BIOCHEMICAL AND MOLECULAR STUDY OF CELLULASE FROM *BACILLUS LICHENIFORMIS* ISOLATED FROM HOT SPRING WATER, GORKHA

M.Sc Thesis
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For partial fulfillment of the requirement for the Master of Science in Biotechnology

Submitted to
CENTRAL DEPARTMENT OF BIOTECHNOLOGY
TRIBHUWAN UNIVERSITY
Kirtipur, Kathmadu, Nepal

Gorkha Raj Giri
Exam Roll No.: BT 032-067
T.U. Regd. No.: 5-2-37-375-2006

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Gorkha Raj Giri

Glossary Acronyms

(List of abbreviation words)

◦C	Degree Centigrade
µg	microgram
µmol	micromole
A	Angstrom
BLAST	Basic Local Alignment Search Tool
CBD	Carbohydrate Binding Domain
CBM_3	Carbohydrate Binding Module III domain
CBP	Consolidated Bioprocessing
CMC	Carboxy-methyl cellulose sodium salt
CMCase	Carboxymethylcellulase
COS	Cellooligosachharides
DNA	Deoxyribonucleic Acid
DNS	Dinitrosalicylic acid
DO	Dissolved oxygen
DP	Degree of Polymerization
EI	Enzyme index
Endo-G	Endoglucanase
Exo-G	Exoglucanase
Fpu	Filter paper unit
GRAS	Generally Recognized as Safe
IPTG	Isopropyl-β-D-thiogalactopyranoside
IU	International Unit
K_m	Michaelis Constant

L	Liter
m	metre
mg	milligram
ml	milliliter
MTBE	methyl tertiary butyl ether
MW	Molecular weight
NAST	Nepal Academy of Science and Technology
NCC	Non-crystalline cellulose
nm	nanometer
PCR	Polymerase Chain Reaction
pNP	*p*-Nitrophenol
pNPG	*p*-nitrophenol-β-D-glucopyranoside
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
U	Unit
V_{max}	Maximal velocity
X- gal	5-bromo-4-chloro-3-indole-β-D-galactoside

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ABSTRACT

Biochemical and molecular study of cellulase from *Bacillus licheniformis* isolated from hot spring water, Gorkha

Cellulases are the kind of hydrolases consisting of exoglucanase, endoglucanase and β-glucosidase that acts in synergy to break down the cellulosic products into useful compounds. The present research is based on cellulase characterization and production from moderately thermophilic *Bacillus licheniformis* strain NAST01 growing from temperature range of 45^{o}C to 60^{o}C with optimum cellulase production at 50^{o}C. It has been found to secrete extracellular cellulase in an aerobic condition at optimal 7.0 pH, 50^{o}C and 200 rpm. The crude cellulase has been partially purified using 60% ammonium sulphate precipitation followed by dialysis and Sephadex G-75 column chromatography. The specific activity of crude enzyme, salt precipitated, dialyzed and column purified was obtained as 0.03956, 0.1029, 0.699, 1.235 U/mg respectively. The molecular weight of enzyme was found to be approximately 55000 Da by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme showed Michaelis Menten kinetics exhibiting K_m 10.73 mg/ml of CMC and V_{max} 42.13 µmol/ml-min. The purified enzyme was found to be active at pH 5.5 and temperature 50^{o}C and stable from pH 5.5 to 7.5 for an hour at room temperature. The enzyme was found to retain its activity from 45 to 50^{o}C for 30 min. The enzyme activity was found to be induced by 5 to 10 mM Ca^{2+} and Fe^{2+} whereas inhibited by even 5 mM Ba^{2+}. Besides CMCase activity the filter paper assay, avicel hydrolysis, cellobiose hydrolysis and *p*-Nitrophenyl-β-D-glucuronide (pNPG) hydrolysis for the dialyzed enzyme that was obtained as 4.07±0.203 fpu/ml, 0.034±0.0017 U/ml, 0.156±0.051 U/ml and 0.007194±0.0003 U/ml respectively. Furthermore, 1.5 kb gene coding endocellulase was amplified and cloned into pTZ57R/T vector and the recombinant plasmid pCelNAST01 restricted with *Nde*I/*Hind*III enzymes showed a single band at 4380 bp. The analysis of partial sequence showed the resemblance of nucleotide to *cel*5 gene of *Bacillus licheniformis* strain GXN151 with presence of conserved domain CBM_3 consisting of 86 amino acid.

Keywords: cellulases, kinetics, endoglucanase, amplification, recombinant plasmid, conserved domain

CHAPTER 1: INTRODUCTION

The last two decades the nations in the world has witnessed of cellulosic waste accumulation along with the industrial inefficiency of raw materials due to the inefficient utilization process. On the other hand, there is increase in fuel consumption with increase in population and global industrializations. This result not only rise in fuel price but also emission of greenhouse gasses, finally leading to oil crisis and shortage of petroleum products as the global oil reserves are expected to exhausted in 30 years (Puppan, 2002). The problem forced many countries in the world to initiate program on biofuel production from renewable resources, and exploring new possibilities for biogas, bioethanol, biodiesel and fuel cells (UCB, 2007). Further, the program seems to be environment friendly and has a global significance, as it is mainly based on biomass conversion from lignocellulose or cellulosic biomass to glucose and eventually bioethanol (Lynd *et al*., 2008). The efficient hydrolysis by microbial enzymes has many attractive possibilities based either on removal of the cellulose or utilization of glucose (Mandels, 1985). The positive aspect is further highlighted with the use of wastes from agriculture or that from industry as pulp and paper, sewage, sludge including forestry that either found from herbs, shrubs, and trees.

Cellulose is the main component of plant cell wall with structural subunit cellobiose. It is the major constituent of raw materials like cotton (over 94%) and wood (over 50%). Although cellulose is of plant origin, it is also present in the outer mantle of marine invertebrates known as tunicates (urochordates). It is the homopolymer of glucose subunits which are linked by β, 1-4 glycosidic bonds and glucose residues tilted by 180^{o} towards its neighbors. Hydrogen bonding within and between multiple layers of closely packed cellulose molecules result in the formation of cellulose micro fibrils. Cellulose exists in a numbers of highly ordered crystalline and less ordered amorphous topologies. It is an ideal for enzymatic hydrolysis rather than chemical or mechanical breakdown due to its heterogeneous and insoluble nature. A variety of microorganisms such as fungi and bacteria convert the insoluble substrate into cello-oligomers and ultimately to cellobiose and glucose. The utilization of cellulose is possible only by screening and isolation of cellulolytic organisms ranging from bacteria to fungi and even that in the gut of termites or that from rumen of ruminants. Microorganisms hydrolyzed cellulose with the help of multi-enzyme system by bioprocessing called consolidated bioprocessing (CBP) using organism with capacity to hydrolyze biomass in a single step in terms of substrate utilization and product formation (Lynd *et al*., 1996).

Cellulase term loosely refers to the group of enzymes that hydrolyze the β-1, 4-glycosidic linkage of cellulose, but complete degradation of cellulose involves the action of three enzymatic activities (Lynd *et al.*, 2002). The enzymes are classified based on the depolymerization of cellulose. Cellulase enzymes are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). Aerobic bacteria produce extra-cellular enzymes bind to different cellulose conformations, act in synergy producing effective hydrolysis products whereas anaerobic bacteria use unique extracellular multienzyme complex, called cellulosome. Cellulosome with mode of action follows binding to non-catalytic structural protein (scaffoldin) which stimulates towards the substrate (Tomme *et al*., 1995). The enzymes to yield cello-oligomers are endoglucanases attack randomly cleave the β-1, 4 glycosidic linkages of cellulose, cellobiohydrolase (CBH, exo-glucanase) that hydrolyzes cellobiosyl units from the reducing or non-reducing end of cellulose to produce cellobiose, and β-glucosidase that further hydrolyzes cellobiose and glucanoligomer to yield glucose. The general scheme for the hydrolysis of cellulose can be depicted as in the below reactions (figure 1) with the multiple sets of enzyme system but may vary with the type of cellulosic material used for hydrolysis and the enzyme system present in the cellulolytic organism.

In nature, cellulose occurs in the form of lignocellulose. The recalcitrant nature of lignocellulosic materials creates an obstacle for the efficient bioconversion process. So the study and screening of different enzymes from microbial source along with the thermostable nature of the produced enzyme is essential. For the objective one can isolate microbes like bacteria, fungi from natural resource such as hot spring water, compost pile and geothermal vents and characterize enzyme from biochemical to molecular level followed by the storage of gene responsible for enzyme production and further genetic manipulation.

The lignocellulosic substrate used in production of commercial cellulase a cocktail of three enzymes together with accessory hemicellulases as xylanase and xylosidase. Among them the fungal species of genera as *Trichoderma* and *Aspergillus* lacking the thermostability as of mesophilic origin and most of the strains lack all sets of enzyme with industrial importance and with the global significance in the biofuel perspectives. For the efficient hydrolysis of cellulose, bacterial cellulase is found to be more efficient than fungal cellulase due slow growth rate of fungi and with less feedback inhibition from hydrolyzed products. Whatever, the microorganisms are present at the close of carbon cycle, all are of great interest with reference to cellulose utilization (Lynd *et al*., 2002). So, screening

microorganisms with higher cellulolytic activity, optimization of fermentation conditions, and directed evolution are common means to achieve industrial goals.

Most cellulase system can be understood by assaying the reducing sugars from a high molecular weight form of cellulose or by measuring the decrease in viscocity of carboxymethylcellulose (Sharrock, 1988). There are different colorimetric methods to assay the cellulase system in microorganisms. Some of the methods such as filter paper assay to determine total cellulase activity on strip made of Whatman No.1 filter paper, avicellase assay to determine exoglucanase activity using a crystalline cellulose avicel, cellobiose assay to determine the cellobiose hydrolase activity on substrate cellobiose (a disachharide) or 4-Nitrophenol β-D-glucopyranoside to produce glucose or *p*-nitrophenol respectively. The production of glucose a reducing sugar can be assayed using dinitrosalicylic assay (DNS) in alkaline condition (Miller, 1959).

During the enzyme production and assay for cellulases the purification of the crude enzyme is essential for the characterization of enzyme. The total protein quantification can be done based on the dye binding method as shift in the absorbance from 365 to 595 nm (using Commassie Brilliant Blue G-250 or R-250) by the Bradford assay (Bradford, 1976). Similarly, the zymogram study and study of cellulases in genetic level by amplification of the cellulase gene and molecular cloning of gene from one organism to another and expression study can increase the production of enzyme. The engineering of organisms with robust ability for mass production of enzyme can add milestone in the industry utilizing cellulases and cellulosic products along with the economic development of country.

In the present day, study of thermophilic microorganisms and their thermostable enzyme is of great significance. Understanding of cellulose hydrolysis can be approached in number of possible ways. It range from isolation of microorganisms, study of component of cellulase enzyme systems, unfractionated cellulase systems, pure culture of cellulolytic microrganisms and mixed culture of cellulolytic microorganisms to even finer level (Lynd *et al*., 2002). But there is need of integration of different components of whole cellulase system from pathways to enzymes to genes for the understanding of cellulose hydrolysis and production of enzyme in particular. The present research focuses partially on such "reductonist" approach and biological solutions for cellulose hydrolysis for the utilization of cellulolytic biomass using commonly distributed cellulolytic microorganisms such as bacteria of genus *Bacillus* isolated from water of hot springs of Gorkha district.

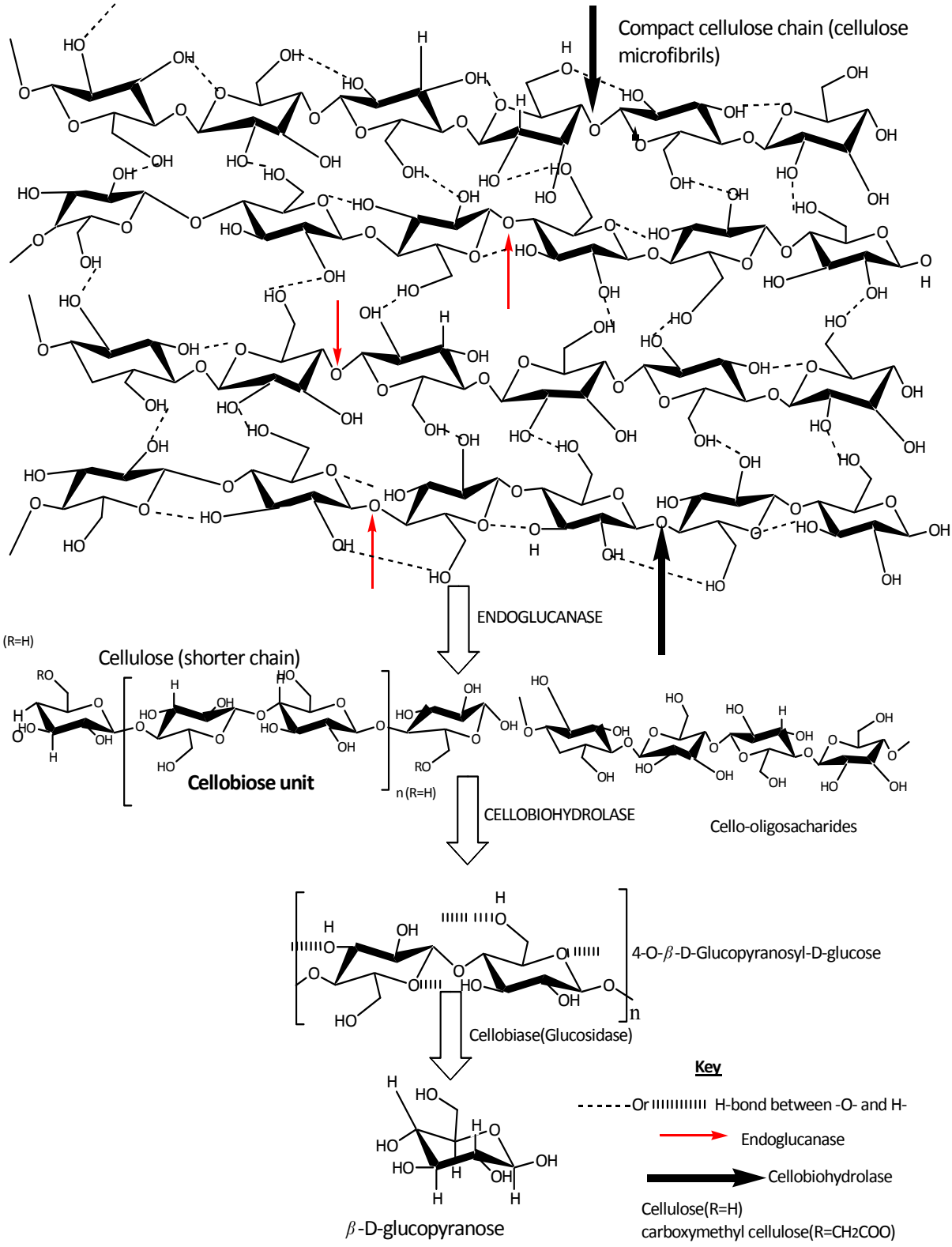


Fig.: General purposed reactions for the cellulose hydrolysis using different sets of enzymes in synergy

1.1 Hypothesis

- There can be different enzymes in bacteria inhabited from hot spring water of Gorkha district that can be screened and characterized.
- Cellulase enzyme can be produce and characterized in lab along with study of gene responsible for the enzyme production to make further genetic manipulation.

1.2 General Objectives

The general objective of this study is to characterize cellulase enzyme from *Bacillus licheniformis* isolated from hot spring water of Gorkha district.

1.3 Specific Objectives

- To screen different industrially important enzymes like protease, pectinase, amylase, lipase, xylanase, cellulase from the *Bacillus* species.
- To optimize the cellulase production by *Bacillus licheniformis* strain NAST01
- To purify and characterize cellulase enzyme
- To clone cellulase coding gene for overexpression in *E.coli*

1.4 Rationale

Nepal is a mountainous country with good biological diversity of microorganisms along with the resources such as flora and fauna, natural heritage, endangered animals, etc. Nepal has hot spring sources at different geographical locations. More than 28 hot water springs are reported in Nepal (Ranjit, 2005). Most of which are located in the land belt stretching between the Himalayan and mountain regions. These hot water springs are locally known as "Tatopani" simply meaning 'hot water'. The sub-surface temperatures are found to vary between 50^{o}C-120^{o}C while the surface temperature of thermal water ranges between 23^{o}C to 73^{o}C. This hot springs are the good sources of thermophiles and hyperthermophilic microorganisms.

In the recent years, thermophiles have been studied most extensively for the production of number of enzymes due to their thermostability and ability to withstand the harsh environmental condition. Cellulase is one of the potent enzymes produced by thermophile that has profound use in much industrial process including in bioethanol production. Many

microbial cellulase enzymes have been studied from different microorganisms including *Bacillus species*. The physiological response of similar organism or species may vary with ecological variations. Therefore, this study focuses on production and characterization of cellulase enzyme from *Bacillus species* isolated from hot spring of Gorkha.

The preliminary screening of different enzymes in the isolates from hot spring water along with cellulase enzyme production and optimization in synergy can hydrolyze cellulosic biomass giving fermentable reducing sugar. The explored organism, conversion strategy and reducing sugar produced can mitigate problem of cellulosic waste accumulation, ensure greater food and fuel security and limit the greenhouse gas emission. The cellulosic biomass conversion and consolidated bioprocessing is possible with the exploration of organisms from different sources and habitat. The biochemical characterization along with study in genetic level seems helpful in the study of enzyme system in organism for further study in molecular level.

1.4 Scope

Cellulosic materials are renewable and present in abundance with relatively low cost. But the search for low-cost technology to overcome the recalcitrance of cellulosic biomass is urgent to utilize the resources. One of the strategies for the efficient cellulose hydrolysis could be the exploration of microbes and production of cellulolytic enzymes, hydrolysis of biomass, and fermentation of resulting sugars to desired products in a single process. The biotechnological conversion of the cellulosic biomass is potentially sustainable approach to develop new bioprocess and products using the microbial cellulase that become focal biocatalyst due to the complex nature and wide spread industrial application. Generation of microbial strains, which are stable, produce the various enzymes involved in the breakdown of cellulosic substances constitutively in high yields and within short time will prove to be helpful in the production of enzymes for industrial use. It is possible by using the process of substrate utilization and product formation properties of microorganism under study.

Bacteria have potential for rapid growth rate, enzyme complexity and extreme habitat variability. The trends of screening of different enzymes along with novel cellulases are essential for industrial use. Search for species of microbial enzyme with resistant to harsh environment condition used in the bioconversion process such as high temperature, acidic or alkali pretreatment is demanding from biofuel and application industry to bioremediation industry utilizing cellulosic materials.

CHAPER 2: LITERATURE REVIEW

2.1 Structure and composition of cellulosic biomass

Cellulose is a native structural biopolymer of plant cell wall and is present in abundance in nature (Guo *et al*., 2008). It differs in composition and anatomical structure across cell wall of different plant taxa. About 7.2×10^{11} tons of cellulose is reserved in plants with yearly production of 4×10^{10} tons (Coughlan *et al*., 1985). It is also a structural component for variety of organisms including fungi, bacteria, and protists as well as wide range of invertebrate animals such as insects, crustaceans, annelids, molluscs and nematodes (Watanabe and Tokuda, 2001; Davison and Blaxter, 2005) besides plant. The content range approximately from 35 to 50% of plant dry weight and embedded in the matrix of other structural biopolymers primarily hemicelluloses and lignin, which comprise 20-35% and 5-30% of plant dry weight respectively (Lynd *et al*., 1999) as shown in fig.2 (Ragauskas *et al*., 2006). The cellulosic content in different lignocellulosic materials is given table 1 (Reshamwala *et al*., 1995; Sun and Cheng, 2002). It is the linear polymer of β-1,4-polyacetal cellobiose (4-O-β-D-glucopyranosyl-D-glucose) also called the polymer of anhydroglucose residue in the chair configuration held together by β-1,4 linkages (Coughlan, 1985). It has the elemental composition 44.4% C, 6.17% H and 49.39% O resulting formula $(C_6H_{10}O_5)_n$. The cellulose chain has a tendency to form intra- and inter-molecular hydrogen bonds through hydroxyl groups on its glucose units, which promotes cellulose aggregations and lead to a supramolecular structure with crystalline and amorphous domains.

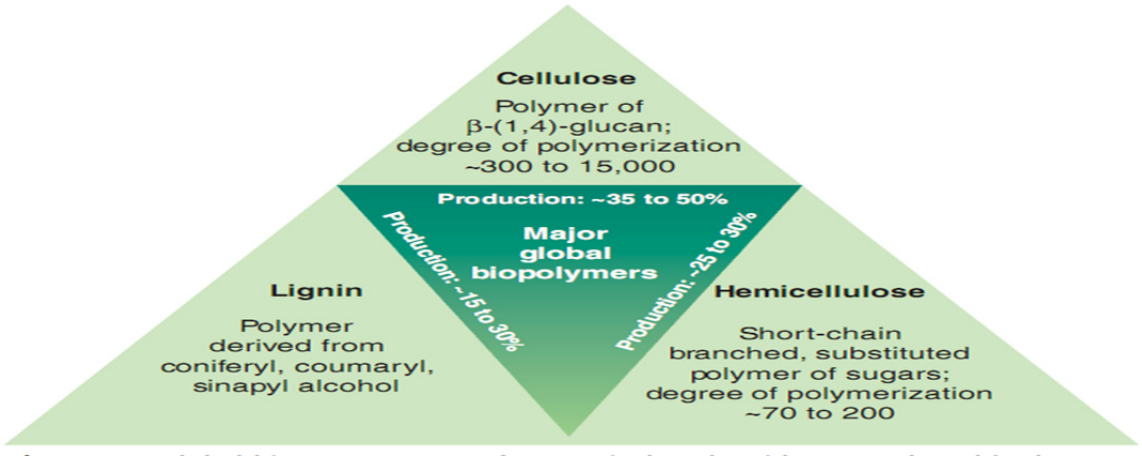


Fig.2: Key global biomass resources from agricultural residues, wood and herbaceous energy crops

Table 1: The general cellulosic content from lignocellulosic materials

Source	**Cellulosic content (%)**
Bagasse	35-45
Bamboo	40-55
Cotton	90-99
Cotton seed hairs	80-95
Flax	70-75
Hemp	75-80
Jute	60-65
Tobacco chops	22-30
Kapok	70-75
Ramie	70-75
Rice Straw	35-45
Hardwood stems	40-55
Softwood stems	45-50
PS sorghum	35.7±0.8
Corn stover	36.1-40.8
Corn cobs	45
Grasses	25-45
Wheat Straw	30
Sorted refuse	60
Arundo donax	30-38
Miscanthus	35-40
Paper	85-99
Newspaper	40-55
Swine waste	6.0
Nut shells	25-30
Leaves	15-20

Each β-D-glucopyranose units within a cellulose chain has three hydroxyl groups; two secondary and one primary. Crystalline and amorphous regions make up 55-75% and 25-45% respectively, in the cellulose fiber. Intramolecular hydrogen bonding is found between adjacent anhydroglucose rings whereas inter-molecular between successive glucose residues (Gardner and Blackwell, 1974; Winterburn, 1974; Rees *et al*., 1982) are responsible for the linear integrity and crosslink of adjacent chain as shown in figure 5 (Coughlan, 1985). The loosely packed molecules remain as the amorphous region of the cellulose macromolecules. The main reactions that cause structural change in cellulose structure are photodegradation, oxidation, acid hydrolysis and biodegradation. Similarly, cellulose undergoes the process of dehydration-decarboxylation in presence of mechanical, chemical and enzymatic transformation to give hexoses, pentoses and other organic acids (Ragauskas *et al*., 2006) as shown in figure 3.

