gene, partial sequence	2453	2453	100%	0.0	99%	NR_114997.1
Bacillus subtilis subsp. *inaquosorum* strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	2452	2452	100%	0.0	99%	NR_104873.1
Bacillus tequilensis strain 10b 16S ribosomal RNA gene, partial sequence	2452	2452	100%	0.0	99%	NR_104919.1
Bacillus subtilis strain BCRC 10255 16S ribosomal RNA gene, partial sequence	2452	2452	100%	0.0	99%	NR_116017.1
Bacillus subtilis strain IAM 12118 16S ribosomal RNA gene, partial sequence	2452	2452	100%	0.0	99%	NR_112116.1
Bacillus subtilis subsp. *subtilis* strain OS-	2450	2450	100%	0.0	99%	NR_115002.1

109 16S ribosomal RNA gene, partial sequence						
Bacillus subtilis strain 168 16S ribosomal RNA gene, complete sequence	2446	2446	100%	0.0	99%	NR_102783.1
Bacillus subtilis subsp. *spizizenii* strain NBRC 101239 16S ribosomal RNA gene, partial sequence	2446	2446	100%	0.0	99%	NR_112686.1
Bacillus mojavensis strain ifo 15718 16S ribosomal RNA gene, partial sequence	2443	2443	100%	0.0	99%	NR_118290.1
Bacillus mojavensis strain NBRC 15718 16S ribosomal RNA gene, partial sequence	2443	2443	100%	0.0	99%	NR_112725.1
[Brevibacterium] halotolerans strain DSM 8802 16S ribosomal RNA gene, complete sequence	2443	2443	100%	0.0	99%	NR_115063.1
Bacillus axarquiensis strain LMG 22476 16S ribosomal RNA gene, partial sequence	2443	2443	100%	0.0	99%	NR_115929.1
Bacillus mojavensis strain IFO15718 16S ribosomal RNA gene, partial sequence	2443	2443	100%	0.0	99%	NR_024693.1
Bacillus subtilis subsp. *spizizenii* strain ATCC 6633 16S ribosomal RNA gene, partial sequence	2443	2443	100%	0.0	99%	NR_112049.1
Bacillus subtilis subsp. *subtilis* strain OS-105 16S ribosomal RNA gene, partial sequence	2441	2441	100%	0.0	99%	NR_115001.1

All the 20 subject sequences were downloaded in FASTA format and then the top 8 sequences of different species of *Bacillus* along with two other sequences of different genus which belongs to the same phylum were aligned with the query sequence to construct the phylogenetic tree with ClustalW using the maximum likelihood method of 500 bootstrap replication (MEGA 6).

3.9 Phylogenetic inference

Phylogenetic tree was constructed subjecting 11 nucleotide sequences (one of which was the query sequence) to maximum likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993) with 500 bootstrap replicates (Felsenstein, 1985) using MEGA 6 (Tamura *et al.,* 2013). The evolutionary tree demonstrated that all the sequences belonging to the same family or order formed cluster. Therefore, the sequences were merged and represented by the same leaf (terminal OTU) for better resolution of the phylogeny. All the *Bacillus* sequences formed one clad (bootstrap value 100). Higher bootstrap value of the query sequence with the sequence of *Bacillus Subtilis* subsp. *Spizizenii* strain indicated higher consistency of the given data for taxonomical bipartioning. While the bootstrap values did not indicate how accurate the tree is but it indicated the stability of the branching pattern. The higher bootstrap value of the braches of *Bacillus Subtilis* subsp. *Spizizenii* with the query sequence (EMBOSS 001) clearly signified the stability of the branching pattern. Thus, it was identified to be the *Bacillus Subtilis*. Also, the distant branches of the two sequences belonging to the two different genuses clearly signify having a distant relationship with the query sequence (Singh *et al*., 2015).