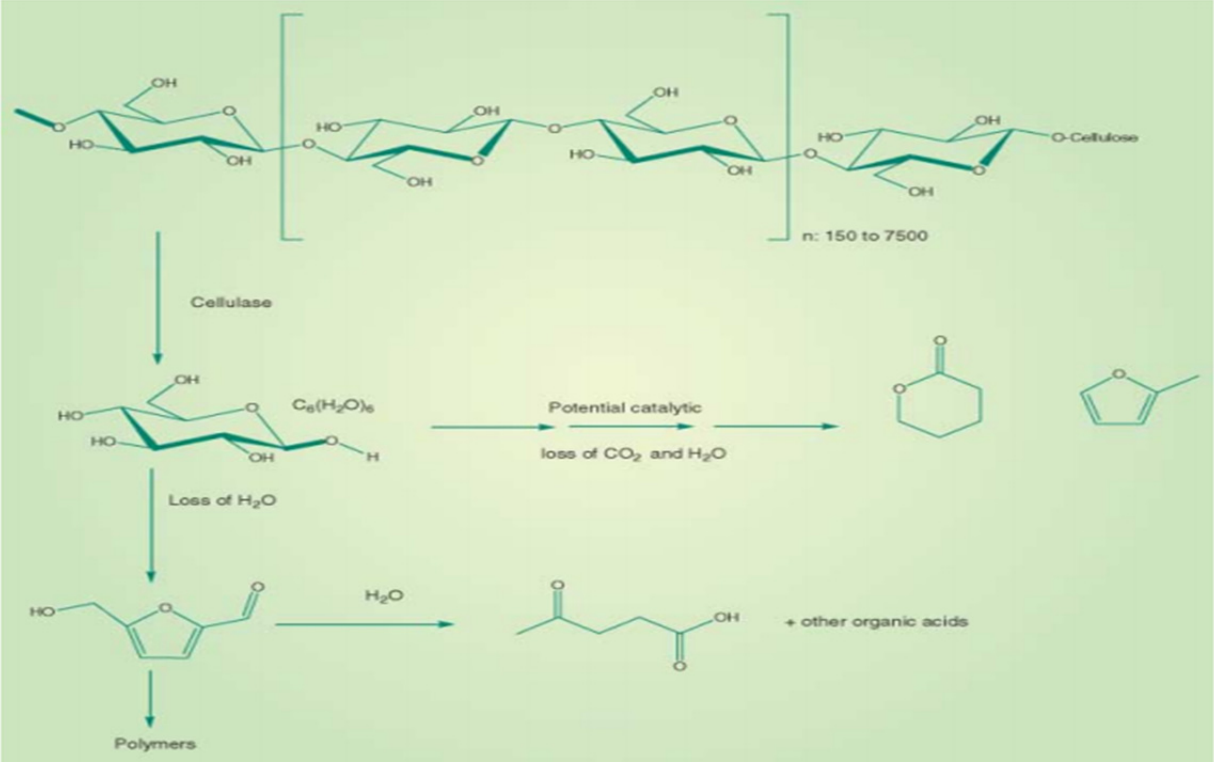


Fig.3: Dehydration-decarboxylation chemistry of hexoses

This reactions can be used for the production of bioenergy and biomaterials to address several societal needs to make advance in genetics, bioethics, process chemistry, and engineering to a new manufacturing concept for renewable biomass to valuable fuels and products referred to as biorefineries (figure 4).

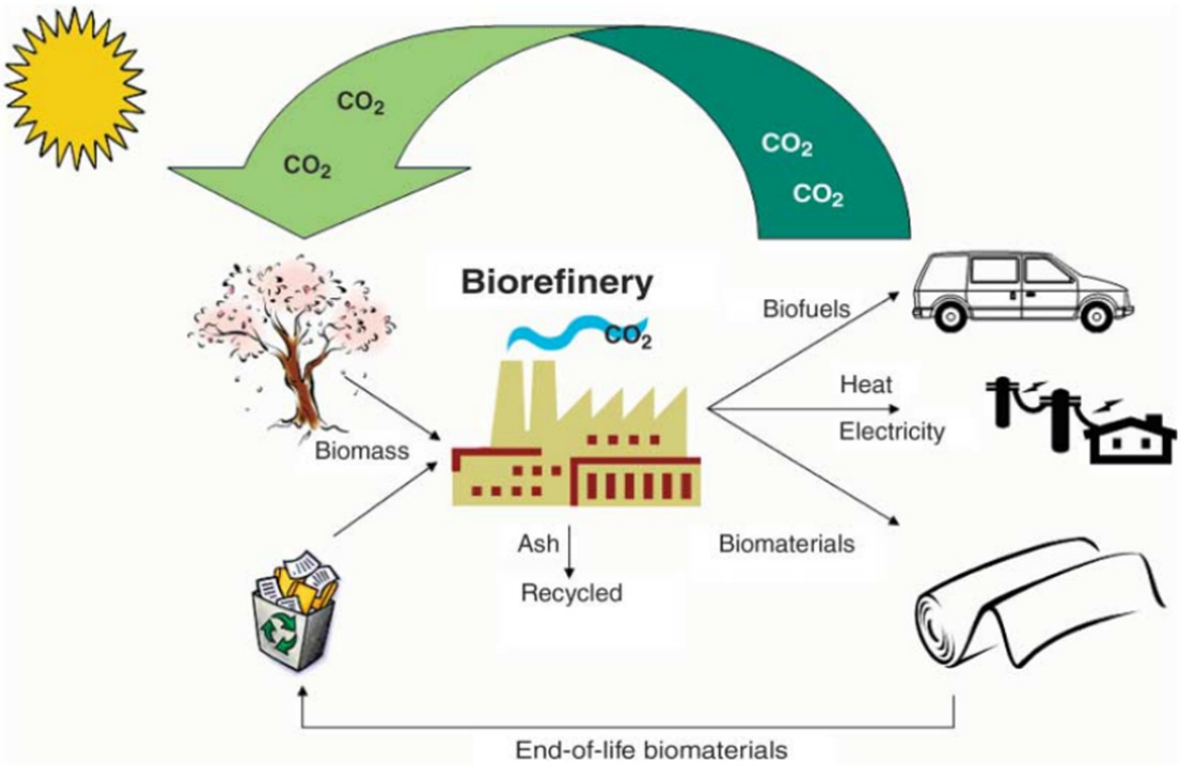


Fig.4: Biorefineries using cellulosic feedstock

For enzymatic hydrolysis of natural celluloses, several determinants of hydrolysis rates have been proposed, including crystallinity, degree of polymerization, particle size, pore volume, and accessible surface area (Converse *et al*., 1988; Mosier *et al*., 2005). The structure of one chain of the polymer is presented in figure 5. Most of the properties of cellulose depend on degree of polymerization (DP), i.e. the number of glucose units that make up one polymer molecule. The DP of cellulose can extend to a value of 17000, even though more commonly a number of 800-10000 units are encountered. For instance, cellulose from wood pulp has a DP between 300-1700 and that from CMC 100 to 1 or even have 170 for untreated sample (Aliyu and Hepher, 2000). The DP of cellulosic substrates determines the relative abundance of terminal and interior β-glycosidic bonds and of substrates for exo-acting and endo-acting enzymes, respectively. Chemical change in the residual cellulose is manifested as change in the DP by attacking interior portion of cellulose molecules. The hydroxyl groups located in the amorphous regions are highly accessible and readily reactive in all chemical reactions, whereas those in the crystalline are not readily accessible and completely inaccessible to some. Enzymatic mechanism for the utilization of cellulose occurs by either removal of

cellulose or utilization of glucose imply enough application and attractive possibilities (Mandels, 1985).

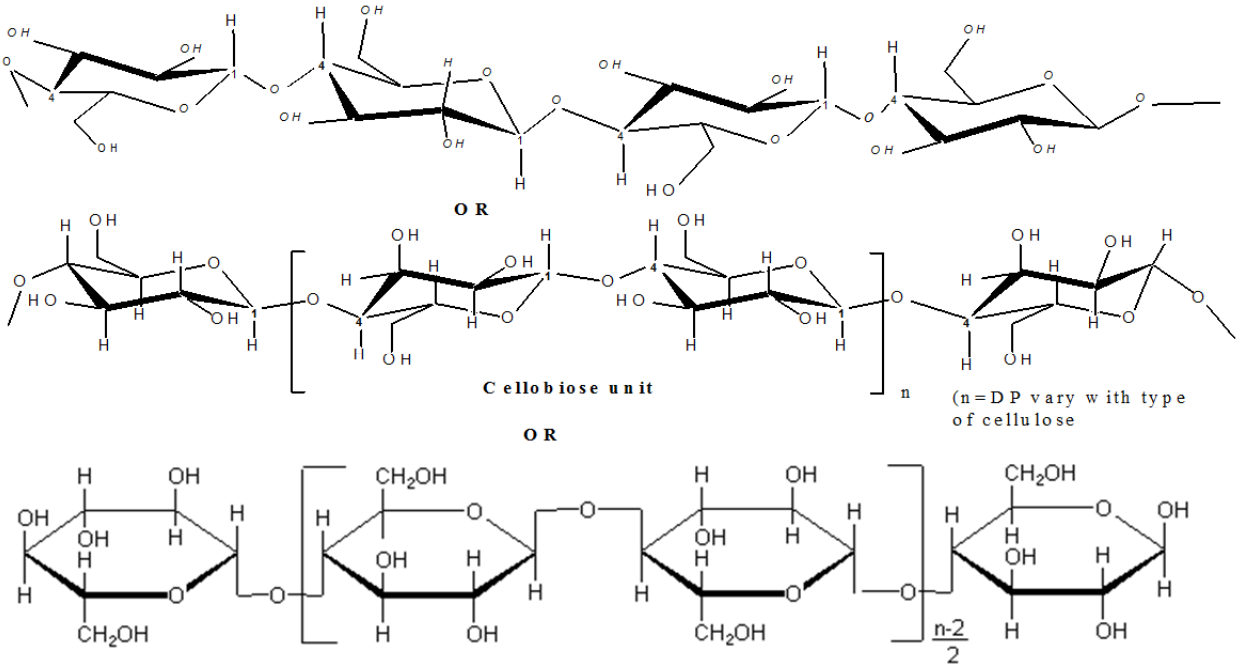


Fig.5: (i) Structure of single cellulose molecule (Chair formula amd Harworth formula)

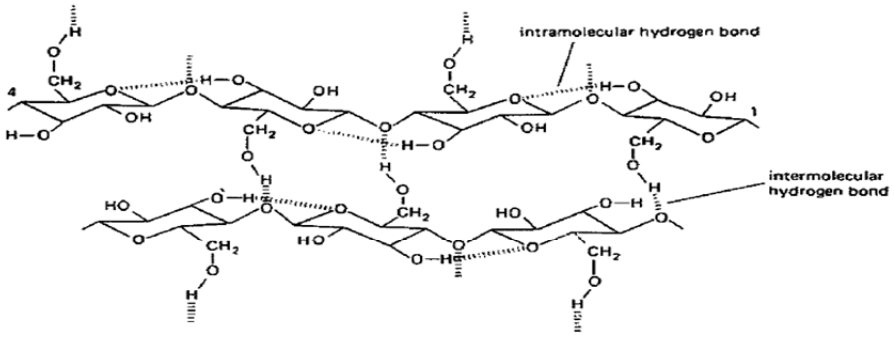


Fig.5: (ii) Cellulose chains showing the *β*- 1, 4-linked residues rotated through 180 degrees with respect to their neighbors in chain and having both intermolecular and intramolecular hydrogen bond

2. 1.1. Application of cellulases

Cellulase enzymes have wide applications. Recently, it has been explored for generation of sugars for the biofuel production. Besides it has many applications in food and animal feed, textile and chemical industries. Some of the applications are mentioned in table 2.

Table 2: The application of cellulases

Applications of cellulases (Mandels, 1985)
(1) Removal of cell walls, crude fibre (a) Release cell contents Flavours Enzymes Polysaccharides (agar) Protein (seeds, leaves) (b) Improve rehydratability of dried vegetables Soup mixes (c) Increase digestibility of high-fibre feeds Oil seed cakes Straws Barley Mesquite (d) Production of plant protoplasts Genetic engineering of higher plants
(2) Production of glucose, soluble sugars (a) Animal feeds Molasses direct or by-product. Increase nutritive value; add sugar to high-fibre feed Single-cell protein: yeasts (b)Industrial feedstock Glues, adhesives Chemicals: ethanol, butanol, etc.
(3)Production of lignin Adhesives Resins Extenders Chemicals raw material
(4)Miscellaneous food applications (a) Cell-free protein High productivity High quality protein (b)Fermentative foods/cellulolytic organisms Addition of mycelial and extracellular protein Removal of crude fibre Conversion of fibre to sugar Removal of other unwanted components (c)Fungal proteases Acid proteases of *Trichoderma* as a meat tenderizer.

Some instances for the application of cellulases

- Cellulases are often used in bioremediation to remove oil spills - a new biotechnological procedure was developed using cellulase as a modifier to produce oil sorbent from corn stalk demonstrated that corn stalk modified by cellulase is an efficient and environment-friendly biosorbent for the removal of spilled oil (Peng *et al*., 2013).
- The enzymes are also used in bioconversion of waste cellulose from paper sludge (Prasetyo *et al*., 2011), water effluents, agriculture residue (Mutreja *et al*., 2011), forest products and even used in deinking dyes and remove stains.
- Enzyme is also used in biostoning process at industrial scale with uniform abrasive effect (Fang and Ouyang, 2010).
- Bofuel production through feedstock's production and biorefining is possible through enzymatic conversion of cellulose to reducing sugar and finally to ethanol to create market for green biofuel with measuring and improving environment performance through cleaner fuel perspectives.
- Cellulases are often used in biocommodity engineering for the production of commodity products (fuels, chemical and materials) and develop of human society in the 21st century is based on sustainable products like cellulose apart from technological revolution to understand and capability related to living system (Lynd *et al*., 1999).
- Cellulases preferably neutral and endoglucanase rich are also used to remove excess dye from denim fabrics, excess microfibrils from surface cotton and non-denim fabrics (Bhat, 2000) and useful even in detergent industry to remove dirt from clothes during washing.
- Cellulases and related enzymes (hemicellulases and pectinases) used as antimicrobial agent during biocontrol, due to their ability to degrade the cell wall of plant pathogen, inhibit spore germination, germ tube elongation and fungal growth (Gijzen *et al*., 1988).

2. 2. Cellulose as a source of Biofuel

The enzymatic research regarding the breakdown of cellulosic products or improvement in hydrolysis is possible by optimization of the cellulase enzymes and the enzyme loading. Engineering organism with novel gene by genetic manipulation can be helpful in generation of second generation ethanol. Campbell and Laherrère used several different techniques to estimate the current known crude oil reserves and the reserves as yet undiscovered and concluded that the decline in worldwide crude oil production begin before 2010. Further, the researcher predicted that the annual global oil production would decline from the

current 25 billion barrels to approximately 5 billion barrels in 2050 (Campbell and Laherrère, 1998). Because the economy in the globe specially US and many other nations depends on oil, the consequences of inadequate oil availability could be severe. Therefore, there is a great interest in exploring alternative energy sources such as the fuel from cellulosic biomass instead of that from corn used during 1980s. These day's ethanol blended fuels are in use, for instance US transportation sector now consumes about 4540 million liters of ethanol annually, about 1% of the total consumption of gasoline (Wang *et al*., 1999). Recently, US automobile manufacturers have announced plans to produce significant numbers of flexible-fueled vehicles that can use an ethanol blend – E85 (85% ethanol and 15% gasoline by volume) – alone or in combination with gasoline. Using ethanol-blended fuel for automobiles can significantly reduce petroleum use and exhaust greenhouse gas emission (Wang *et al*., 1999). It acts as a safer alternative to methyl tertiary butyl ether (MTBE), the most common additive to gasoline used, to provide cleaner combustion of cellulose to bioethanol.

Extensive research has been completed on conversion of lignocellulosic materials to ethanol in the last two decades (Dale *et al*., 1984; Wright, 1988; Azzam, 1989; Cadoche and López, 1989; Reshamwala *et al*., 1995; Bjerre *et al*., 1996; Duff and Murray, 1996). The conversion of cellulose involves different stages from hydrolysis of cellulose in the lignocellulosic materials to fermentable reducing sugars, and fermentation of the sugars to ethanol. The hydrolysis is usually catalyzed by cellulase enzymes, and the fermentation is mainly carried out by yeasts or bacteria. The factors that have been identified to affect the hydrolysis of cellulose include porosity (accessible surface area) of the waste materials, cellulose fiber crystallinity, and lignin and hemicellulose content (McMillan, 1994). The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose difficult, thus reducing the efficiency of the hydrolysis. Removal of lignin and hemicellulose, reduction of cellulose crystallinity and an increase of porosity in pretreatment processes can significantly improve the hydrolysis (McMillan, 1994).

2.3. Hydrolysis of cellulose

Cellulose hydrolysis can be done by chemically or enzymatic ways besides some extent through mechanical breakdown by milling and ultrasonication. Chemical hydrolysis is purely initiated by chemical reactions for disruption of biomass structure. Weak acid/ alkali, strong acid/alkali, organoslov, oxidative delignification etc are some of the chemical pretreatment methods. Similarly, the combination of mechanical and chemical such as steam explosion,

ammonia fiber explosion etc are often used for cellulose hydrolysis. The main aim of pretreatment or hydrolysis before actual utilization of cellulose to make improvement in hydrolysis of lignocellulosic materials (Harmsen *et al.*, 2010) by:

- Increasing of the surface area and porosity
- Modification of lignin structure
- Removal of lignin
- Partial depolymerization of hemicellulose
- Removal of hemicellulose
- Reducing the crystallinity of cellulose

Although there are obstacles in the existing pretreatment and chemical hydrolysis processes with insufficient separation of cellulose and lignin and formation of by-products that inhibit ethanol fermentation (e.g. acetic acid from hemicellulose, furans from sugars). So, these days research has been diverted in enzymatic pretreatment and hydrolysis.

Enzymatic hydrolysis of cellulose is carried out by cellulase enzymes which are highly specific (Beguin and Aubert, 1994). The products of the hydrolysis are usually reducing sugars including glucose. Utility cost of enzymatic hydrolysis is low compared to acid or alkaline hydrolysis because enzyme hydrolysis is usually conducted at mild conditions (pH 4.8 and temperature 45–50^{o}C) and does not have a corrosion problem (Duff and Murray, 1996) .

2.3.1. Sources of cellulase

Cellulose hydrolyzing enzymes are widespread in fungi and bacteria. They produce three types of cellulases responsible for the hydrolysis of cellulose either separately or in the form of complex (Bhat and Bhat, 1997; Lynd *et al*., 2002). These microorganisms can be aerobic or anaerobic, mesophilic or thermophilic. Most commonly available commercial cellulase is produced by *Trichoderma spp.* Bacteria belonging to *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces* can produce cellulases (Bisaria and Martin, 1991). *Cellulomonas fimi* and *Thermomonospora fus*ca have been extensively studied for cellulase production. Although many cellulolytic bacteria, particularly the cellulolytic anaerobes such as *Clostridium thermocellum* and *Bacteroides cellulosolvens* produce cellulases with high specific activity, but do not produce high enzyme titres (Duff and Murray, 1996). Anaerobic fermentation of cellulose results in the production of low-molecular-weight fatty acids as well as carbondioxide, water and cell biomass. But anaerobes degrade cellulose primarily via

complexed cellulase systems localized directly on the surface of the cell or the cell-glycocalyx matrix without release of measurable amount of extracellular cellulase. Unlike aerobic cellulase degraders, both bacteria and fungi utilize cellulose through the production of substantial amounts of extracellular enzymes that are freely recovered from culture supernatants (Rapp and Beerman, 1991; Schwarz, 2001). The main products of cellulose degradation under aerobic condition are carbondioxide, water and cell biomass. Some of the cellulolytic bacteria based on oxygen relationship are grouped as (Lynd *et al*., 2002) in table 3.

Table 3: Classification of cellulolytic bacteria

Bacteria	**Gram-positive**	**Gram-negative**
Aerobic	*Acidothermus*, *Bacillus*, *Caldobacillus*, *Cellulomonas*, *Micromonospora*, *Streptomyces*, *Thermobifida*	*Cellvibrio*, *Cytophaga*, *Erwinia*, *Pseudomonas*, *Sporocytophaga*
Anaerobic	*Anaerocellum*, *Butyrivibrio*, *Closteridium*, *Eubacterium*, *Ruminococcus* and *Spirochaeta*	*Acetivibrio*, *Caldicellulosiruptor*, *Ferbidobacterium*, *Fibrobacter*, *Halocella*, *Thermotoga*

Many research for commercial cellulase production focused on fungi (Duff and Murray, 1996) although fungi play minor role in the cellulose degradation during anaerobic condition. But aerobic cellulose utilization is distributed across entire kingdom from primitive, protist-like Chytridomycetes to the advanced Basidomycetes. Fungi that have been reported to produce cellulases include *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* (Sternberg, 1976; Fan *et al*., 1987; Duff and Murray, 1996). Of all these fungal genera, *Trichoderma* has been most extensively studied for cellulase production (Sternberg, 1976). During the enzymatic hydrolysis, cellulose is degraded by the cellulases to reducing sugars that can be fermented by yeasts or bacteria to ethanol.

2.3.1.1. *Bacillus* as cellulolytic organism

Focusing on wide applicability of cellulase enzyme and cellulosic products, it is essential to initiate hydrolysis. It is easily achieved through amorphous zone of cellulose through sets of enzyme known as endoglucanases (Dominguez *et al*., 1992). The enzymes are easily

obtained through bacteria of genus *Bacillus*. *Bacillus species* are gram-positive and endospore forming organism. They have short generation time and ability to grow through high cell density using inexpensive carbon and nitrogen source. *Bacillus species* also has capacity to produce several hydrolytic enzymes (Mawadza and Zvauya, 1996) such as cellulase, protease and amylase, etc. The cellulase has been reported from bacteria is mainly secreted by *Bacillus cereus*, *Bacillus licheniformis*, *Bacillus subtilis* and *Bacillus polymyxa* (Han *et al*., 1995). Most of the *Bacillus species* can be isolated from soils and plant material. Besides this extreme habitat such as hot springs, geothermal vent, etc. can be the place for isolation of moderately thermophilic and thermophilic organisms. One of the important facts about *Bacillus species* is the organism is easier for genetic manipulation unlike fungi. Next thing is the organism was never reported to be pathogenic i.e. GRAS status for either animals or plants (Lam *et al*., 1998). The availability of genome sequence of *B. licheniformis* ATCC 14580 facilitates direct genetic manipulations (Waldeck *et al*., 2007) and thus maximum exploitation of gene with possibility for higher level expression of endogenous cellulase i.e. more easily achieved in bacteria (Li *et al*., 2009). Thus, the organism is extensively used for large-scale industrial production of exoenzymes as it can secrete large quantities of proteins (Veith *et al*., 2004).

2.3.2 Classification of cellulolytic enzymes

Cellulolytic enzymes are synthesized by large number of microorganisms ranging from fungi, actinomycetes and gliding bacteria to true bacteria of different genus. Fungal enzymes have been most extensively studied and secreted in large amounts in the media and advances have been made recently in the understanding of the enzymes and their modes of operation. It is well established that rapid dissolution of cellulose requires the independent and co-operative action of mixture of enzyme although there is speculation regarding the mechanism how they interact and what factors control the interactions on the face of the cellulose crystallite. Fungal cellulases consists of three major enzymes involved in the hydrolysis of native cellulose (Wood and McCrae, 1979) namely endo-1,4-β-glucanase (endo-1,4-β-D-glucan-4-glucanohydrolase, EC 3.2.1.4), cellobiohydrolase (1,4-β-D-glucan 4—glucohydrolase, EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). Some cellulase system a minor component a glucohydrolase (1,4-β-D-glucan-4-glucohydrolase, EC 3.2.1.74). Cellobiohydrolase, a major constituent present in fungal cellulase system with ability to degrade crystalline cellulose; thus the exoglucanase would appear to be distinguishing feature.

Some of the generalization of classification made for the fungal enzyme system which are equally applicable to most of the bacterial cellulase system are:

Table 4: General classification of cellulolytic enzymes

Enzyme Type	**EC Code**	**Synonym**	**Mode of Action**
Endo-(1-4)-β-D-glucanase	EC 3.2.1.4	CM-cellulase or CX enzyme Endoglucanase or endocellulase	Attack CM-cellulose or amorph-ous region of cellulose (Mosier *et al*., 2005) in random manner with increase in reducing groups (Wood and McCrae, 1979)
Exo-(1-4)-β-D-glucanase	EC 3.2.1.91	Cellobiohydrolase or exocellulase	Release cellobiose either from reducing or non-reducing end (Bhat and Bhat, 1997; Lynd *et al*., 2002)
1,4-β-D-glucan-4-glucohydrolase	EC 3.2.1.74	Exoglucanase or glucohydrolase	Releases glucose from non-reducing end (Wood and McCrae, 1972; Berghem and Pettersson, 1973) prefer substrate of longer chain length and hydrolyzed by inversion of their products.
β-Glucosidase	EC 3.2.2.1	Cellobiase	Releases glucose from cellobiose and short chain cello-oligosacchafides, transferase activity with retention of β-configuration and hydrolyze low DP faster (Reese *et al*., 1968)

The hydrolysis of cellulose through enzymatic mode require enzyme to be adsorbed on the surface of insoluble substrate. It results in the loosening of bulk of the substrate. The molecular disorder of the tightly packed regions increases to expose the cellulose chains

buried within the microfibrils while they remain almost unchanged as shown in figure 6a (Coughlan, 1985). Once the cellulose network is accessible to the enzymes, the synergistic action of endo- and exo-glucanases promote the fragmentation of accessible molecules to soluble cello-oligosaccharides (figure 6b), which are quickly hydrolyzed, mostly to cellobiose (Figure 6c). β-glucosidase is usually added to completely hydrolyze the cellobiose to glucose (Figure 6d). This component of the proposed mechanism seems likely to occur in most of the cellulase system.

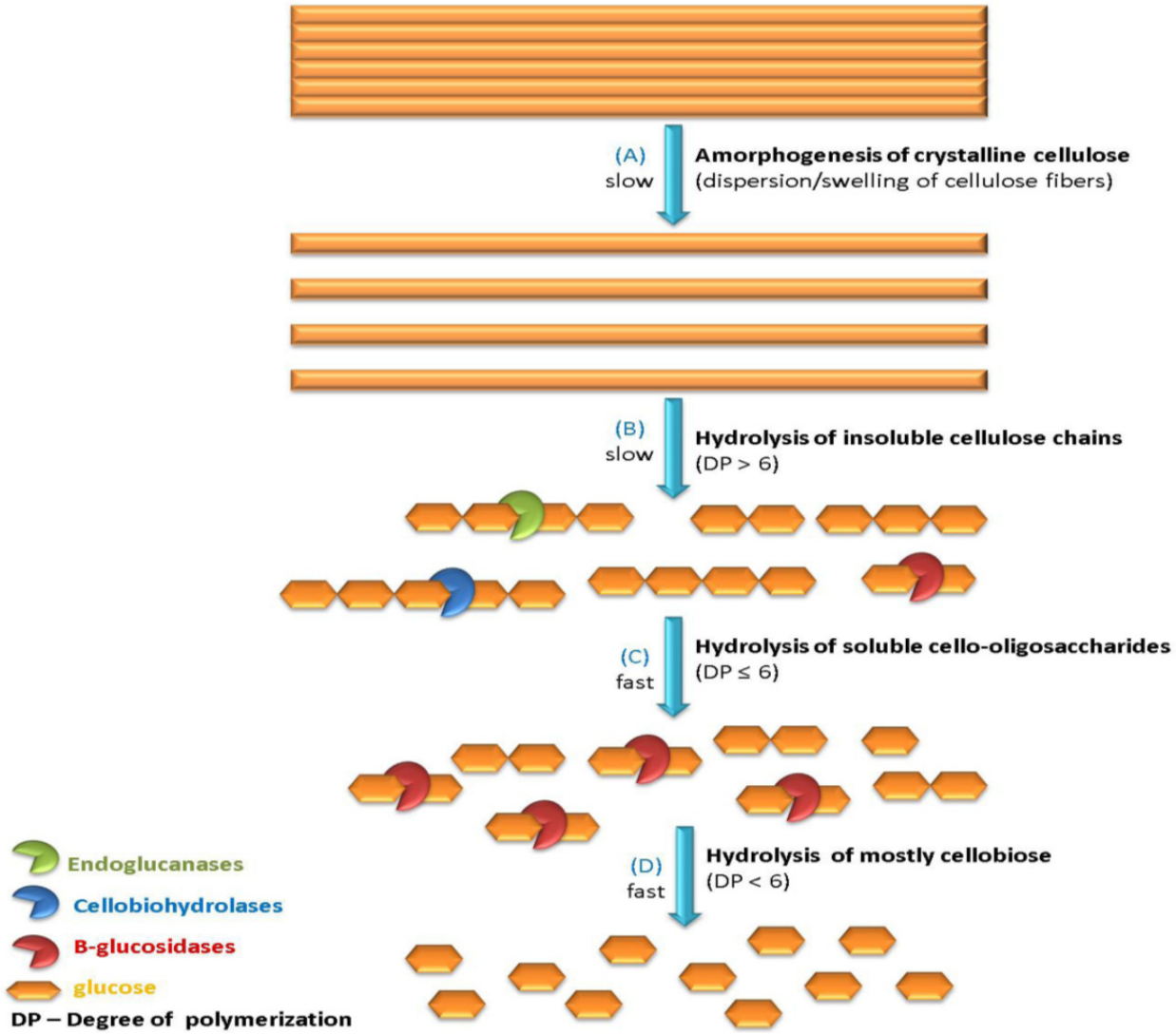


Fig.6: Purposed Mechanism for cellulose hydrolysis adapted from(Arantes and Saddler, 2010)

Unlike fungal cellulases, most of the bacterial cellulases either cell-free and cell-bound unable to solubilize highly ordered cellulose significantly. But they can hydrolyze CM cellulose and H_3PO_4 swollen cellulose to varying degrees. Such enzyme preparation contains

endoglucanase but little exoglucanase activity. *Closteridium thermocellum* is an example. It produces an yellow substance with low molecular weight (Dermoun and Belaich, 1985) affinity for endoglucanase also comprises a multi-subunit with both exo-endoglucanase activity called celulosome. It imply involvement of enzyme-enzyme complexes (Lamed *et al*., 1983) and perhaps gives some credence to the speculation that these may also exist in fungal cellulose.

2.4. Enzyme production and characterization

The factors responsible for optimum cellulase enzyme production include microbial strains, media composition with C-source, nitrogen source and process control (Ghosh *et al*., 1984) such as effect of metal ions, pH, temperature and agitation, etc. Although cellulases are produced by number of microorganisms, the yields are still low due to catabolite repression and end product inhibition (Amano and Kanda, 2002). Cellulase activity is regulated by induction and repression (Lin and Wilson, 1987). To improve the yield and rate of the enzymatic hydrolysis, research throughout the world are focused on optimizing the hydrolysis process, purification of enzymes and studying enzyme kinetics and enhancing cellulase activity (Adlakha *et al*., 2012; Yeh *et al*., 2013). For instance, in the production of endoglucanase (Ariffin *et al*., 2008) from oil palm empty fruit branch by locally isolated aerobic *Bacillus pumilus* EB3, the effects of the fermentation parameters such as initial pH, temperature, and nitrogen source on the endoglucanase production were studied using carboxymethylcellulose (CMC) as the carbon source and optimum condition obtained as 37^{o}C, initial pH 7.0 with 10 g/l of CMC as carbon source, and 2 g/l of yeast extract as organic nitrogen source. The activity recorded during the fermentation was 0.076 U/ml. The productivity of the enzyme increased two fold when 2 g/l of yeast extract was used as the organic nitrogen supplement as compared to the non-supplemented medium suggesting an oil palm can act as good substrate for enzyme production. A novel bacteria MG7 with maximum production of enzyme observed at pH 7.0 and temperature 35^{o}C whereas, purified enzyme showed maximum activity at pH 6.0 and 65^{o}C and degrade cellulosic substrate as CMC, avicel, filter paper and beta glucan (Asha *et al*., 2012). Therefore, it is very important to characterize the biochemical character of enzyme to investigate the specific activity.

Besides this enzyme production in the industrial scale require bioreactor in which one can produce enzyme according to the requirement. A great number of articles with the production of cellulases on different carbon sources and optimization of growth conditions

in laboratory and pilot fermenters have appeared (Sternberg, 1976; Gong *et al*., 1979; Ryu and Mandels, 1980; Coughlan, 1985; Doppelbauer *et al*., 1987; Suto and Tomita, 2001). But it is necessary to design solid state or submerged state fermentation, feed-batch or continuous fermentation. Next thing is it is necessary to optimize culture condition (Asha Poorna and Prema, 2007) inside fermenter by varying different parameters from substrate to dissolved oxygen, agitation, aeration rate and even control of pH and temperature until the production reach optimum.

2.4.1. Substrate for study of enzyme

Both fungal and bacterial cellulolytic organisms produce multiple cellulases when grown on cellulose. Among most of the previously studied mechanism of hydrolysis reaction of cellulase, substrate with crystalline property are used (Zhang and Lynd, 2006). The surface area of crystalline substrates is lower than non-crystalline substrate; hence the cellulase accessibility is also low for crystalline substrates and catalytic activity of the enzyme is controlled by the chemical characteristics of cellulose (Zhang and Lynd, 2004). But the elimination of the physical barrier for cellulase access is necessary to understand the effect of true intrinsic chemical features such as crystallinity and DP on the reactivity of various cellulolytic components. To understand the diverse characteristics of ezyme on different cellulosic substrates ranging from Avicel, filter paper and CMC are often used. So that one can monitor release of glucose, cellobiose or other reducing sugar (Eriksson *et al*., 2002) using colorimetric method (Gan *et al*., 2003) or weight loss of the substrates (Valjamae *et al*., 1998). Monitoring cello-oligosachharides (COS) in cellulase-NCC reaction provides an additional tool to study and separate between individual enzymes such as endo-glucanase (Endo-G) and exo-glucanase (Exo-G) (Valjamae *et al*., 1998; Kojima *et al*., 2012). Similarly, for cellobiase or β-glucosidase production, substrate such as cellobiose or *p*-nitrophenol-β-D-glucopyranoside (pNPG) can be used. The release of *p*-nitrophenol can be checked to make kinetic analysis of cellobiase or β-glucosidase (Chauve *et al*., 2010). However, the individual enzyme may behave differently depending on whether they exist singularly or in mixture in which different supplementary non-hydrolytic proteins are also present. Synergistic action of different cellulolytic proteins has been observed with hydrolytic as well as non-hydrolytic proteins (Lynd *et al*., 2002; Robledo *et al*., 2012). Endo-G is known to react with the amorphous region of the cellulose, generating reducing ends and the COS whereas; Exo-G action can be studied by glucose and cellobiose data. As long as the different components of celluase system in crude cellulase have not been separated, the determination of their activity is a difficult task, well reflected in the diversity of analytical

procedures so far devised (Mandels *et al*., 1976). There are mainly three types of assay (Canevascini and Gattlen, 1981) so far used to study the activity of cellulase such as:

1) Assay based on determining the decrease in viscosity of solutions of soluble cellulose derivatives (carboxymethyl-, hydroxyethylcellulose) during the enzymatic reaction,

2) Assays in which the increase in reducing power during the enzymatic reaction toward soluble CMC is monitored by means of a reducing sugar determination and

3) Measuring the reducing power as in 2) but using insoluble (un-substituted) cellulosic substrates.

As an alternative to the viscometric method, endo-cellulase activity can be determined by monitoring the increase in reducing power during the enzymatic reaction toward a soluble CMC although exo-cellulase and β-glucosidase eventually contribute in an unpredictable manner to the final result.

Some of the substrates used for measuring cellulase activities are mentioned in table 6 (Bhat and Bhat, 1997).

Table 6: Substrate used for measuring cellulase

Enzyme	**Substrate**	**Assay**
Total cellulase	Cotton	Solubilization: 1. Estimation of cellulose residue 2. Reducing sugar released 3. Weight loss 4. Loss in tensile strength
	Filter paper, Hydrocellulose, Avicel and Solka Floc	Solubilization: 1. Release of reducing sugars
	Dyed Avicel	1. Release of dyed soluble fragments

Exo-1,4-β-D-glucanase (cellobiohydrolase, exocellulase or Avicelase	**Avicel** Hydrocellulose Dyed Avicel Amorphous cellulose Substituted and unsubstituted cello-oligosaccharides	Solubilization: 1. Release of reducing sugars 2. Release of dyed cellobiose Solubilization: 1. Release of reducing sugar 2. Decrease in turbidity 1. Increase in reducing power 2. Analysis by HPLC
Endo- 1,4-β-D-glucanase (endoglucanase, CM- cellulase, endocellulase)	**CMC** Hydroxyethylcellulose Substituted and Un-substituted cello-oligosaccharide Cotton Amorphous cellulose	1. Release of reducing sugars 2. Decrease in viscosity 1. Increase in reducing power 2. Analysis by HPLC 1. Swelling in alkali 2. Solubilization: release of reducing sugars 3. Decrease in turbidity
β-Glucosidase or Cellobiase	**Cellobiose** Cello-oligosaccharides o- or ***p*-Nitrophenyl-β-D-glucosides**	1. Release of glucose 2. Increase in reducing power 1. Release of o- or *p*-nitrophenol

2.4.1.1. Carboxymethylcellulose (CMC):

Carboxymethylcellulose (CMC) is a derivative of cellulose, containing carboxymethyl groups that are generated via the reaction of cellulose with chloroacetate in alkali to produce substitutions in the C2, C3, or C6 positions of glucose units. The molecular structure of CMC is shown in Figure 7 (Gelman, 1982). CMC was first produced in Germany in the 1930s'. Since 1947, it was manufactured in the USA. It was used for the production of synthetic laundry detergent. Meanwhile CMC began to be used in many other industries. As a result, CMC is water soluble and more amenable to the hydrolytic activity of cellulases. CMC is therefore a useful additive to both liquid and solid medium for the detection of cellulase activity (Mandels *et al*., 1976), and its hydrolysis can be subsequently determined by the use of the dye Congo red, which binds to intact β-D-glucans. Zones of clearing around

colonies growing on solid medium containing CMC, subsequently stained with Congo red, provides a useful assay for detecting hydrolysis of CMC and therefore, β-D-glucanase activity (Teather and Wood, 1982).

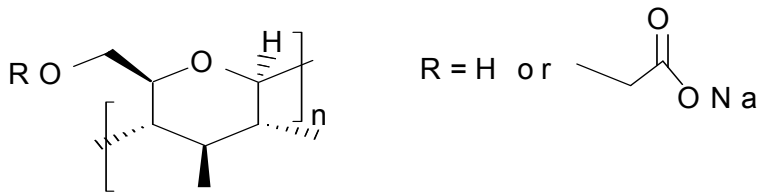


Fig.7: Molecular structure of carrboxymethyl cellulose

2.5. Thermophilic bacteria and thermostable enzyme

Thermophiles are found in all domains as multicellular and unicellular organisms, such as fungi, algae, cyanobacteria, bacteria and protozoa and they grow best at temperatures higher than 45°C. In contrast, the extreme thermophiles, or hyperthermophiles, grow best at temperatures higher than 80°C and are almost exclusively restricted to the Archaea, with only two hyperthermophilic orders in the Bacteria, namely, the Thermotogales and Aquificales (Reysenbach and Rising, 2001). There are ongoing research around the globe on thermophilic organisms, their habit, habitat, enzyme production, metabolite secretion and even evolution from biochemical to molecular level. Thermophilic microorganisms have been of great scientific interest for several decades, principally in regard to their overall inherent stability, biotechnological potential and also of the thermostable enzymes they produce (Yang *et al*., 1969). The enzymes can be produced from the thermophiles through either optimized fermentation of the microorganisms or cloning of fast-growing mesophiles by recombinant DNA technology (Haki and Rakshit, 2003). There is a general relatedness evident between catalytic domains, cellulose binding domains and other ancillary domains in thermophiles, which suggests that there may have been significant lateral gene transfer from mesophiles during the evolution of these microorganisms (Bergquist *et al*., 1999).

In the present days, along with other organisms the research on cellulose degrading microorganism specially bacteria through different extreme habitat from hot-springs to hydrothermal vent has been going on, to study broad potential of organism to decompose a set of various carbon substrates. Further, study on thermostable cellulases offer many advantages in the bioconversion process including increase in specific activity, higher levels of stability, inhibition of microbial growth, increase in mass transfer rate due to lower fluid viscosity, and greater flexibility in the bioprocess (Anbar and Bayer, 2012). Thermophilic bacteria and thermostable enzyme also help to overcome the limitations of existing

lignocellulosic biomass conversion processes (Bhalla *et al*., 2013). Most studies on the thermophilic CBP concentrate on co-cultivation of the thermophilic cellulolytic bacterium *Clostridium thermocellum* with non-cellulolytic thermophilic anaerobes at temperatures of 55^{o}C to 60^{o}C result in screening of cellulolytic bacteria growing at temperatures >70 degrees C to enable direct conversion of lignocellulosic materials into ethanol and not requiring additional cellulase (Svetlitchnyi *et al*., 2013).

2.6 Molecular study of cellulase gene

2.6.1. Identification of bacteria

Beginning in the 1990s, the application of molecular ecological methods, especially those based on surveys of genes after PCR amplification, has allowed easier identification of species of bacteria (Janssen, 2006). The power of method relies on the targeting of functional genes or phylogenetically informative genes, or RNA. In particular, 16S rRNA and its gene have proven to be useful powerful marker for the elucidation of bacterial phylogeny as well as bacterial identification. Phylogenetic investigation made by targeting alternative phylogenetic markers such as large subunit rRNA, elongation factor, and ATPases have shown the 16S rRNA-based trees reflect the history of corresponding organisms globally (Ludwig *et al*., 1998). Apart from this bacterial identification can be made by using different phenotypic and genotypic methods including fatty acid methyl ester and rep-PCR profiling, BOX-PCR (Adiguzel *et al*., 2009).