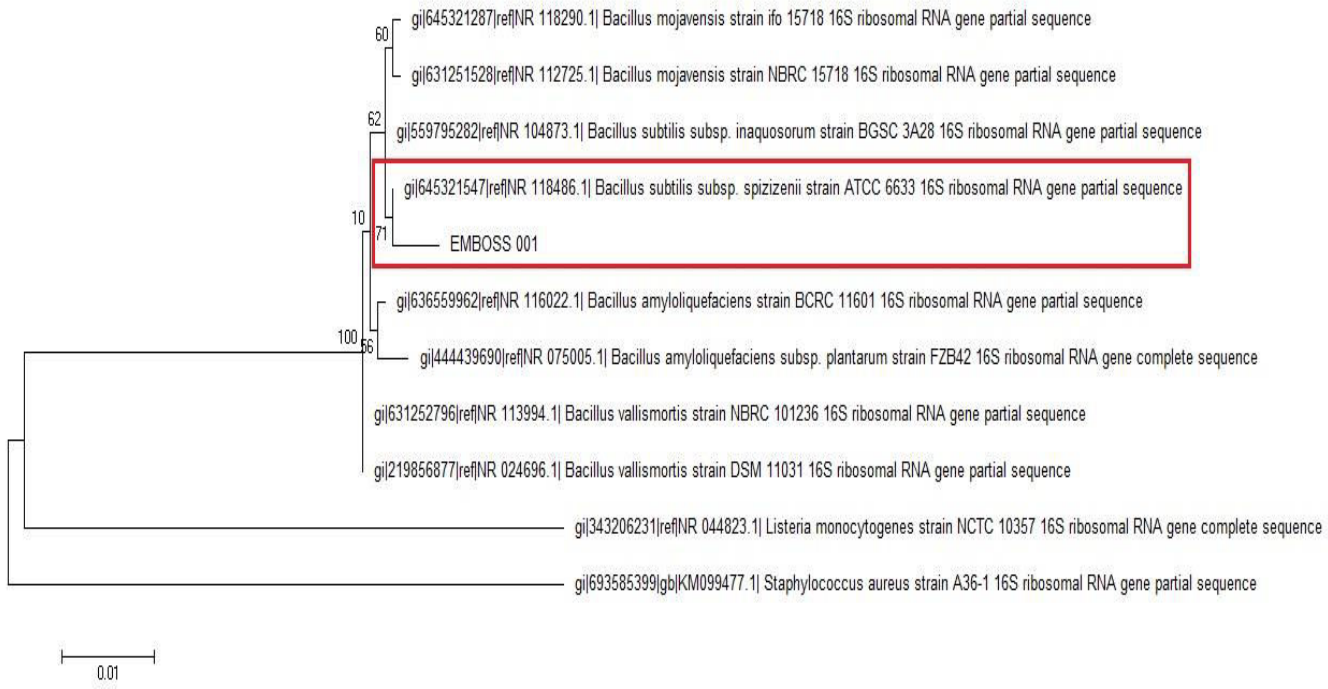


Figure 3.9.1: Molecular Phylogenetic analysis by Maximum Likelihood method illustrating the query sequence (EMBOSS 001) to be identified as *Bacillus subtilis* with a bootstrap value of 71.

Chapter 4

4. Discussion

The present study aims for isolation and identification of potent cellulose degrading bacteria that can hold a high demand in the industry to overcome the limitation of energy and reduce the cost of biofuel production. At the beginning of this project, out of 68 colonies that was isolated from the sample soil mixed with cow manure, 31 colonies had the capability of cellulose hydrolysis. Only two of them were later selected to be the potent cellulose hydrolyser according to their highest clear zone ratio. After the enzyme assay, finally one of them was selected to carry out further study comparing their enzyme activity along with specific activity and the one with the better result was selected. After that, biochemical characterization along with determination of colony morphology and cell morphology was carried out for the tentative identification of the isolate and then for the confirmation of the isolate, the 16S rRNA gene of the isolate was amplified using universal primers. The gene was then sequenced and a phylogenetic tree was constructed. Finally the strain was identified to be *Bacillus subtilis* based on the 73 % boot strap value on the phylogenetic tree.

The cow manure mixed soil sample that was used to carry out this study was collected from Keraniganj. The percentage of cellulolytic bacteria collected from this sample was 45.59%. In some of the previous studies, the percentage was 34.7% in farming soil, 52.4% in forest soil (Hatami *et al*., 2008), 42.86% in cow dung (Bai *et al*., 2012), 46.15% in retting ponds and estuary (Shenkani &Sundara, 2015), 32.35% in soil, wood furnishing region and sugar cane farm and water samples that were collected from paper industry waste and municipal waste (Shaikh *et al.*, 2013). Compared to these results, the collected soil proved to be a good source of cellulase producer. It was slightly better than previous study done from cow dung by Bai *et al*., 2012. Cellulase producing bacteria are found commonly in all environments which enables them to degrade the cellulose found prevalent in waste materials. For example: The natural habitat of *Bacillus subtilis* is soil, which contains a wide range of carbohydrates and polysaccharides from microorganisms, plants and animals, and so it can utilize a wide range of such substrates and possesses a large number of enzymes which degrade polysaccharides. The most common natural habitats of *Bacillus subtilis* is soil and environments that are directly or indirectly contaminated by soil such as: air, compost, emperor moth caterpillars, feathers, inner tissues of plants including cotton, grape, pea, spruce and sweet corn having important roles in growth promotion and plant protection, leather, poultry litter and manure, gemstones, stone surfaces of ancient

monuments, rhizosphere of tea bushes. Natural fibers and vegetable products in several traditional fermented foods are dominated by *Bacillus Subtilis* (Logan and Vos, 2009).These sources can also be studied to obtain an even better cellulase producer.