2.6.2. Molecular cloning

Nobel bacteria encoding cellulase producing gene can be identified based on cellulase specific primer through polymerase chain reaction from different habitat and can be clone into easily grown host for the higher level expression of functional protein by genetic and protein engineering technology with aim of overall increase in the production of enzyme in industrial scale. For the fulfillment of the purpose, there has been work going on cloning of individual genes that help in characterization and sequencing of genes and to study gene regulation under different condition. The cloned genes are also useful source of cellulase components devoid of contamination by other gene products (Ghangas and Wilson, 1987). Some of the cloned endoglucanase that initiate cellulose degradation are given in the table 7. Besides above, the cellulase genes from fungi, bacteria and actinomycetes have been cloned, expressed and recombinant enzymes purified and characterized (Beguin and Aubert, 1994). In addition, using genetic engineering techniques, attempts have been made to change the proportion of different cellulase and *invitro* modification of gene incapable of

coding for active enzyme has been reported in *T. reesei* (Harkki *et al*., 1991). The molecular study also made for gene from bacterial enzyme with exo-cellobiohydrolase. A specific endo-β-1, 4-D-glucanase (Avicelase-II) resembling exo-cellobiohydrolase (CBH) was purified from *Bacillus circulans* F-2. Both Avicelase and carboxymethylcellulase activity was found to reside on a monomeric protein of 72 kDa (Kim and Kim, 1995). Gene coding for particular enzyme was cloned and sequenced followed by molecular analysis of protein. Similarly, the *cel*-BL11 gene from *Paenibacillus campinasensis* BL11 was cloned and expressed in Escherichia coli as a His-tag fusion protein (Ko *et al*., 2010) and the gene, *cmc*I, encoding an enzyme of approximately 74 kDa was cloned as a 2.7 kb fragment and expressed in Escherichia coli (Kahler and Pemberton, 1996). It is capable of degrading CMC and xylan but did not attack the microcrystalline cellulose substrate avicel. A second cellulase capable of degrading avicel, encoded by *exo*I, was found 5.5 kb downstream of *cmc*I. Two translation products of 53.7 kDa and 51.5 kDa were produced in *E. coli* strains expressing *exo*I. This kind of trend for the characterization of cellulase system in microorganisms has been developed in molecular cloning and genetic manipulation by different researcher (Bergquist *et al*., 1999; Endo *et al*., 2001; Aftab *et al*., 2012; Amore *et al*., 2012).

Table 7: Endoglucanase gene cloned and expressed from some microorganisms

Microorganisms	Genes	Host	Reference
Beetle -*Batocera horsfieldi*	β-1,4-endoglucanase	Baculovirus-infected insect BmN cells and *Bombyx mori* larvae	(Xia *et al*., 2013)
Myceliophthora thermophila	GH7 endoglucanase	*Pichia pastoris*	(Karnaouri *et al*., 2013)
Strptomyces	*celstrep*	*Escherichia coli*	(Amore *et al*., 2012)
Bacillus licheniformis ATCC 14580	endo-1, 4–β-glucanase	*Escherichia coli* BL21 (DE 3	(Aftab *et al*., 2012)
Aspergillus niger BCRC31494	Endoglucanase B (EGLB)	*Pichia pastoris*	(Li *et al*., 2012)
Closteridium thermocellum	*celA*	*Bacillus subtilis*	(Liu *et al*., 2012)
Paenibacillus strain(ICGEB2008)	β-1,4-endoglucanase (*endo*5A)	*Escheichia coli*	(Adlakha *et al*., 2011)
Bacillus spp.	β-1,4-endoglucanase	*Escheichia coli*	(Afzal *et al*., 2010)

CHAPTER 3: MATERIALS AND METHODS

3.1. Microbial strains, culture condition, and enzyme production

3.1.1. Microbial strain

A total of 16 bacterial strains were assessed for the production of cellulase enzyme. All these bacterial strains were isolated from the hot spring of Gorkha, Manaslu Conservation Area located at 953 m height from sea level, extending on latitude of 28^{o}16.598' N and longitude of 84^{o}53.861' E. The pure cultures of each isolated bacteria were inoculated into sterile Nutrient agar (NA) slants and nutrient broth (NB) for further use.

3.1.2. Preservation of Isolates

Glycerol stocks were prepared and stored at -80°C for long term preservation. Cultures were grown on NA plates. They were transferred in NB until the optical density reached 0.5 to 0.6 at 600 nm and 0.5ml of each culture broth was transferred into the cryotubes and 0.5 ml broth containing 20% glycerol were added. Tubes were mixed gently and thoroughly freeze and were stored at -80°C freezer.

3.1.3. Screening of extracellular enzymes

3.1.3.1. Crude enzyme preparation

For the screening of different extracellular enzyme, crude enzyme preparation was used. Crude enzyme was prepared from all the sixteen bacterial isolates by transferring the culture into Nutrient broth (NB) separately in 15 ml culture flask under aseptic condition. It was incubated in an incubator (100 rpm) at 50^{o}C for 24 hours. On the following day, broth was centrifuged at 4100 rpm for ten minutes at 4^{o}C and the supernatant was used as crude enzyme.

3.1.3.2. Screening of extracellular enzymes

The ability of isolates to hydrolyze different substrate was tested by agar plate diffusion method. The NA plate incorporated with different substrates (CMC, xylan, pectin, skim milk, casein and starch 0.5% each; tween 80 (2%), tributyrin (2%) were used for the enzyme screening. Using cork borer the wells were made aseptically and about 50 µl the supernatant was put into each well. Equal volume of media and water were used as negative control. The inoculated plates were incubated at 50^{o}C overnight as mentioned above.

After incubation, for screening cellulase and xylanase activity of isolate, the agar medium was flooded with aqueous solution of Congo red (1 mg/ml) for 15 min (Teather and Wood, 1982). The Congo red solution was then poured off from plate containing CMC or xylan as substrate. It was further treated by flooding with 1 M NaCl for 15 min. The diameter of the well and that of hydrolyzed zone were measured followed by the calculation of hydrolyzed zone diameter to well diameter. Similarly, for amylase activity, the plate was flooded with iodine solution. For pectinase, caseinase and protease activity, the clearing zone of hydrolysis was observed of respective substrate. For lipase activity of isolates, tween-80 and tributyrin plates were flooded with methyl red and methylene blue as indicator respectively (Samad *et al*., 1989). Above process was done in triplicates. The isolate with maximum hydrolyzed zone in CMC plate was chosen for further biochemical characterization of cellulase enzyme.

3.2. Identification of cellulase producing bacterial isolate

3.2.1. Phenotypic identification

3.2.1.1. Gram staining

A clean grease-free slide was taken and a thin smear of 24 hours bacterial culture grown in 50^{o}C in a NA plate was prepared and the smear was allowed to dry in a sterile zone followed by heat fixation. It was flooded with crystal violet, a primary stain for one minute followed by quickly and gently rinsing off the stain from the slide. Gram's iodine was applied as a mordant for one minute. One drop of alcohol (95%) was used as a decolorizer for 20 seconds and the slide was rinsed gently with water. The slide was counterstained with safranin for one minute and rinsed with water. It was air dried and finally observe in low power (10X) followed by oil immersion (100X) microscope.

3.2.1.2. Oxidative/Fermentative Test

For the test, two tubes of O/F medium with the name of isolate were labeled and the media semi-solid uniformly mixed was prepared according to the manufacture instruction and cotton plugged. It was autoclaved at 121^{o}C with 15 psi pressure for 15 min and was cooled down in sterile zone. With a sterile inoculating wire, the organism incubated overnight in NB at 50^{o}C was stabbed in both tubes. One of the tubes was sealed with 1 ml of sterile paraffin oil to create anaerobic condition. Both the tubes were incubated at 50^{o}C and the color development in both the tubes was observed after 16 hours incubation.

3.2.1.3. Effect of temperatures on bacterial growth

The temperature tolerance of selected bacterial isolate (above) was tested by growing the isolate at different temperature from 40^{o}C to 65^{o}C for 16 hours. At first bacterial isolate was grown at respective temperature in NB. Absorbance was taken at 600 nm. About 50 µl of organism was spread onto NA plate using spread plate method. All the plates were incubated at their respective temperature and the colony forming units were counted using colony counter.

3.2.1.4. Growth curve and study of growth pattern

For the study of growth pattern, bacterial culture of selected isolate was used to inoculate a single colony to sterile 50 ml NB media in aseptic condition. It was incubated in aerobic incubator at temperature with maximum number of colonies. Absorbance was taken in spectrophotometer (Thermo Scientific, UK) in optical density 600 nm with respective blank of media. Absorbance was taken in different time intervals till the growth reach constant decline phase.

3.2.2. Genotypic identification

3.2.2.1. DNA isolation

DNA was extracted through modified SDS-based DNA extraction method (Zhou *et al*., 1996). Bacterial culture in NB was centrifuged at 8000 rpm for 5 min. About 0.1 g cell pellet was resuspended completely with 564 µl TE buffer. Approximately 10 µg lysozyme (crystalline) was mixed by inverting. The mixture was incubated for 10 to 60 min at 37^{o}C followed by addition of 6 µl proteinase K (10 mg/ml) and 30 µl of 20% SDS. The suspension was mixed and incubated at 37^{o}C until relatively clear and viscous. About 100 µl of 5 M NaCl was added and was mixed thoroughly with suspension. It was incubated at 65^{o}C water bath for 2 min. It was preheated with 80 µl 1 M CTAB/1.5 M NaCl and mixed thoroughly with incubation at 65^{o}C for 10 min. The supernatant were collected after centrifugation at 6000*g* for 10 min at room temperature and transferred to 1.5 ml microfuge tubes. The supernatants were combined and mixed with equal volume of chloroform-isoamyl alcohol (24:1, vol/vol). The aqueous phase was recovered by centrifugation at 10000*g* for 5 min. The process was repeated at 15,000*g* with phenol-chloroform-isoamyl alcohol. The aqueous phase was recovered again and the supernatant was extracted with equal volume of chloroform-isoamyl alcohol by centrifugation at 10,000*g* for 5 min. It was precipitated with 0.7 volume of isopropanol at room temperature for 1 hour. The pellet of nucleic acids was obtained by centrifugation at 12,000*g* for 20 min at room temperature, washed with cold ethanol and

resuspended in 60 µl Tris-EDTA (TE) buffer. DNA was quantified using Biophotometer (Eppendorf, Germany).

3.2.2.2. Amplification of 16S rDNA sequence

The 16S rDNA was amplified using the following set of forward and reverse primers: 8t (FD1) 5'AGTTGATCCTGGCTCA3' and U2-88 (rp2) 5'ACGGCTACCTTGTTACGACT3' respectively (Weisburg *et al*., 1991). The 25 µl reaction mixture contain 2.5 µl (10x) PCR buffer, 0.5 µl (10 mM) dNTP mixture, 2.5 µl (25 mM) $MgCl_2$, 0.5 µl (10 pmol/µl) of each primer, 0.2 µl (5U/µl) Taq DNA polymerase, 1 µl of genomic DNA (20 ng/µl) and 17.3 µl double distilled autoclaved water. The reaction mixture was incubated in a thermal cycler (Eppendorf, Germany) at 95^{o}C for 5 min and then underwent 30 cycles of 1 min at 95^{o}C, 1 min at 55^{o}C and 1 min at 72^{o}C, followed by final extension of 7 min at 72^{o}C.

3.2.2.3. Electrophoresis of amplified PCR product

The PCR product was analyzed on 1.5% agarose gel, prepared by dissolving 0.75 g agarose in 50 ml of TAE buffer (242 g/l Tris acetate, pH 8.0, 57.1 ml glacial acetic acid, 100 ml sodium salt of 1mM EDTA) and boiling. About 1.8 µl ethidium bromide (0.5 µg/ml) was added to it. The gel upon cooling to 50^{o}C was poured to the gel caster of electrophoresis apparatus (Major Science, USA). The gel was then allowed to solidify. TAE buffer was filled in the chambers of the electrophoresis apparatus and gel was placed in it allowing the buffer to make a thin film covering the gel. The DNA sample (6 µl) was prepared by mixing with 6x loading buffer (5:1 ratio). Samples were loaded and the gel was electrophoresed at a constant voltage of 80 V. DNA bands were visualized on Gel Documentation System (Syngene, UK).

3.2.2.4. Sequencing and sequence analysis

The PCR product from 16S rDNA based PCR was sent to Macrogen (Korea) for sequencing. The sequence was then aligned and analyzed for phylogenetic relatedness of raw sequence using Condon Code Aligner 4.0.4. Based on the similarity search tool BLAST, the isolate was identified and the sequence was submitted to NCBI.

3.3. Cellulase assay

The cellulase enzyme was assayed for hydrolysis of CMCase using modified CMCase assay method (Ghose, 1987; Wood and Bhat, 1988) with some modification using International Union of Pure and Applied Chemistry (IUPAC) guidelines. The reducing sugar produced during enzymatic reaction was determined by dinitrosalicylic acid (DNS) method (Miller,

1959). The reaction mixture contained 0.5 ml enzyme, 1% substrate (CMC), and 0.5 ml buffer at pH 7.0 (0.1 M) . The reaction mixture containing 0.5 ml boiled enzyme and 0.5 ml water instead of enzyme were used as an enzyme and substrate blank respectively. Both test and blank samples were incubated at 50^{o}C for 15 min to perform the enzyme substrate reaction followed by addition of 3 mL DNS. It was heated to boil for 10 min and immediately cooled. After cooling, the absorbance of the contents was measured at 540 nm in UV spectrophotometer (Thermo Scientific, U.K). The activity of enzyme is expressed in international unit (IU/ml or U/ml). The standard curve was simultaneously drawn for reducing sugars with glucose as a standard (Appendix I). One unit is defined as the amount of enzyme required to produce one micromole equivalent of glucose per minute. The absorbance obtained during enzyme assay was transferred in terms of IU/ml or U/ml.

3.4. Total protein content

The amount of protein was determined by Bradford assay (Bradford, 1976) with Bovine Serum Albumin (BSA) as a standard protein. A standard curve of absorbance λ_{max} versus concentration (mg/ml) BSA was plotted. It was used to find the protein content in sample (Appendix I).

3.5. Optimization of cellulase production

For the optimization of cellulase production by bacterial strain NAST01, the innoculum was prepared by transferring a loopful of organisms into autoclaved 15 ml NB in culture flask. It was incubated in an aerobic incubator with adjusted temperature of 50^{o}C at 100 rpm (shaking) till the organisms reach logarithmic phase (optical density at 600 nm with ≥0.150 A). For each optimization, during the enzyme production a control 250 mL flask with 50mL sterile media without innoculum for cross check was also incubated along with 0.1% innoculum transferred test flask. For both test and control except innoculum all the parameters for optimization were kept constant. During each optimization, the optimum parameter obtained was used for the subsequent optimization parameter.

3.5.1 Effect pH and incubation time

Prior to other parameter optimization, the media for screening enzyme was used in broth with 1% CMC (without agar) to optimize pH. The pH of the media used was set, range from 6.5 to 8.0 with 0.5 unit of pH interval. The innoculum at log phase (0.1%) was inoculated into the 50 ml broth of respective pH media, and then incubated in aerobic condition with an adjusted temperature 50^{o}C for different time intervals ranging from a day to 4th day. DNS

and Bradford assay for cellulase and total protein content were done. The pH range for media was again set from 5.0 to 8.5 along with 6.8, 7.0 and 7.2 to find exact pH value for enzyme production. Plate screening was done to calculate ratio of hydrolyzed zone to well diameter as described in section 3 (3.1.3.2) for different pH produced enzyme.

3.5.2 Effect of agitation rate

For the optimization, 50 ml preliminary screening media (appendix III) in broth was used. 0.1% inoculum at log phase was transferred to flask in consecutive optimum time interval and incubated at 50^{o}C with agitation rate of 50 rpm. The process was repeated for 100, 150 and 200 rpm with preparation of crude enzyme and assay for enzyme production by DNS method.

3.5.3. Effect of temperature

For the optimization of optimum temperature of enzyme for cellulase production, the preliminary screening media was inoculated with 0.1% innoculum at log phase. It was incubated at 40^{o}C with the optimize agitation rate for optimize time interval followed by DNS assay for enzyme after scanning the λ_{max}, the wavelength in which the absorbance was found maximum. The process was repeated with fresh media and innoculum for temperature 50^{o}C, 55^{o}C, and 60^{o}C.

3.5.4. Effect of pH

The pH optimization of a production media was done again from pH 6.5 to 8.0 as in section 3.5.1 and the enzyme activity was assayed by DNS method at λ_{max}.

3.5.5. Effect of Inorganic nitrogen sources

The nitrogen source optimization was made with inorganic nitrogen source supplemented in above media with 0.5% each ammonium nitrate, ammonium oxalate, ammonium molybdate, ammonium chloride and ammonium sulphate respectively. Each media was inoculated by innoculum and incubated in incubator with an adjusted optimum temperature and optimum shaking condition. Crude enzyme was prepared as above was used to assay the activity by DNS method.

3.5.6. Effect of organic nitrogen sources

For the organic nitrogen source optimization, the production media was incorporated with each 0.5% yeast extract, urea, peptone, malt extract in separate flask instead of optimized inorganic nitrogen source. The innoculum was transferred and enzyme production was assayed as mentioned above.

3.5.6.1. Effect of concentration of yeast extracts

For the optimization of the concentration of yeast extract required for the optimum production of enzyme, yeast extract was incorporated in the media as nitrogen source with 0.1%, 0.25%, 0.4% and 0.5% in each 50 ml production media. Transfer of innoculum and assay for enzyme was done as above.

3.5.7. Effect of different carbon source

3.5.7.1. Effect of different carbon substrates

The comparative enzyme production was made on different types of substrate with optimize percentage of carbon source above *viz*. CMC, avicel and cellobiose respectively in three 250 ml flask with 50 ml production media. Furthermore, the production was monitored in presence of different concentration of CMC.

3.5.8. Effect of % salt ions

The salt (sodium chloride) optimization was done by incorporating % NaCl from 0, 0.25, 0.5 and 1 in the production media. The enzyme production and assay was done as described above.

3.6. Enzyme production using optimized media

The optimized production media was prepared with following constituents:

Table 8: Constituents in optimized media

Constituents	g/l	3.5L	
KH_2PO_4	1.0	3.5g	
K_2HPO_4	1.145	4.0075	
$MgSO_4.7H_2O$	0.4	1.4	
Yeast extract	4	14	pH 7±0.2
$CaCl_2.2H_2O$	0.05	0.15	
$FeSO_4.7H_2O$	0.00125	0.004375	
NaCl	2.5	8.75	
CMC	10	35	

For the production of enzyme each components were dissolved uniformly and the pH was maintained 7.0±0.2 using 0.1 M NaOH. Finally, the volume was made 3.5 L by adding additional quantity of water.

3.6. 1.Scale up of enzyme production

Enzyme production was scaled up in large scale from 50 ml Erlenmeyer flask to 3 L bioreactor using optimized media.

3.6.1.1. Comparative study of enzyme production in bioreactor and Erlenmeyer flask

In two Erlenmeyer flasks, 50 ml media was poured followed by cotton plugging. Similarly 3 L media was poured to the bioreactor and autoclaved along with 0.1 M NaOH and 0.1 M HCl (300 ml each) at 121^{o}C, 15 psi pressure for 20 min as described in fermenter manual (Electrolab, UK). After cooling down of media, 0.1% innoculum at log phase was inoculated into the bioreactor using syringe DO was adjusted to 25%, temperature 50^{o}C, pH 7.0, agitation 200 rpm, and aeration 0.6 L/min. The broth was sampled and activity of crude enzyme production was assayed for two days.

In parallel, innoculum was also inoculated into one of the flask and the rest was kept as negative control to ensure contamination. Both the flasks were incubated at 50^{o}C with 200 rpm in shaking incubator. The crude enzyme and total protein production in the flask and that of fermenter were assayed for two days.

3.6. 2.Purification of crude enzyme

3.6.2.1. Ammonium sulphate precipitation

The bacterial isolate was grown in optimized media mentioned above at 50^{o}C for 48 hr. After 48 hr, the bacterial cell was removed by centrifugation at 4100 rpm and the supernatant was used as the crude enzyme. All procedures of the crude enzyme purification were carried at 4^{o}C. The crude enzyme obtained as supernatant (120 ml) was salt precipitated by addition of ammonium sulphate to the crude in sequential order from 30%, 40%, 50%, 60%, 70% saturation. After 12 hr resulting precipitate was collected by centrifugation at 13000 rpm for 30 min at 4^{o}C. It was dissolved in minimum amount of 0.1 M McIlvaine buffer buffer (pH 7.0±0.2). Enzyme activity of each precipitation was measured.

3.6.2.2. Dialysis

The ammonium sulphate precipitated enzyme with maximum activity was taken for dialysis (after dissolving in minimum amount of buffer) to remove sulphate ion during precipitation. For the dialysis, dialysis bag (average flat width=24.26 mm, average diameter=14.3 mm,

capacity=1.61 ml/cm) about 10 cm was pretreated using 0.2% sodium hydrogen carbonate ($NaHCO_3$) and pinch of EDTA. It was cooled to room temperature and lower end was tightened using thread. The enzyme was loaded in bag through an open end and again tightened with thread. The beaker was filled with 80 ml buffer (pH7.0±2). The dialysis bag was held onto buffer with slow motion at cool condition followed by changing buffer at certain interval of time till the buffer contain no any $SO4^{--}$ ion. The enzyme so purified was dissolved in minimum amount of buffer (section 3.6.2.1) and was used for characterization.

3.6.2.3. Gel filtration on Sephadex G-75

Gel-filtration (permeation) chromatography was done using Sephadex G-75 (Sigma) with 10-12 ml bed volume. About 3 g of Sephadex G-75 was suspended in 40 ml of 0.001M sodium phosphate buffer (pH 7.0± 0.2). It was stirred intermittently for 2 hours and left to swell for overnight. Sephadex G-75 slurry was packed into a column (1.5 x 18.5 cm). The beads were allowed to settle down and the column was washed using a slow flow of 50 ml phosphate buffer. A large amount of solvent (Mcllvaine buffer) was used to ensure efficient column packaging as well as elimination of air bubbles. The equilibration of column was done with 300 ml of 0.1 M Mcllvaine buffer (pH 7.0±0.2) with 0.1 M NaCl and checked for elimination of chloride ion. Dialyzed enzyme was applied to the column. Fractions (0.5 ml) were collected at a flow rate of 10 ml/hr. The elution was monitored for protein concentration at 280 nm and was also assayed for enzyme activity. Fractions with high protein activity were pooled together and precipitated with solid ammonium sulphate. Again, the precipitate was collected by centrifugation at 4,000 *g* at 4°C for 30 min, re-dissolved in minimal amount of 0.1 M Mcllvaine buffer, pH 7.0 and then dialysed against the same buffer for 6 hr. The purified enzyme thus obtained was stored at –20^{o}C for further use.

3.6.3. Zymogram analysis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight of the cellulase enzyme and the degree of the purity of an enzyme (Laemmli, 1970). Protein sample (20 µl each) was denatured by heating with equal volume of sample loading buffer (pH 6.8) for 2 min at 95^{o}C. The entire samples were cooled and loaded into the lane along with Broad Range Protein Molecular Weight Marker (Promega, USA). Protein samples were analyzed by 12% PAGE by staining with comassie brilliant blue (appendix III).

3.7. Characterization of cellulase enzyme

The purified enzyme after dialysis was used for biochemical characterization.

3.7.1. Effect of pH on enzyme activity and stability

The suitable pH for activity of cellulase enzyme was investigated in 0.1 M McIlvaine buffer (appendix III) solutions of various pH (i.e. 4.0 to 8.0). In 0.1 M McIlvaine buffer of different pH, 1% CMC was dissolved. Enzyme was incubated in the buffer for 15 min and 0.5 ml 1% CMC was added. The samples were incubated at 50^{o}C for 15 min. Three mililitre DNS was added and activity was assayed using DNS method.

Similarly, the stability of cellulase enzyme on different pH was investigated by incubating diluted enzyme in the buffer of pH 4.0 to 8.0 for 1 hour in room temperature as above and the % residual activity was calculated in terms of activity at 15 min.

3.7.2 Effect of temperature on enzyme activity and stability

The optimum temperature for enzyme activity of cellulase enzyme was also investigated at different temperature from 35 to 55^{o}C. The enzyme samples were incubated in 0.1 M McIlvaine buffer (at optimum pH for enzyme) at different temperature for 15 min to 60 min and residual activity was determined by incubating enzymes with the substrate (1% CMC) at 50^{o}C for 15 min. Three milliliters DNS was added and activity was assayed using DNS method.

3.7.3 Effect of various additives on cellulase activity

The effect of various additives on the purified cellulase activity was determined by the presence of metal ions and other reagents. The additives used in this study were $FeCl_3$, lead acetate, cobalt chloride, EDTA, $MnCl_2$, $CaCl_2$, NaCl, $ZnSO_4$, $BaCl_2$ and $FeSO_4$. Concentration of each additive was 1mM, 5 mM and 10 mM. The reaction mixtures with various additives with control without any additives but with enzyme, substrate and buffer were incubated for 15 min at 50^{o}C and cellulase activity was assayed by DNS method.

3.7.4. Determination of kinetic parameter

The apparent kinetic parameters (V_{max} and K_m) of the cellulase were determined by varying the concentration of carboxymethylcellulose from 3 mg/ml to 15 mg/ml in 0.1 M McIlvaine buffer, pH 7.0. The assays were performed with the enzyme, which had been diluted appropriately with buffer. The apparent kinetic parameters were determined by fitting data into Michaelis-Menten kinetics model (using GraphPad Prism 6.0) using non-linear regression and value were compared with that from double reciprocal plot (Lineweaver and Burk, 1934).

3.8. Assay for various kinds of cellulase system

Besides carboxymethyl cellulose hydrolyzing activity, the enzyme capacity to hydrolyze other substrates such as avicel, filter paper, cellobiose and pNPG were assayed using avicellase assay, filter paper assay, cellobiase assay and pNPG assay. During each type of assay 1% of substrate was used along with buffer and diluted enzyme with their respective blank and looked for the activity in terms of µmol/min-ml glucose equivalent (U/ml).

Avicellase assay

Suspension of avicel of about 1% (wt/vol) was prepared in water and uniformly mixed. During the assay, reaction mixtures (1.5 ml) were prepared using 0.5 ml avicel, 0.5 ml buffer and 0.5 ml diluted enzyme and the substrate blank and enzyme blank were prepared parallel. The activity (synonymous with exoglucanase or CBH) was assayed by incubation for 1 hour at 50^{o}C in shaking incubator at pH 5.5. After removal of solids by centrifugation, aliquots of the supernatant were assayed for the release of reducing sugar by DNS method (Miller, 1959).

Cellobiase assay

Cellobiose of concentration 1% (wt/vol) was used as substrate as in avicellase assay along with buffer (pH 5.5). The enzyme to be tested was diluted to make reaction mixture of 1.5 ml final volume. The substrate blank and enzyme blank were also made parallel in triplicate manner. Cellobiase (β-glucosidase) activity was assayed by incubation for 15 min at 50^{o}C in shaking incubator followed by assay for the release of glucose by DNS method (Miller, 1959).

pNPG hydrolase assay

The assay is based on the determination of the continuous hydrolytic release of *p*-nitrophenol from pNPG and the coloration of this yellow compound further deepens in (0.1 mol/L) Na_2CO_3. For the assay 5 mM *p*-nitrophenol-β-D-glucopyranoside in water was used as substrate. *p*-Nitrophenol standard curve was plotted using different concentration of *p*-Nitrophenol (0.0005, 0.001, 0.0014, 0.002, 0.003, 0.004) diluted from 0.01 mg/ml in water (Chapdelaine *et al*., 1978). Standard contains 0.5 ml diluted *p*-Nitrophenol, 0.5 ml buffer along with blank. The assay contains 0.5 ml enzyme diluted in water, 0.5 ml buffer (pH 5.5) and 0.5 ml of substrate. The test, standards, substrate blank and enzyme blank and blank for standards were incubated at 50^{o}C for 15 min followed by the addition of 2.0 ml of 0.1 mol/L Na_2CO_3 were measured spectrophotometrically at 400 nm (after scanning the λ_{max}).

One unit of enzyme activity is defined as the amount of enzyme that liberated 1 µmol of *p*-nitrophenol in 1 min per ml solution at 50^{o}C.

Fiter paper-hydrolyzing assay

Filter paper assay for saccharifying cellulase activity (FPase) was assayed according to the method explained by (Ghose, 1987; Wood and Bhat, 1988) with some modifications using International Union of Pure and Applied Chemistry (IUPAC) guidelines. Briefly, the methods are similar to the CMCase assay method, but the substrate used to be 50 mg Whatman no. 1 filter paper strip (1 x 6 cm). The filter paper strip was added in 1.0 ml of 0.1 M Mcllvaine buffer (pH 5.5) along with 0.5 ml of crude or diluted partially purified enzyme. The Reagent blanks, Enzyme blank, Substrate blank, and glucose standards were incubated at 50^{o}C for 60 min along with the enzyme assay tubes. The reaction was terminated at the end of 60 min by the addition of 3.0 ml of 3, 5 dinitrosalicylic acid (DNS) reagent. The reagent tubes were subsequently placed in a water bath at 100°C for 5 min. All samples, controls, blanks, and glucose standards were boiled together. After boiling, all the tubes were transferred to a cold ice water. The absorbance was recorded at 540 nm against the enzyme blank. The absorbance of the sample tubes were translated into glucose production during the reaction by constructing a linear glucose standard curve using the absolute amounts of glucose plotted against A_{540}. One unit of FPase activity was determined as one µmol of glucose liberated per ml enzyme per minute.

Fpase activity = 0.37/enzyme concentration to release 2 mg of glucose

Where, concentration of enzyme= volume of enzyme in dilution/Total volume after dilution

3.9. Molecular cloning of cellulase gene of bacterial strain NAST01

3.9.1. DNA isolation

Genomic DNA was isolated as described in section 3.2.2.1 above.

3.9.2. Amplification of cellulase gene

Cellulase(endo-1,4-β-glucanase) gene was amplified by polymerase chain reaction (PCR) using: forward primer-5' GCCATATGCGTTCCATCTCTGTCTTCAT 3'; reverse primer- 5' TTATTTAGGTTCAGTGCCC 3' (Aftab et al., 2012). The 25 µl reaction mixture contained 2.5 µl of 10 × PCR buffer, 0.5 µl of 10 mM dNTP mixture, 2 µl of 25 mM magnesium chloride, 1 µl of 10 µM of each primer, 0.3 µl of 5 units of Taq DNA polymerase and 1 µl of genomic DNA (25 ng/µl). Before the final reaction mixture the PCR optimization was done varying

template concentration 15 ng/µl to 35 ng/µl, primer concentration 5 pM/µl to 20 pM/µl, dNTP from 0.1 mM to 0.32 mM and $MgCl_2$ from 1.5 mM to 4.5 mM. The reaction mixture was incubated in a thermal cycler at 94°C for 5 min and then underwent 35 amplification cycles of 30 s at 94°C, 1 min at 54°C and 90 s at 72°C, followed by a final incubation of 20 min at 72°C. The PCR product was analyzed using 1.5% agarose gel and purified by ethanol precipitation.

3.9.3. Sequencing and sequence analysis of amplified cellulase gene

The PCR product from endoglucanase specific PCR was sent to Macrogen (Korea) for sequencing and sequence analysis of raw sequence was done using Codon Code Aligner 4.0.4 .The similarity of endocellulase was compared using NCBI BLAST. The sequence alignment was done by using Bioedit Sequence Alignment Editor and Mega 5.2. It was further analyzed using expasy, prosite, cazy to determine conserved amino acid in the sequence in C-terminal region.

3.9.4. Ligation reaction

The PCR product was purified (appendix III) and ligated with the vector pTZ57R/T based on Thermo Scientific Ins TAclone PCR cloning Kit (#K1213) with cloning strategy as shown in the figure 8. The reaction mixture contained 1.7 µg (3µl) vector DNA, 2.3 µg (4µl) insert DNA, 5U (1 µl) T4 DNA ligase, 6µl 5X Ligation buffer in a total volume 30 µl. The ligation mixture was vortexed briefly and centrifuged for 3 to 5 s. It was incubated at room at 22^{o}C for 1 hour.

3.9.5. *E. coli* transformation

Preparation and transformation of competent *E. coli* was done using Calcium chloride based method (Sambrook and Russell, 2001)

3.9.5.1. Preparation of competent cell

A single colony of *E. coli* XL1 Blue host strain was inoculated in 3 ml of LB media. It was incubated at 37^{o}C overnight and 25 ml LB medium was inoculated with 0.25 ml of cells from the overnight culture. The culture was incubated at 37^{o}C in a shaker with 100 rpm speed until the absorbance reached 0.35 at 600 nm. The culture was transferred to a screw capped falcon tube and chilled on ice for 10 min. It was centrifuged at 4100 rpm at 4^{o}C for 10 min and the supernatant was discarded. The tube was left standing on an inverted position on a pad of paper towels for 1 min to allow last traces of media to drain away. The pellet was resuspended in 15 ml ice-cold $MgCl_2$-$CaCl_2$ solution (80mM $MgCl_2$, 20 mM $CaCl_2$). The cells were recovered by centrifugation at 4100 rpm at 4^{o}C for 10 min and the medium was decanted to drain traces of media as above. Finally, the cells were resuspended in 1 ml

of ice-cold 0.1 M $CaCl_2$. The cells were dispensed in eppendorf tubes and stored at 4^{o}C and -80^{o}C.

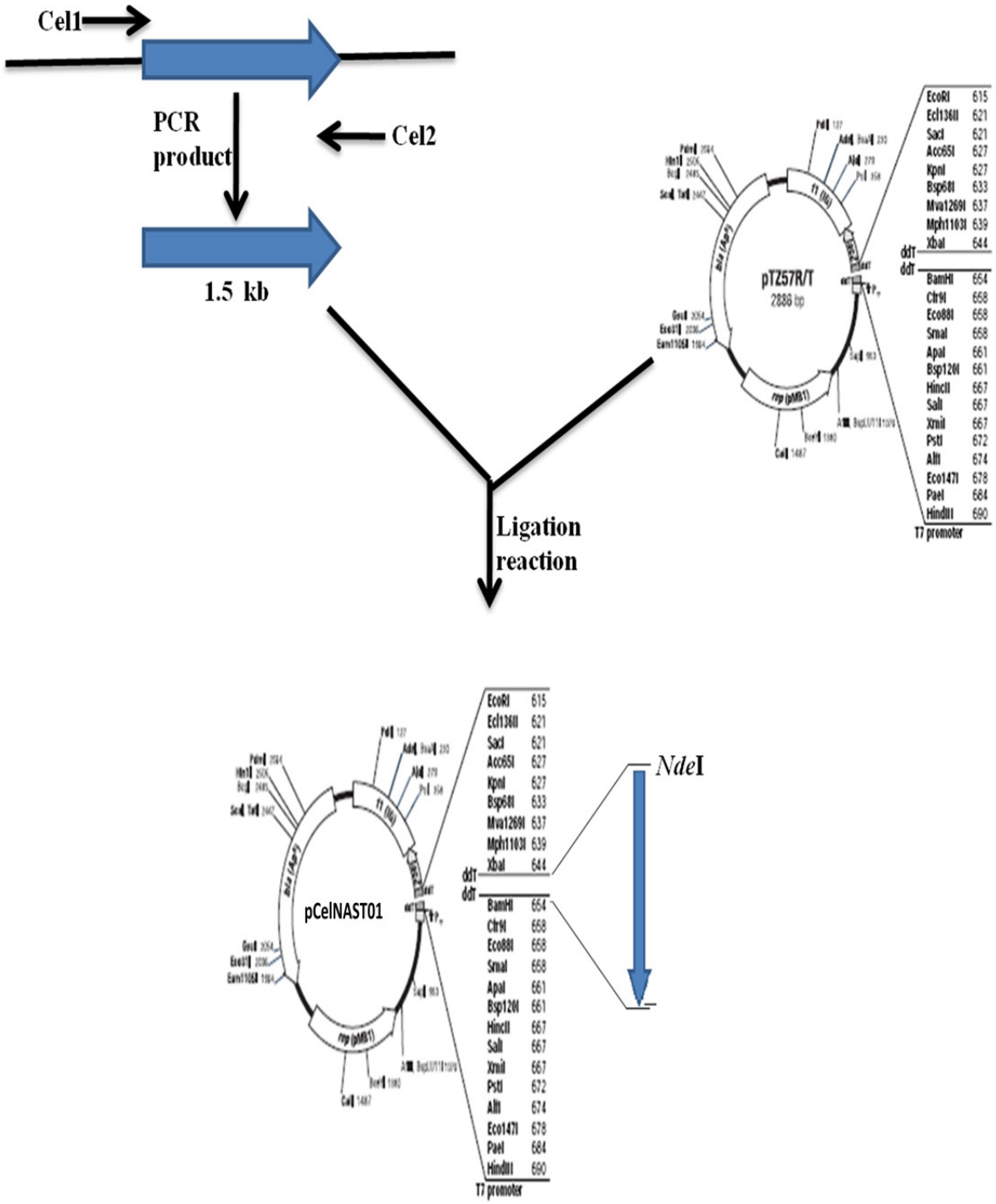


Figure 8: Cloning strategy for 1, 4- *β*-endoglucanase from *B. licheniformis* NAST01 strain into pTZ57R/T.

3.9.5.2. Transformation

To 200 µl of competent cells of *E. coli* XL1 Blue, 7 µl ligation mixtures was added and incubated in ice for 30 min. It was then transferred to water bath at 42^{o}C for 90 s and then immediately transferred to ice to chill for 2 min. To it 800 µl of LB medium was added and left for incubation at 37^{o}C for 45 min in shaker (50cycles/min). After incubation, 200 µl cultures was spread on LB agar plates containing 50 µg/ml ampicillin, 20 % IPTG and 2% X-gal. The plates were incubated at 37^{o}C overnight.

3.9.5.3. Screening of transformants

Single white colonies picked and transferred to 3 ml LB broth with 100 µg/ml ampicillin through sterile toothpick were simultaneously streaked on to LB agar plate containing ampicillin (50 µg/ml). The plate as well as broth was incubated at 37^{o}C overnight.

3.9.5.4. Preparation of plasmid

Recombinant plasmid DNA from transformants LB broth was prepared with the help of Sigma-Aldrich GenElute^{TM} Plasmid Minipreparation Kit (Sigma-Aldrich USA) according to the instruction of the manufacturer and the quantification of DNA was made with BioPhotometer (Eppendorf, Germany).

3.9.5.5. Restriction analysis of recombinant plasmid

The recombinant plasmid DNA was digested by two restriction digestion enzymes-*Nde*I and *Hind*III. For the double digestion, the plasmid DNA (approx 1 µg) was at first digested as above by *Nde*I enzyme followed by heating at 65^{o}C for 20 min. It was digested by 10 U of *Hind*III with its buffer solution recommended by the manufacturer for 1 hour at 37^{o}C. The gel electrophoresis was done using 1.5% agarose gel as above.

CHAPTER 4: RESULT

4.1. Screening different enzymes

Among the sixteen isolates that were screened for different enzymes; the maximum zone of hydrolysis based on the triplicate data of hydrolyzed zone to well diameter inferred isolate NAST01 with highest cellulase activity. NAST01 also had highest protease activity. NAST05 and NAST15 had almost equal amylase activity. NAST14 and NAST12 had highest hydrolysis of substrate tributyrin and tween 80 (substrate for lipase) respectively. NAST04, NAST11 and NAST02 had highest hydrolysis of substrate pectin, casein and xylan (substrate for pectinase, caseinase and xylanase) respectively. The result of crude enzyme from different isolates on hydrolysis of varied substrate is shown in figure 9 (appendix II and IV, table 14).

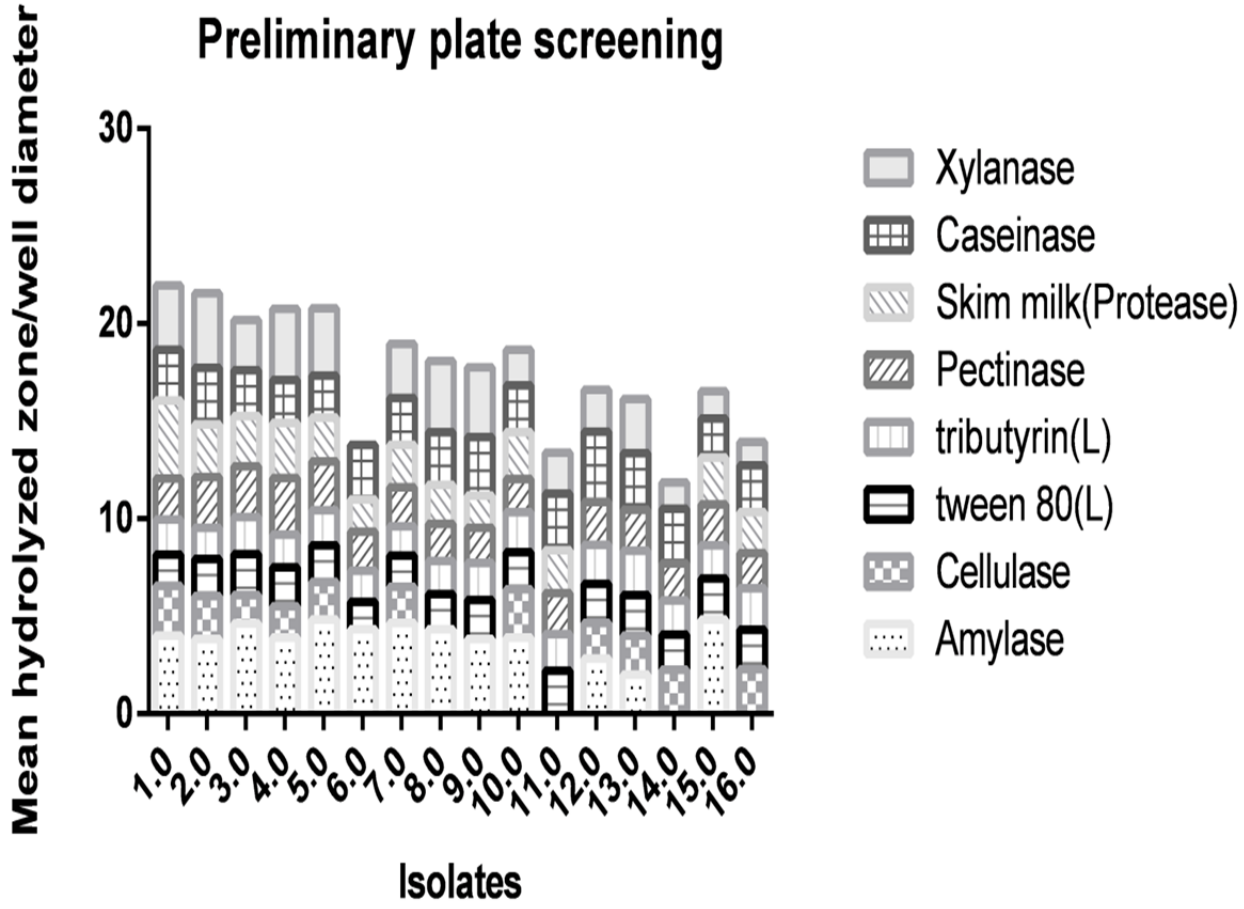


Fig.9: Screening for different enzyme from 16 isolates from hot spring water of Gorkha

NAST01 with maximum cellulase activity was chosen for characterization of cellulase enzyme.

4.2. Identification of cellulase producing bacterial isolate

4.2.1. Phenotypic identification

4.2.1.1. Gram staining

The strain (NAST01) under light microscope (oil immersion) were seen rod shaped, violet in color indicating gram positive nature as shown in figure 10a.

4.2.1.2. Oxidative/Fermentative Test

The two tubes of O/F medium with one seal using paraffin oil and other open but both cotton plugged showed yellow color in the open tube but light green color in the closed tube revealed the fact that the isolate (NAST01) is oxidative as shown in figure 10b.