Out of 31 isolates, only two were selected to carry out enzyme assay on the basis of their better clear zone ratio. The ratio of the two isolates were found to be 3.85 (isolate 66) and 4.87 (isolate 47) which was 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 reason for better clear zone was due to the variety of microorganism present in the soil as well as the type of organic matter in these soils. Cow dung is a rich source of cellulose degrading bacteria due to the diet of ruminent which consists of high amounts of cellulosic material. Hence, the soil mixed with cow dung refers to the superior strains with more ability for survival and production of cellulase.

Enzymatic activity of the two isolates was found to be 0.165 U/ml (isolate 66) and 0.101 U/ml (isolate 47) by DNSA method after submerged fermentation process of 48 hours at 150 rpm and 37◦C. Since cellulases are active at 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. Although, Irfan *et al.,* 2012, reported that cellulases from some species of *Bacillus subtilis subsp Subtilis A-53, B. subtilis YJ1* and *Bacillus strains RH68* and *CH43* have optimum temperature of 50˚C, 60˚C (Yin & Lin, 2010) , 70˚C (RH68) and 65˚C (Mawadza *et al.,* 2000) respectively. Besides, according to Shaikh *et al.*, 2013, at 50◦C *Bacillus* species showed the EU 3.5 U/ml to be the highest cellulase activity and at pH 7 it was 3.5 U/ml whereas at pH 7.5 it showed the highest activity of 4.9 U/ml. Again, after 48 hours of incubation it was recorded to be 1.87 U/ml. Another study revealed enzymatic activity of *Bacillus* spp. to be 32.48 U/ml at 30°C from cow dung (Bai *et al.,* 2012). A work done by Abdelnasser and Ahmed in 2007 reported that 75°C was found to be the optimum temperature for *Bacillus* sp. and maximum production of the enzyme (31.87 U/ml) was obtained at the pH 7.0. The pH of the selected organism was closely related to the optimum pH values of most of the *Bacillus* spp. All of the records regarding the enzyme activity mentioned above are much higher than the result of the present study. Analysis of the optimum temperature, pH, suitable substrate and other parameters is necessary before the full potentiality of the isolate can be determined.

The extracellular protein concentrations of the crude enzymes of the two potential isolates were 0.495 mg/ml with specific activity of 0.333 U/mg for isolate 66 and 0.372 mg/ml with specific activity of 0.271 U/mg for isolate 47. According to Shaikh *et al.*, 2013, *Bacillus spp*. had specific activity of 8.4 U/mg while others reported to be 7.1 U/mg at 48 hour of fermentation, 3.6 U/mg at pH 5.5, 17 U/mg with protein content of 3 mg/ml (Vijayaraghavan & Vincent, 2012), 0.73 U/mg (Sadhu *et al.,* 2013) and 24.2 U /mg with total protein content of 6211.0 mg/ml for *Bacillus subtilis* YJ1(Yin *et al.,* 2010); all of which were much greater than the result of this project. On the other hand, 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*. from isolated sugarcane bagasse and corn steep liquor (Ladeira *et al*., 2014). Specific activity along with protein concentration differs due to the substrate concentration available, type of organism and hours of incubation along with other parameters such as optimum temperature and pH for the enzyme to activate. The lower amount of protein concentration and the less of amount of specific activity of the protein of two isolates of this study compared to previous studies might be due to the shorter hours of incubation and lower concentration of substrates.

From the result of enzymatic activity and specific activity of the two isolates, the one with the better performance, that is isolate 66, was chosen for identification.

The study of colony morphology along with sporulation revealed that the colonies were moderate in size with no pigmentation, they were opaque and white and with circular form, undulated margin, convex elevation and of mucoid texture (with an underlying mucoid matrix). According to Logan & Vos in Bergey's Manual of Systematic Bacteriology, 2009, the colony morphology of *Bacillus subtilis* was reported to be irregular in shape with moderate diameter; margins varying from undulate to fimbriate; they become opaque; color is whitish; 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; pigments, varying from cream through yellow, orange, pink and red, to brown or black, may be formed on potato or agar media containing glucose. The cultural characteristics demonstrated by Logan & Vos, 2009, is indistinguishable from the characteristics expressed by the isolate 66. Hence, the isolate could be predicted to be *Bacillus*. Considering the outcome of spore staining, it could be

observed that the isolate 66 is capable of producing spore. In order 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. Also, no swelling of the sporangia could be seen. As Logan &Vos, 2009, mentioned about the position of the spores of *Bacillus Subtilis* to be central as well as ellipsoidal, paracentral and subterminal spores along with no swelling the sporangia indicates the bacteria to be of the genus *Bacillus.* However, using the colony morphology and sporulation nature, only the genus of the bacteria could be predicted to be *Bacillus*. Since, *Bacillus* species showed a very wide range of colonial morphologies, both within and between species, it might give the appearance of a mixed culture considering medium composition and other incubation conditions having a strong influence. Despite this diversity, however, *Bacillus* colonies on routine media are not generally difficult to recognize. Further confirmation of the species was carried out on the next part.

The biochemical characteristics of the isolate 66 was subsequently found to be positive for sucrose, fructose , glycerol utilization test, 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 while negative for maltose and D-xylose utilization test, methyl red test, motility test, urease test, oxidase test , and growth at 65◦C. The result of the biochemical tests then interpreted from Bergey's Manual of Systematic Bacteriology (Logan & Vos, 2009) revealed it to be mostly similar with the characteristics of *Bacillus*. Furthermore, in order to receive a full tentative 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 be used to predict the genus of the isolated bacterium. From the previous studies, biochemical characteristics were positive for indole, MR, VP, citrate, catalase, oxidase, gelatin, nitrate and negative for urease which was identified as *Bacillus spp*. (Shenkani & Sundara , 2015). Another report was found giving biochemical test results to be positive indole, catalase, oxidase MR, VP, citrate, nitrate , gelatine, starch and casein. Among sugar fermentation, glucose, lactose, maltose, xylose, mannitol and fructose fermentations were positive 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 belonging to the genus *Bacillus* without performing any molecular analysis.

For the molecular characterization, the DNA of the isolate 66 was then extracted using the wizard® Genomic DNA Purification Kit. 16S r RNA gene was amplified by thermal cycler (Applied Biosystems, USA) using the universal primers 27F - AGAGTTTGATCCTGGCTCAG and 1492R- GGTTACCTTGTTACGACTT. The amplified 16S rRNA PCR product was purified using the purification kit Wizard® SV Gel and PCR Clean-Up System and sequenced using ABI Genetic Analyzer (Model:3700) by Sanger sequencing method. A continuous stretch of 1381 nucleotide long sequence of 16S rRNA gene was used to search for similar sequences using online search tool called BLAST (http://www.ncbi.nlm.nih.gov/blast/). The isolate number 66 had closest sequence identity mostly with *Bacillus subtilis* having 99.0 % identity along with few other species belonging from the same genus in the top 20 list given by NCBI database. In the absence of overall genome relatedness, chemotaxonomic data, the strain could be identified as belonging from the genus *Bacillus* so far. After that, the top most reference sequences along with the sequences of two other species from the same phylum "firmicutes" and the query sequence were then aligned by CLUSTALW program using MEGA 6 (Tamura *et al.,* 2013). Initially, similarity values or the distances were estimated after pair wise alignment by CLUSTALW program applying Neighbor-Join and BioNJ algorithms and then the topology was selected with superior log likelihood value. Thus, a phylogenetic tree was constructed showing relationship between the query sequence of isolate number 66 (named EMBOSS 001) and other reference strains by Maximum Likelihood method based on the Tamura-Nei model (Tamura & Nei, 1993). Similar method of identification of the isolates from cow dung was reported by Bai *et al.,* 2012 and Sadhu *et al.,* 2013, by 16S rDNA analysis and 16S rRNA analysis, respectively. On the phylogenetic tree 71% bootstrap value of the branches of *Bacillus subtilis* with the query sequence (EMBOSS 001) which were in the same node, clearly indicated the stability of the branching pattern. Hence, it could be identified to be the *Bacillus subtilis*.