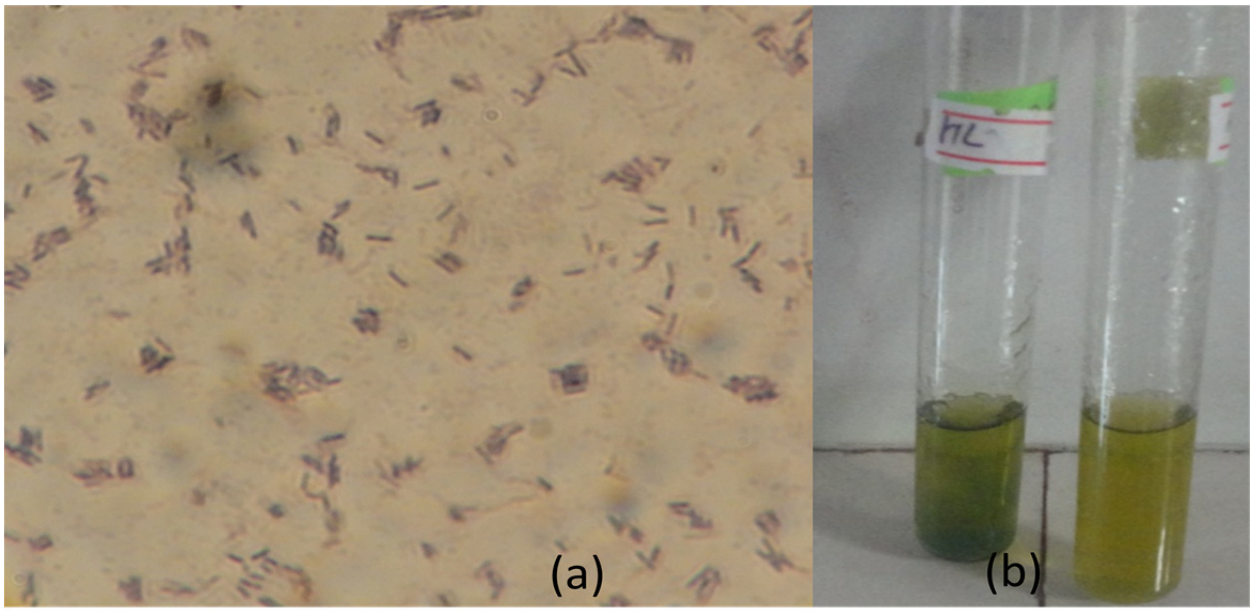


Fig. 10: (a) Gram positive bacteria under light microscope (b) Oxidative metabolism

4.2.1.3. Effect of temperature on bacterial growth

The isolate NAST01 grow at different temperature in NB from 50-65^{o}C shows the optimum temperature for the growth of organism is 50^{o}C (table 9). The optical density at 600 nm shows significant turbidity at 50^{o}C with too numerous organism to count (TNTC). Thus, for the innoculum preparation and crude enzyme preparation 50^{o}C is selected as growth temperature and the organism is considered as moderate thermophile.

Table 9: Bacteria isolate NAST01 growth at different temperature

Temperature (^oC)	Optical Density at 600 nm	Colony number
45^oC	0.45	TNTC
50^oC	0.35	TNTC
55^oC	0.10	220
60^oC	0.08	100
65^oC	0	0

4.2.1.4. Growth curve and study of growth pattern

The bacterial growth pattern at 50^oC, 100 rpm shaking showed indistinct lag with time below 2 hours and more pronounced and stepper log phase till 5.33 hours, indistinct plateau phase, a decline phase till 9.55 hours and a dormant phase after 9.55 hours as shown in below figure 11. Generally, the optical density of logarithmic phase for innoculum preparation during enzyme production was taken from 0.15 to 0.25 A (at 600 nm).

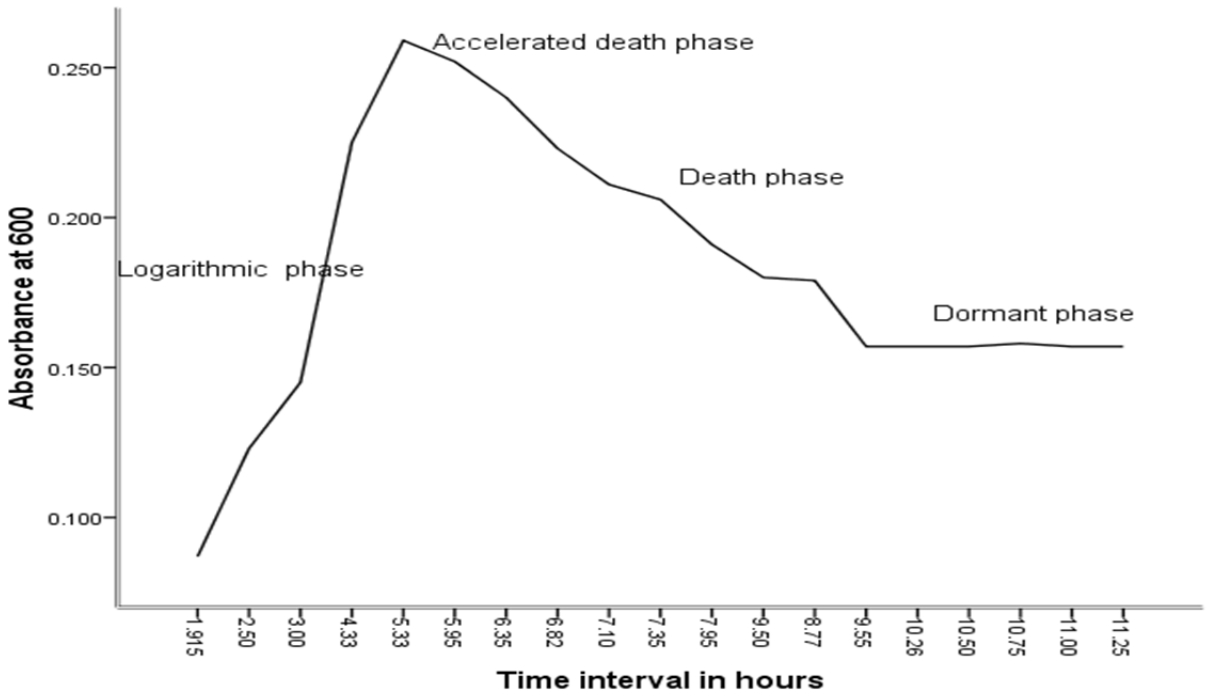


Fig.11: Growth curve showing different phase of growth of NAST01 strain.

4.2.2. Genotypic identification

The chromosomal DNA was isolated as described in materials and methods and the concentration of DNA was obtained 29.1 ng/µl with ratio of absorbance 260/280 (purity) 1.50.

The gel electrophoresis of amplified PCR product from 16S rRNA specific PCR had given a product of approximately 1500 bp as shown in the figure 12.

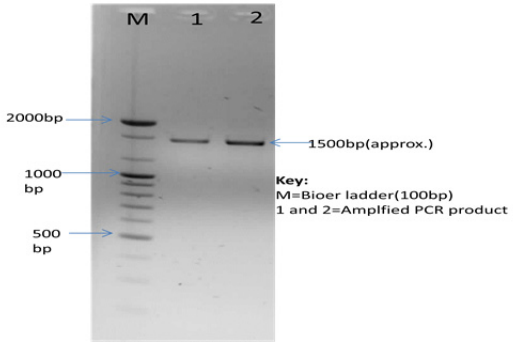


Fig.12: Gel electrophoresis of 16S rRNA specific PCR using 1.5 % agarose gel

The sequencing and sequence analysis of a 16S rRNA coding partial sequence (1404 bp) showed 99% homology with most of the *Bacillus licheniformis* strains (Appendix II). Further, the phylogenetic tree (http://www.ncbi.nlm.nih.gov/blast/treeview/treeView.cgi) drawn showed the evolutionary position closer to *Bacillus licheniformis* 14580 complete genome (figure 13). Based on these results, the bacterium was designated as *Bacillus licheniformis* strain NAST01 and submitted to NCBI with GenBank accession number JX281689.

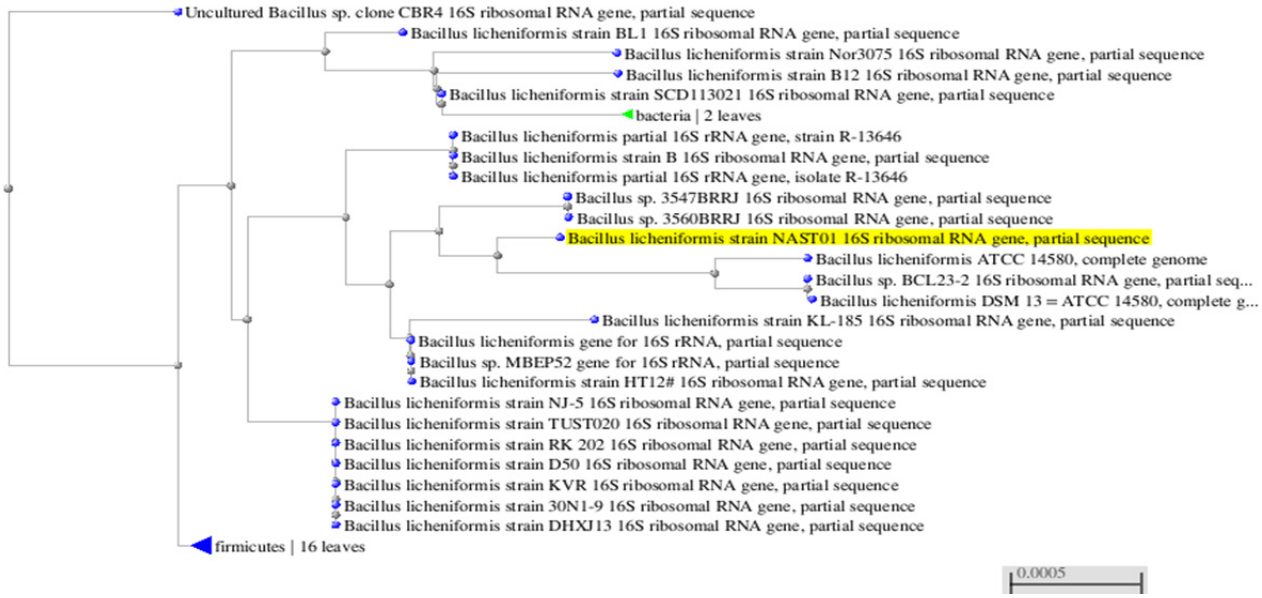


Fig.13: Phylogenetic tree showing evolutionary position of strain NAST01 among the genus *Bacillus* and different genera

4.3 Enzyme assay and optimization

4.3.1. Effect of pH and incubation time

The color development during DNS assay was highest after 2 days at pH 7.0 (figure 14, table 10) with optimum production of reducing sugar. Absorbance from DNS method and hydrolyzed zone to well diameter both gave highest activity of enzyme after 2 days and pH 7.0 (figure 15).

Table 10: Effect of pH and incubation time

pH	Absorbance at 600 nm after DNS assay (A600)					Absorbance at 595 nm (Bradford assay)	
	1st day	2nd day	3rd day	4th day	Mean	1st Day	2nd day
6.5	0.04	0.056	0.043	0.023	0.0324	0.335	0.44
7.0	0.095	0.144	0.059	0.054	0.0814	0.393	0.393
7.5	0.035	0.038	0.021	0.01	0.0208	0.477	0.589
8.0	0.047	0.032	0.0	0.0	0.0158	0.405	0.527

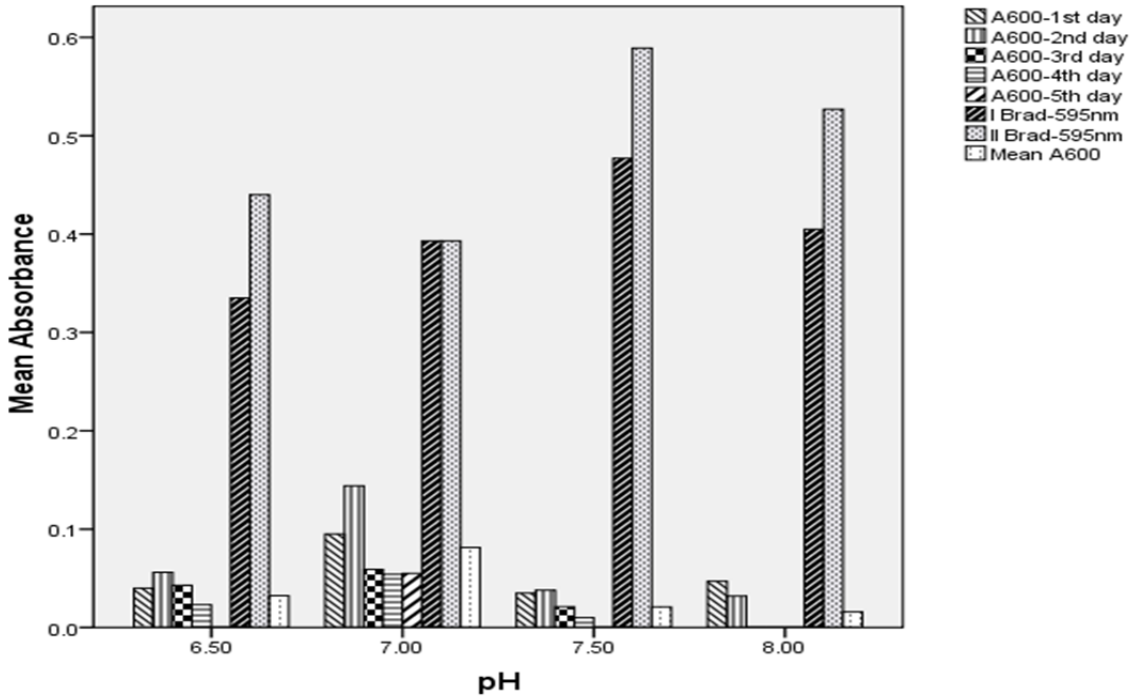


Fig.14: Enzyme production and total protein production from 1 to 5 days

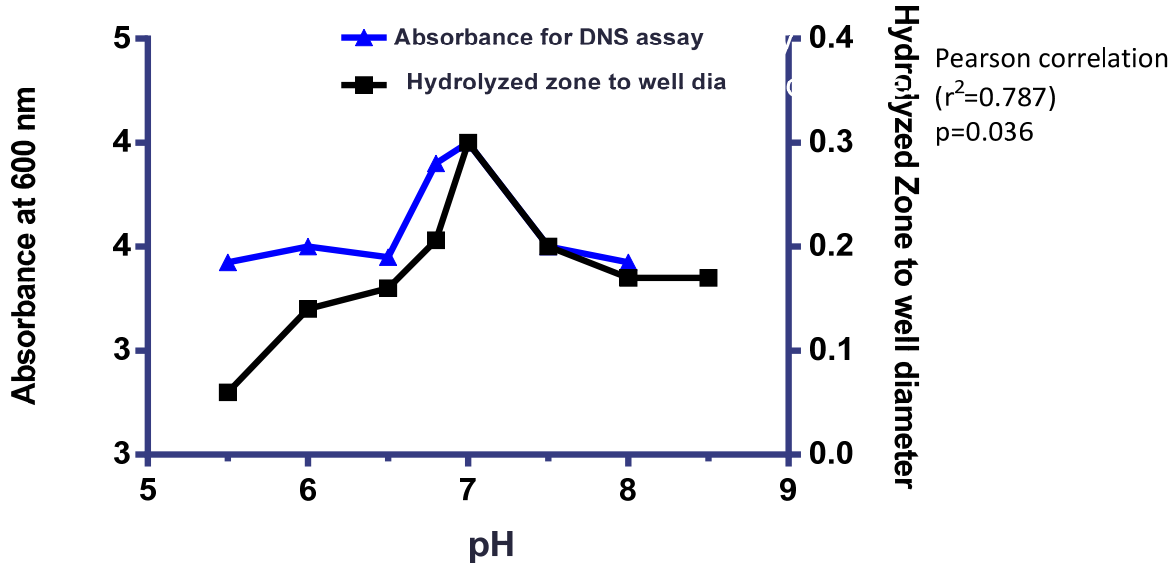


Fig.15: Celllase activity after 48 hours: DNS method vs. Plate screening

4.3.1.1 λ_{max} suitable for DNS and Bradford assay

The λ_{max} (i.e. the wavelength in which the absorbance was maximum) after scanning the test was obtained 540 nm for DNS assay and 595 nm for Bradford assay is used to assay cellulase activity and total protein content in each assay and optimization of parameter.

4.3.2. Effect of agitation rate

The agitation rate was optimized as 200 rpm for the production of enzyme as shown in below figure 16.

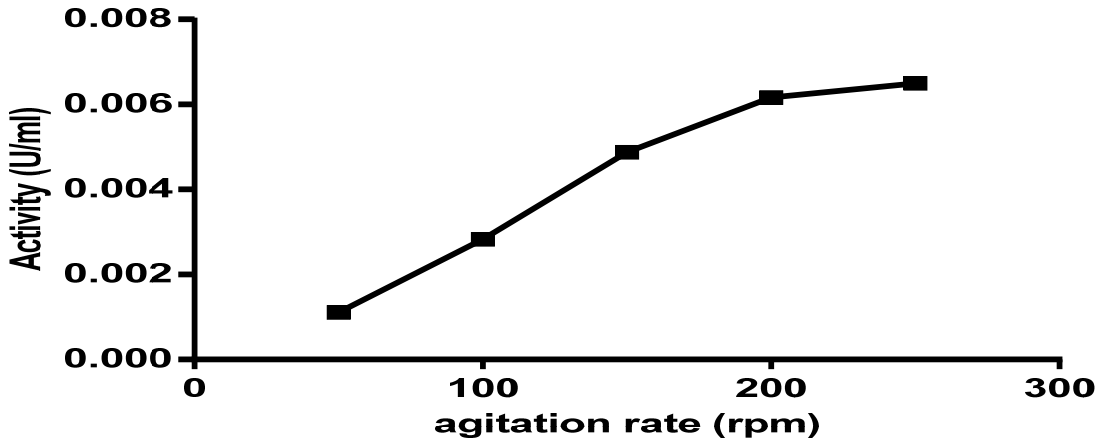


Fig.16: Effect of agitation on cellulase activity

4.3.3. Effect of temperature of incubation

From the range of temperature from 40^{o}C to 60^{o}C, the optimum temperature was obtained for enzyme production was 50^{o}C as shown in the below figure 17.

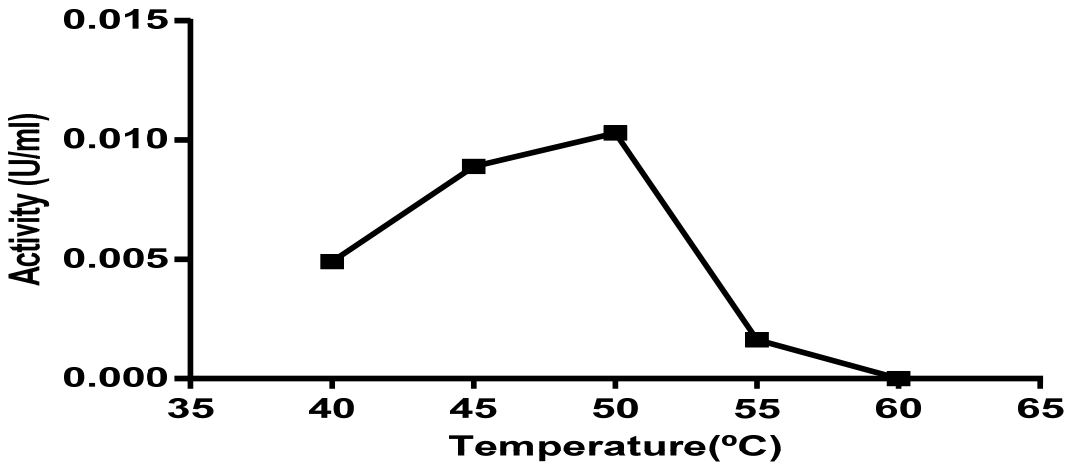


Fig.17: Temperature optimization for enzyme production

4.3.4 Optimization of production media

The production media was optimized sequentially by incorporating optimized condition for optimum enzyme production. From the pH optimization, optimum pH was obtained 7.0 during re-optimization in λ_{max} 540 nm as shown in the figure 18. Among the inorganic nitrogen source supplemented 0.5% NH_4SO_4 incorporated media was best (figure 19). In the organic nitrogen source optimization yeast extract was found best (figure 20). The optimization made for the concentration of yeast extract from 0.1 to 0.5%, 0.4% was chosen for the enzyme production (as in figure 21).

Three substratre CMC, cellobiose and avicel all were hydrolyzed by the enzyme but the hydrolysis of CMC was highest (figure 22). The optimization of C-source as carboxymethylcellulose, 1% and 1.5% has almost similar activity and it was difficult to dissolve 1.5% C-source easily and 1% CMC was chosen as enzyme production C-source (figure 23). Finally, the % NaCl incorporated media that increases the stability of enzyme; the production with above optimum parameter along with different concentration of NaCl showed enzyme production is most favorable with 0.25% NaCl (figure 24).