However, four of the biochemical tests including motility, anaerobic, xylose and maltose utilization test gave anomalous results than that of *Bacillus subtilis* mentioned by Logan & Vos, 2009. It has also been mentioned that *Bacillus subtilis* has been long regarded as strict aerobe but now it is established to be capable of growing anaerobically, not only with nitrate as electron

acceptor but also by fermentation in the absence of electron acceptors making them facultative anaerobes. Again, negative result is considered to be 0-15% positive (Logan & Vos, 2009). The cause of the anomalous results of motility, xylose and maltose utilization test could be justified as it had been mentioned positive result to range from > 85% (Logan & Vos, 2009). So, there are still chances of variable results to be found depending on the strain type. 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 and refrigeration period. In addition to that, Logan & Vos (2009) reported that *Bacillus subtilis* subspp. *spizizenii* gives no pigmented colonies, is positive for starch hydrolysis, nitrate reduction, growth in 7% NaCl and negative for growth at 65 which is identical with the result of the isolate of present study.

In this study, the species had been chosen upon its highest enzyme activity and specific activity compared to others and then carried out with the identification of the species. The identification of the organism would help to control the fermentation according to the requirement of the specific organism. Also, knowledge of the genome of the identified species would allow genetic manipulation such as site directed mutations to bring modifications as well as strain developments for better industrial performance. A comprehensive study of the factors affecting the mechanism of cellulase-cellulose interaction which includes substrate adsorption, accessibility, processibility, synergism and mechanistic details of cellulose depolymerizations can lead to many opportunities to utilize the enzyme for specific purposes (Hamid et al., 2015). Besides cellulase production, a better understanding of the physiology of the microorganisms, pre treatment of cellulosic biomass for better microbial attack, processes for cost effective production of cellulases, treatment of biomass for production of hydrolytic products which can serve as substrates for downstream fermentative production of various necessary metabolites and finally utilization of the knowledge of metabolic and protein engineering principle is a must for this purpose (Sukumaran et al., 2005). However, a quantitative analysis on characterization of both the producer organism *Bacillus subtilis* and its cellulase will justify the potentiality of the strain to be used in biofuel and other cellulase based industries.

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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

Nutrient Broth

Component	Amount (g/L)
Nutrient Broth	13.02

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

CMC (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

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.

EDTA (Disodium Ethylene Diamine Tetra-Acetate, pH 8.0) 500mM/0.5M EDTA solution (1000ml)

In a 1000ml beaker, 181.6 g of $Na_2EDTA.2H_2O$ was added to 800 ml H20. Then, pH 8.0 was adjusted while stirring using a glass stirrer with pellets of NaOH (about 20 g NaOH). The solution was transferred to a 1000ml Duran. After that, it was sterilized by autoclaving and stored at room temperature.

In order to prepare 50mM/0.05M EDTA 15 ml, 1.5 ml of 0.5M EDTA was mixed with 13.5 ml of dH_2O.

Ethidium Bromide (100 ml)

To 100 ml distilled water, 1 g ethidium bromide was mixed. The solution was carefully stored at room temperature.

Lysozyme solution (20 ml)

To 20 ml of distilled water, 0.2 g of lysozyme powder was added and dissolved. The solution was stored at 4°C.

Tris Boric Acid EDTA (TBE) Buffer (500 ml)

To 500 ml distilled water, 5.4 g Tris HCL powder, 2.75 g boric acid and 0.5M EDTA of 2 ml were dissolved. The pH of the buffer was adjusted to 8, autoclaved and stored at room temperature.

Citrate Buffer (0.05 M 50 ml)

To make 1 M 50 ml citrate buffer, 14 g citric acid was added to 50 ml distilled water. From there, 2.5 ml buffer was added to 47.5 ml distilled water to make 50 ml of 0.05 M buffer. The pH

was adjusted to need (3,4,5) using NaOH and HCL. The buffer was autoclaved and stored at 4°C.

Sodium Phosphate Buffer (0.05 M 50 ml)

For 50 ml of 0.05 M NaH_2PO_4, 0.35 g was added to 50 ml distilled water. For 50 ml of 0.2 M Na_2HPO_4, 0.67 g was added to 50 ml distilled water. To make sodium phosphate buffer of pH 6, 1.28 ml of Na_2HPO_4 was mixed with 3.73 ml NaH_2PO_4 and the volume was adjusted to 50 ml using distilled water. For pH 7, 2.89 ml Na_2HPO_4 was added to 2.12 ml NaH_2PO_4 and the volume was adjusted to 50 ml by adding distilled water. The buffer was autoclaved and stores 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
Agarose Gel Electrophoresis Apparatus	Cleaver Scientific Ltd, Denmark
PCR Machine, Model: 2720 Thermal Cycler	Appliead Biosystems, Singapore
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