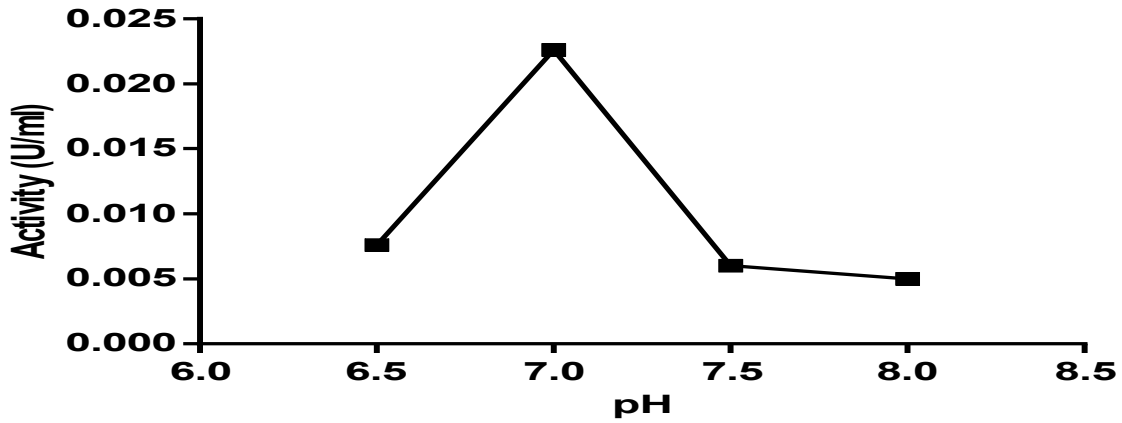


Fig.18: Optimization of pH of production media

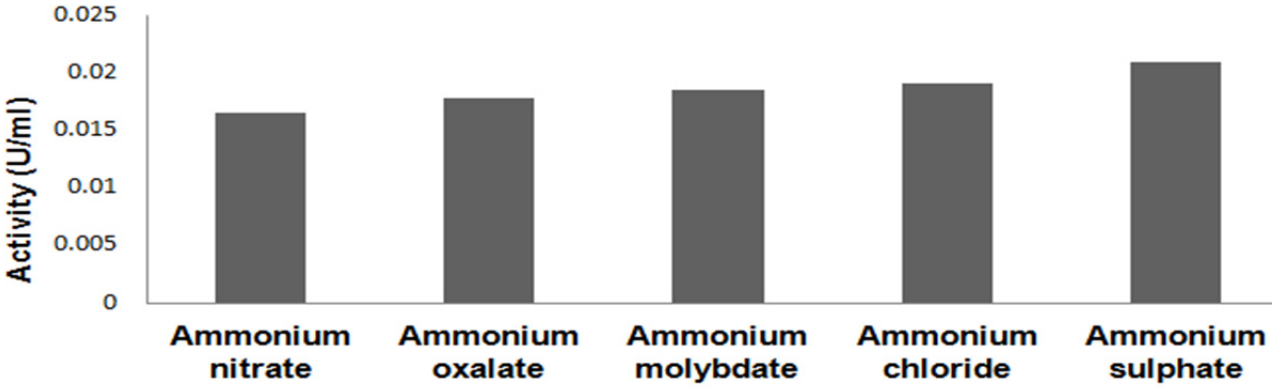


Fig.19: Inorganic nitrogen source optimization for cellulase enzyme production

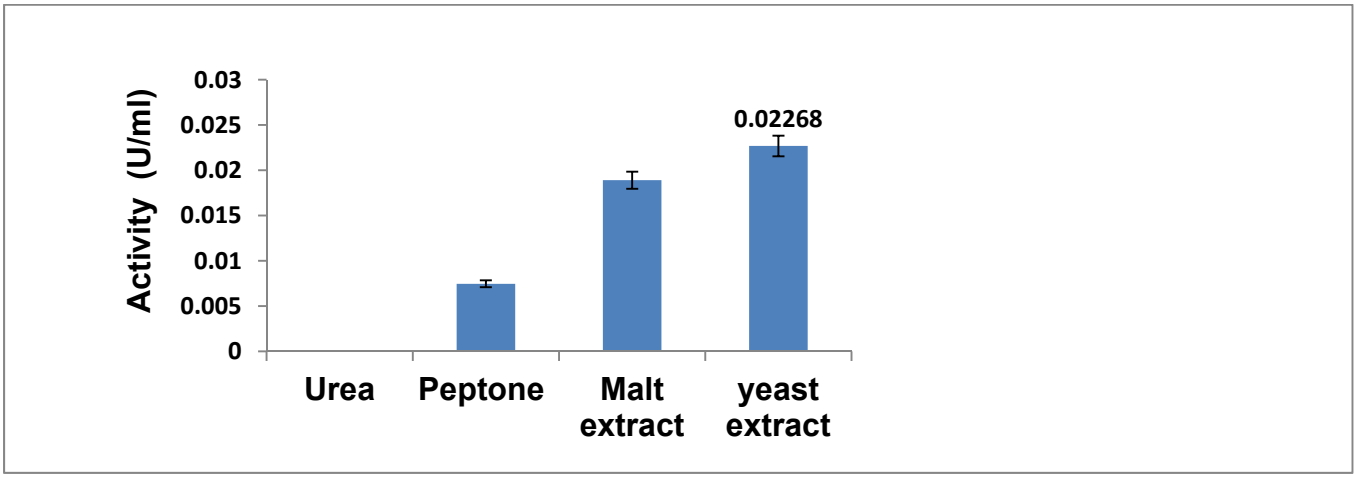


Fig.20: Optimization of organic nitrogen source

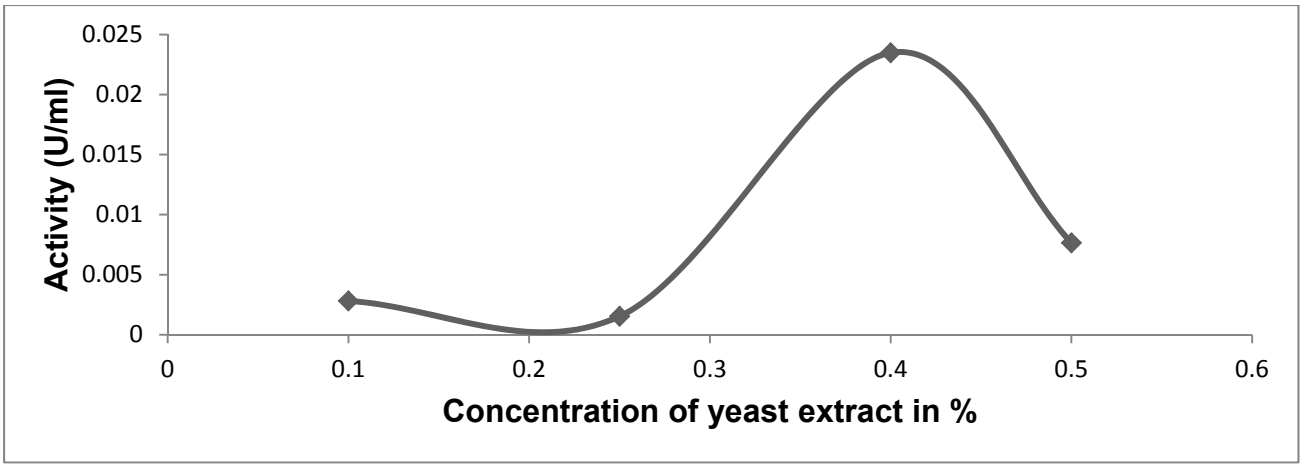


Fig.21: Optimization of concentration of yeast extract (%)

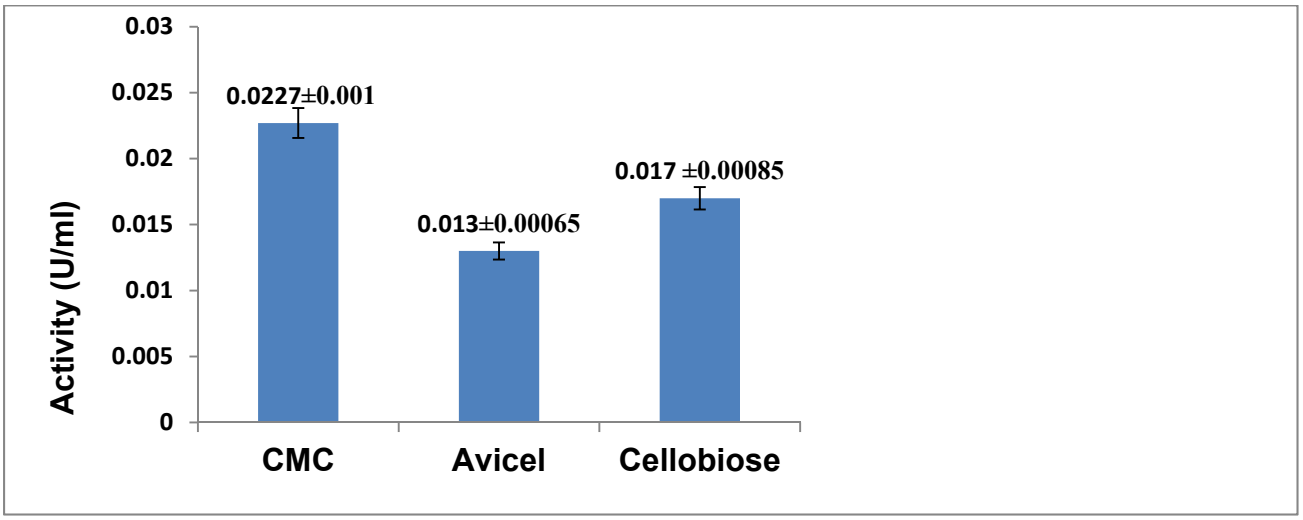


Fig.22: Comparative enzyme production in different substrate

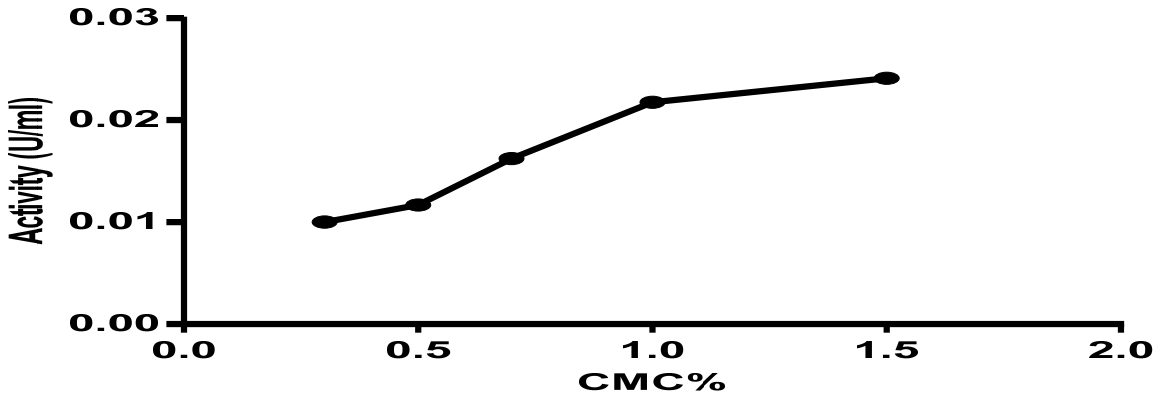


Fig.23: Optimization of % CMC as C-source

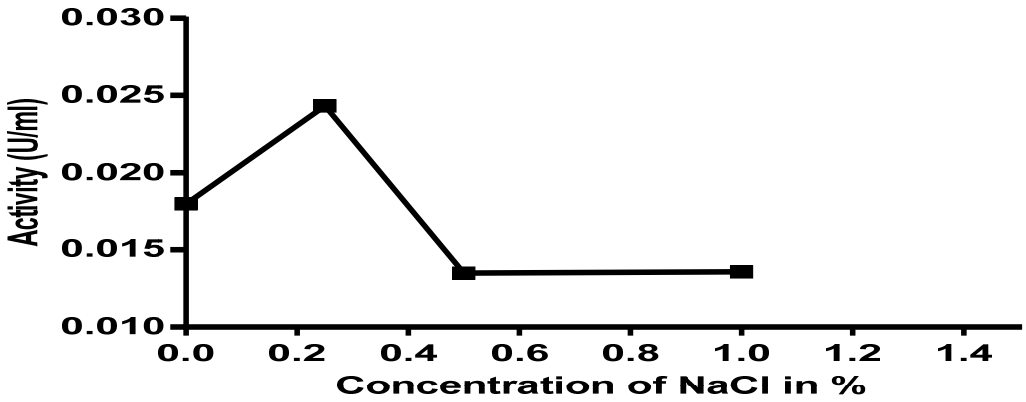


Fig.24: Optimization of % NaCl as salt

4.4. Enzyme production using optimized media

4.4.1 Scale up of enzyme production

Three litre media, 200 rpm agitation, 0.6 L/min aeration rate and DO value 25% could produce enzyme in terms of glucose equivalent 0.0211 U/ml after two days time interval.

4.4.1.1 Comparative study of enzyme production in bioreactor and Erlenmeyer flask

The enzyme production done in optimized 250 ml flask with 50 ml media and other with bioreactor with 3 L media is shown below (figure 25). The activity in both the case is comparable with optimum production of enzyme 0.029667 and 0.0215 U/ml respectively and both reach to optimum after nearly two days (in flask after 41 hours and in bioreactor after 48 hours).

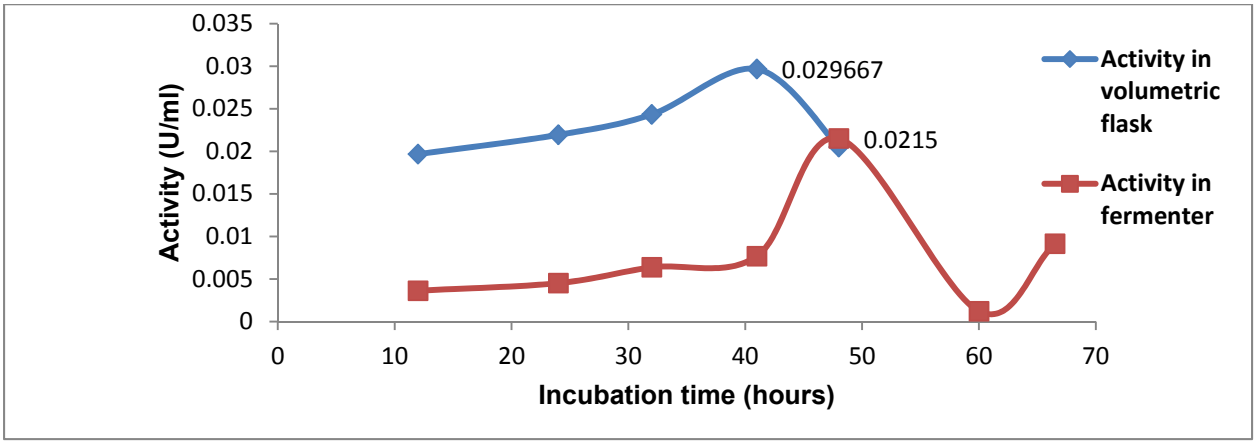


Fig.25: Incubation time vs. activity in bioreactor and 250 ml Erlenmeyer flask

4.5 Determination of specific Activity

4.5.1 Purification of crude enzyme

Salt precipitation reached saturation with 60% ammonium sulphate addition. Activity of 60% salt precipitated enzyme was highest (figure 26). It was taken for further purification step dialysis and gel filtration on Sephadex G-75 column. During gel-filtration (gel-permeation chromatography) through Sephadex G-75, active enzyme was eluted in 32nd fraction (figure 27). The specific activity of cellulase from crude enzyme, salt precipitated, dialyzed and column purified enzyme was obtained as 0.03956 U/mg, 0.1029 U/mg and 0.699 U/mg and 1.235 U/mg respectively .The yield and purification fold for the four phases is shown in the table below.

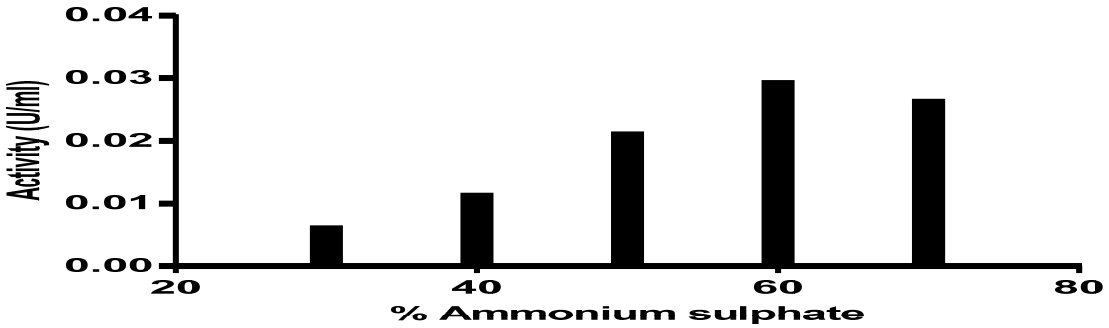


Fig.26: Ammonium sulphate for salt precipitation of crude cellulase

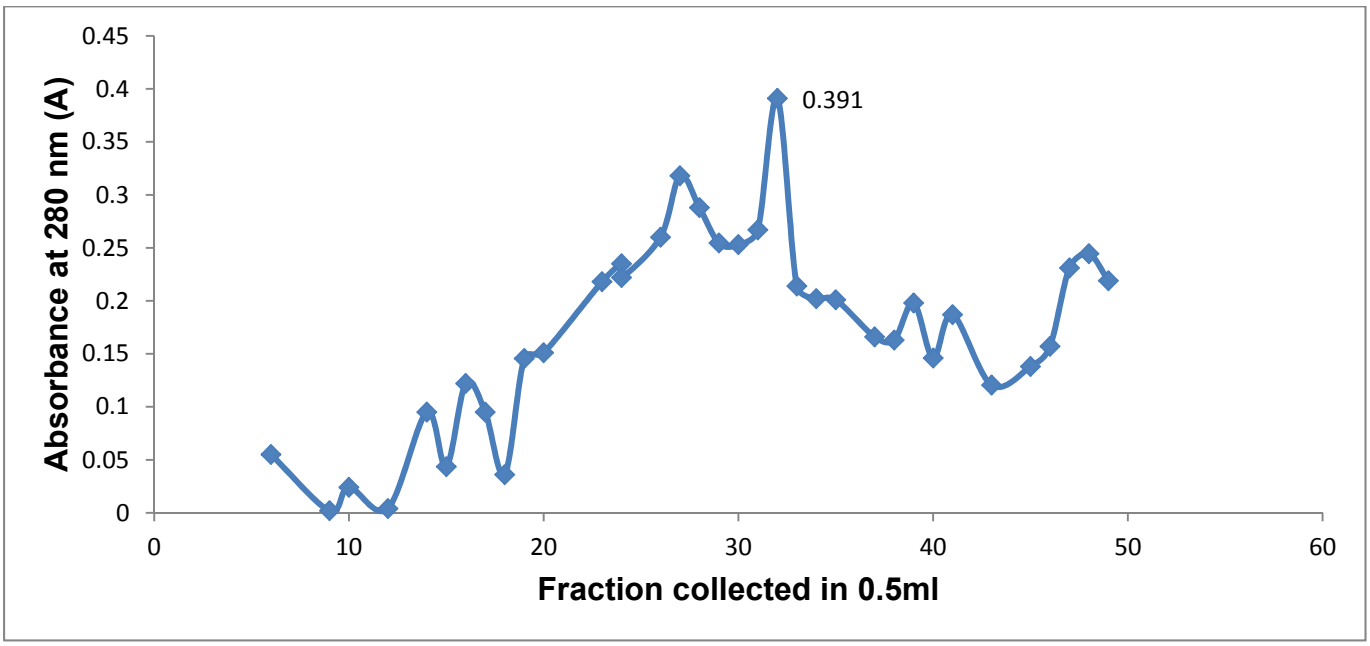


Fig.27: Absorbance of fraction (0.5ml each) eluted from Sephadex G-75 at the flow rate 10ml/hr

Table 11: Characterization of crude, salt precipitated, dialyzed and column purified enzyme

Phase	Volume (ml)	Total activity(U)	Protein (mg/ml)	Total Protein (mg)	Specific activity(U/mg)	Yield (%)	Purification fold (X)
Crude extract	120	3.56004	0.75	90	0.03956	100	1
salt ppt.	10	0.9300	0.903	9.03	0.1029	26.12	2.603
Dialyzed	4	0.259	0.37	1.48	0.699	7.275	17.67
Column purified	1	0.105	0.085	0.085	1.235	2.94	31.21

4.5.2. Zymogram analysis (SDS-PAGE)

The protein band for purified enzyme was obtained with size approx 55 kDa on 12 % SDS-PAGE (figure 28a and 28b.

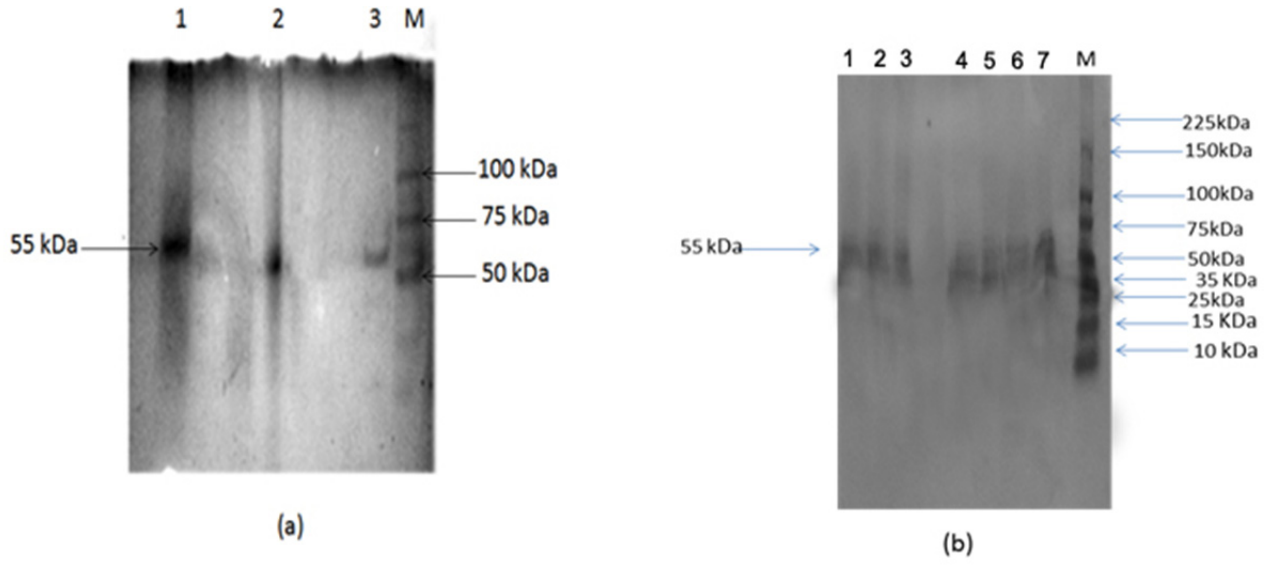


Fig.28: (12%) SDS-PAGE of cellulase enzyme from isolated *Bacillus licheniformis* NAST01 strain (a) M=protein marker, 1=salt precipitated, 2=dilaysed, 3=column purified enzyme (b) 1, 2, 3= dialysed, 4, 5, 6= salt precipitated, 7= crude cellulase, M= Broad Range Protein Marker

4.6. Biochemical characterization of cellulase enzyme

4.6.1 Effect of pH on enzyme activity and stability

The extracellular enzyme activity of partially purified enzyme at various pH from 4.0 to 8.0 shows the optimum pH for cellulase activity as 5.5 (figure 29).

From the residual activity (%), cellulase enzyme was found stable over a pH 6.0 to 7.5 at room temperature for 1 hour (figure 30). Before pH 6.0 and after 7.50 there was sudden decrease in stability and only 60% activity retained when it was incubated at pH 4.0.

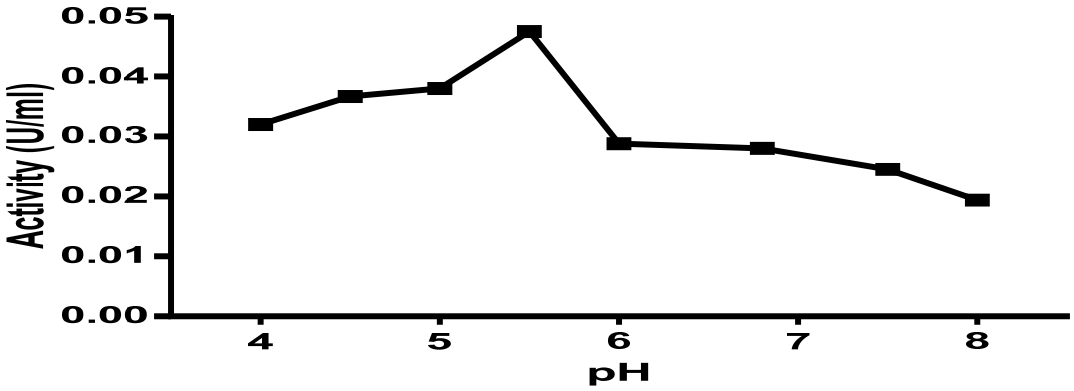


Fig.29: Effect of pH on activity of cellulase enzyme

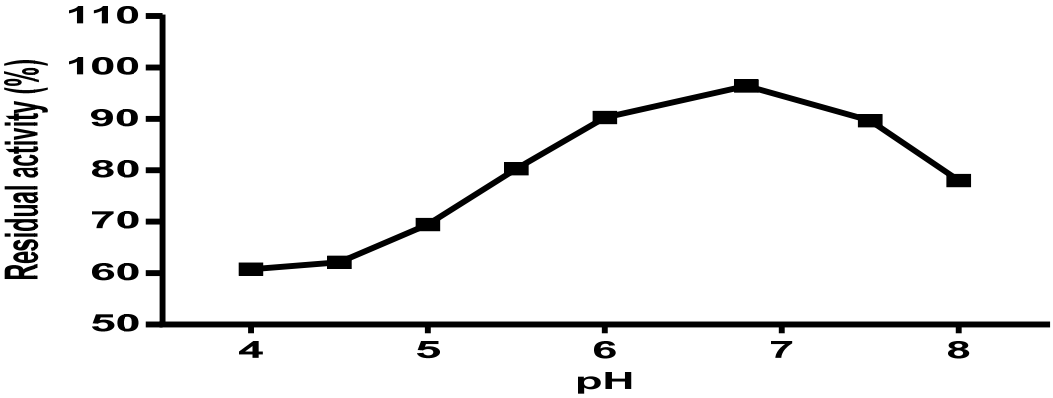


Fig.30: Effect of pH on stability of cellulase enzyme

4.6.2. Effect of temperature on enzyme activity and stability

The optimum temperature for cellulase activity was determined by measuring the cellulase activity after incubating the enzyme with 0.5 ml, 1% CMC as substrate at various temperature

from 35-55°C at pH 5.5 (0.1M McIlvaine buffer) using standard assay conditions. The enzyme was most active at 50°C (activity = 0.1330±0.001U/ml) as shown in figure 31.

The thermostability of cellulase determined by incubating cellulase enzyme in 0.005 M McIlvaine buffer (pH 5.5) at various temperatures for 15 min to 60 min. Temperature profile showed that cellulase enzyme retained its activity from 40 to 50°C for 30 min incubation at optimum enzyme pH 5.5 and gradually lost its activity when incubated more than 30 minutes. But at 55°C the activity of enzyme at first slightly decreased up to 30 min and it remains almost constant (figure 32).

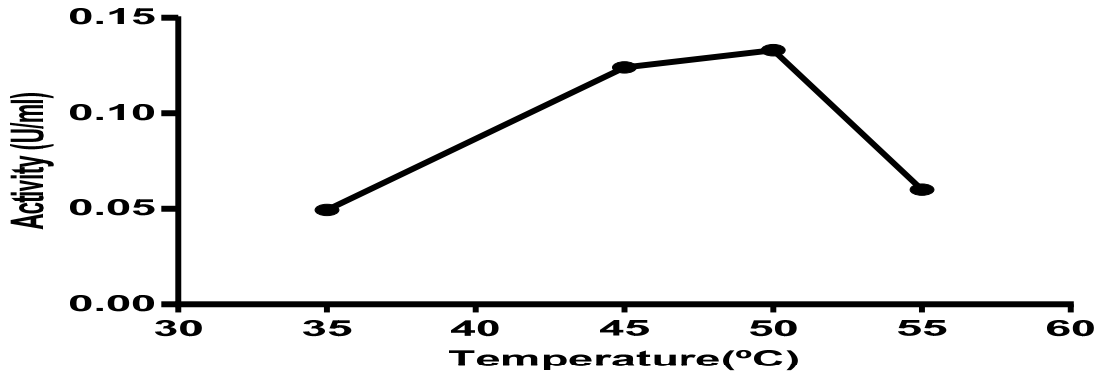


Fig.31: Effect of temperature on activity of cellulase

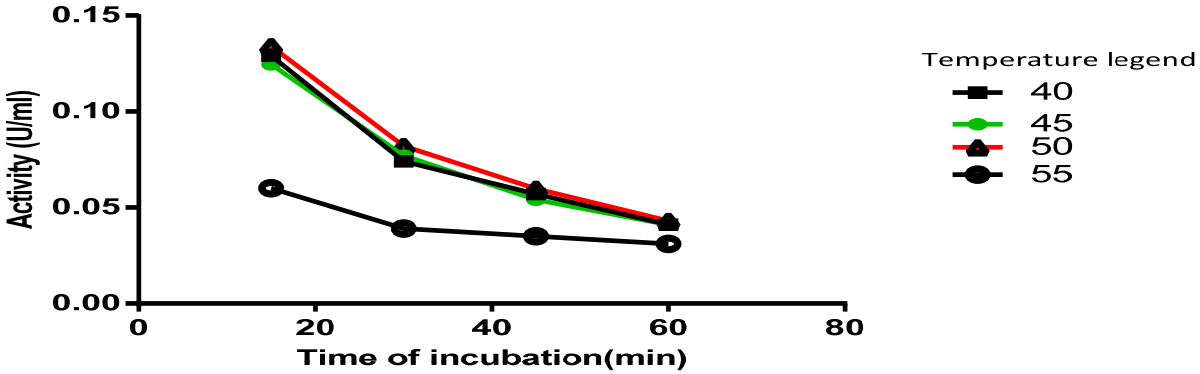
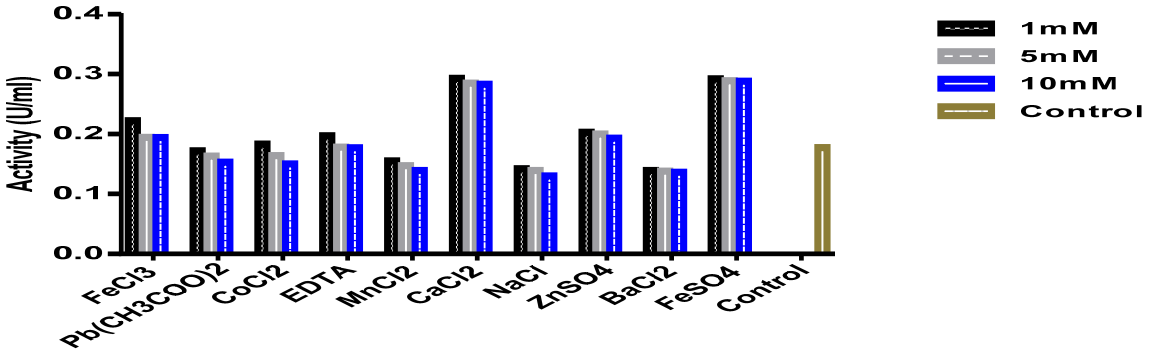


Fig.32: Effect of temperature on stability of cellulase

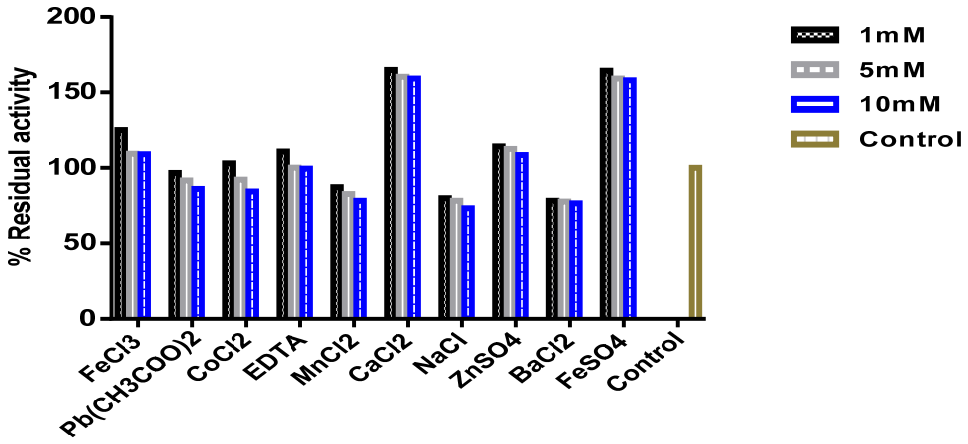
4.6.3. Effect of various additives on cellulase activity

Among the tested metal ions and additives on the partially purified cellulase, the enzyme activity was found enhanced by use of (1 mM to 10 mM) each $FeSO_4$, $CaCl_2$, $ZnSO_4$ and $FeCl_3$ and EDTA as shown in the figure 33. The activity of enzyme was found to increase by use of 1

mM each of Ca^{2+} and Fe^{2+} ion. It was recorded to be highest with 0.293±0.002 U/ml (164.9%) and 0.292±0.002 U/ml (164.4%) respectively. But use of NaCl, $MnCl_2$, $BaCl_2$, Cobalt chloride and lead acetate generally inhibited the activity of cellulase produced by the bacteria. The minimum activity of cellulase was observed with $BaCl_2$ 0.139±0.002 U/ml. Similarly, it was found that activity of enzyme increased from use of 1 mM metal ions and then gradually decreases. In case of cobalt chloride activity of enzyme enhanced up to 0.183±0.002 U/ml (103%) compared to 0.1776±0.002 U/ml (100%) of control without the metal ions although 5 mM and 10 mM metal ions act as inhibitor.



(a)Different additives used (with control) versus activity



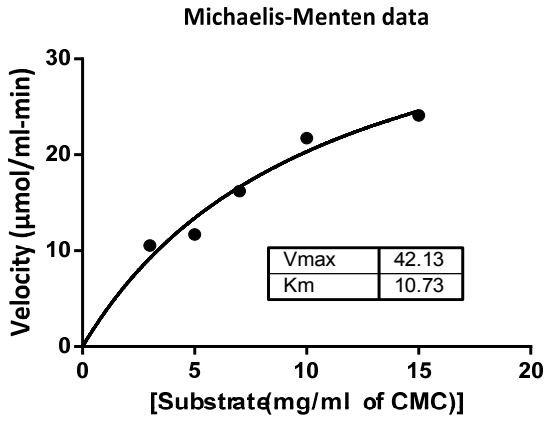
(b)Different additives used (with control) versus % Residual activity

Fig.33: Effect of various additives on cellulase activity

4.6.4. Determination of kinetic parameter

The data obtained from velocity of enzyme to produce µmol per min using different concentration of substrate is fitted to Michaelis-Menten model using GraphPad Prism 6. V_{max}

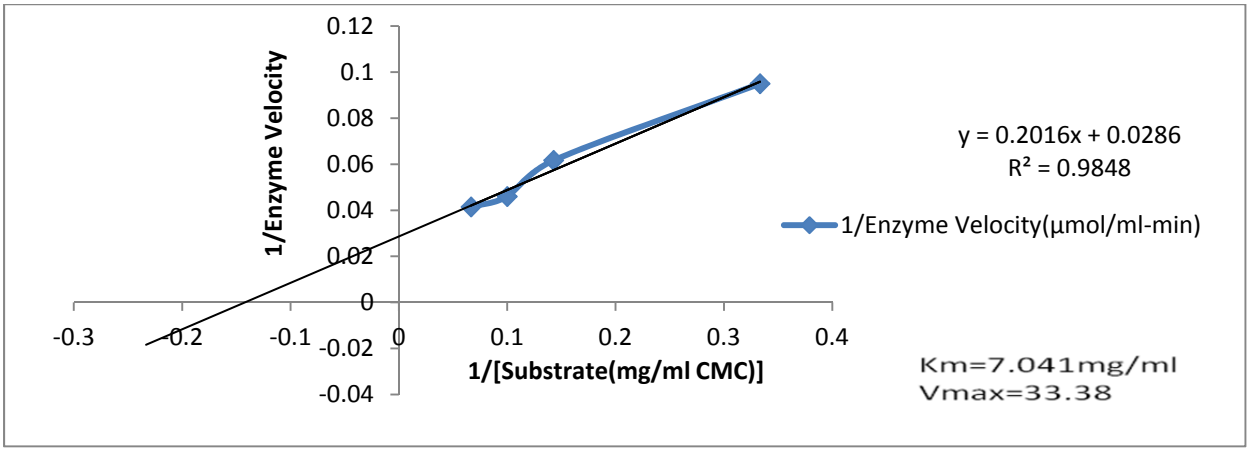
i.e. the maximal velocity without inhibitor was obtained as 42.13 µmol per ml per minute and K_m i.e. the substrate concentration that yield a half-maximal velocity was obtained as 10.73 mg/ml (1.073%) as in figure 34(a) using non-linear regression and Michaelis-Menten model. Whereas, that from Lineweaver-Burke plot V_{max} and K_m were 33.38 µmol per ml per minute and 7.041 mg/ml respectively (figure 34b). The data from Lineweaver-Burke plot lies within 95% confidence interval of V_{max} and $K_{m.}$




```
        Enzyme Velocity (µmol/ml-min)
Michaelis-Menten
Best-fit values
     Vmax          42.13
     Km 10.73
Std. Error
     Vmax          3.852
     Km 1.821
95% Confidence Intervals
     Vmax          33.81 to 50.45
     Km 6.798 to 14.66
Goodness of Fit
     Degrees of Freedom 13
     R square    0.9509
     Absolute Sum of Squares     21.14
     Sy.x        1.275
Constraints
     Km Km > 0.0

Number of points
     Analyzed    15
```

(a) Michaelis-Menten kinetics of enzyme using GraphPad Prism6



(b) Lineweaver-Burke plot

Fig.34: The kinetics study of enzyme using Michaelis-Menten kinetics and Lineweaver-Burke plot

4.7. Asaay for other kinds of cellulase system

Besides CMCase (hydrolyzing CMC as substrate), the enzyme from bacteria could hydrolyze other substrate such as filter paper, avicel, cellobiose and *p*-Nitrophenol α-D-glucopyranoside

(figure 35). The enzyme was found to hydrolyze filter paper with significant amount. The filter paper unit fpu of dialyzed enzyme was obtained 4.07±0.203 fpu/ml which shows total cellulase activity. But the avicel hydrolysis, cellobiose hydrolysis and PNPG hydrolysis for the dialyzed enzyme was obtained as 0.034±0.0017 U/ml, 0.156±0.051 U/ml and 0.007194±0.0003 U/ml respectively. This might probably be due to enzyme system of strain with avicellase and cellobiase and β-glucosidase besides CMCase but need further characterization.

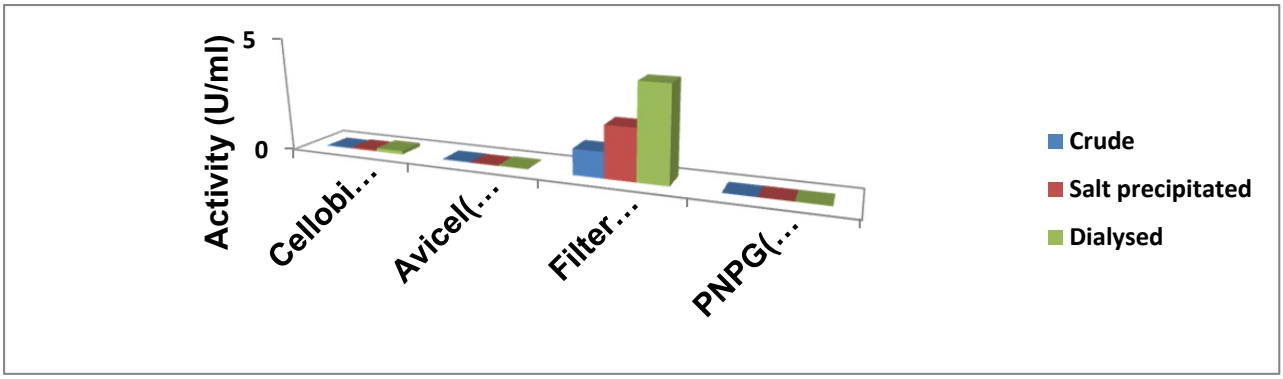


Fig.35: Assay for different kinds of cellulase system

4.8. Molecular cloning of cellulase gene of bacterial strain NAST01

4.8.1. DNA isolation

The DNA isolated as described in materials and methods through modified SDS-based DNA extraction method with concentration range from 24.1 ng/µl to 28 ng/µl and the purity range from 1.64 to 1.75

4.8.2. Amplification of cellulase gene

The cellulase gene was amplified with approximately 1500 bp size. The optimum condition for template DNA, primer, $MgCl_2$ and dNTP was obtained as 25 ng/µl, 10 µM (10 pM/µl), 2.5 mM and 0.2 mM respectively for each optimization. One of the optimization with dNTP from 0.1mM to 0.32mM is given in figure 36.

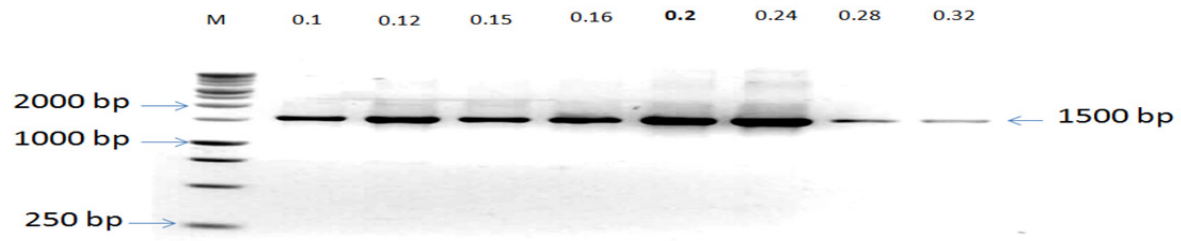


Fig.36: 1.5% agarose gel electrophoresis of cellulase specific PCR

4.8.3. Sequencing and sequence analysis of amplified cellulase gene

The raw sequences were obtained from forward and reverse primer specific sequencing. No contig could be created using codon code aligner. Only the sequence obtained from reverse primer based sequencing could be analyzed for similarity search using NCBI BLAST (blastn, blastx) of endocellulase from *Bacillus licheniformis* strain NAST01. Through blastn it was found similar with that from putative endocellulase *cel*5A from *Bacillus licheniformis* GXN151 strain (appendix II, figure 46). The blastx and blastp shows the translated nucleotide was found with conserved domain of Carbohydrate-binding module family 3 (CBM_3). Further, conserved region was found most similar with endo-1, 4-β- glucanase glycoside hydrolase family 5 *Bacillus licheniformis* with 99% identity as shown in figure 37. The region was found to share with 86 conserved amino acid (figure 38).

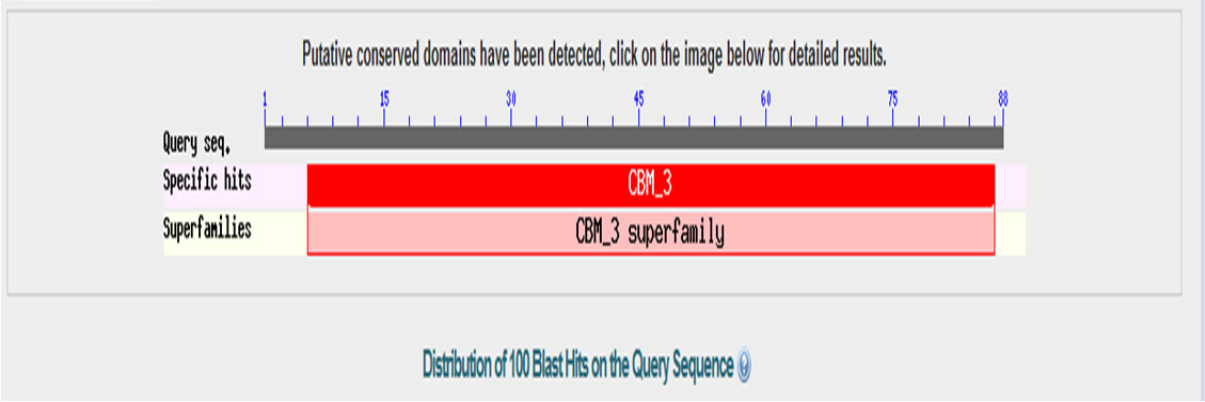


Fig. 37: (a) Putative conserved domain of CBM_3 found in NAST01.

Select: All None Selected:0

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident	Accession
endo-beta-1,4-glucanase precursor [Bacillus licheniformis] >emb|CAJ70714.1| endo-beta-1,4-glucanase precursor [Bacillus licheniformis]	179	179	97%	1e-51	99%	CAJ70710.1
CelC [Bacillus sp. CY1-3]	179	179	97%	1e-51	99%	ABG91147.1
endo-1,4-beta-glucanase, glycoside hydrolase family 5 [Bacillus licheniformis DSM 13 = ATCC 14580] >ref|WP_011198020.1| endoglucanase [Bacillus lic	179	179	97%	1e-51	99%	YP_079251.2
cellulose hydrolase [Bacillus licheniformis]	179	179	97%	1e-51	99%	ACY72379.1
cellulase BglC [Bacillus licheniformis DSM 13 = ATCC 14580] >ref|WP_011201662.1| endoglucanase [Bacillus licheniformis] >gb|AAU40977.1| cellulase	179	179	97%	1e-51	99%	YP_006713454.1
endoglucanase [Bacillus licheniformis] >gb|AAP51020.1| Cel5A [Bacillus licheniformis] >gb|EQM27813.1| endoglucanase [Bacillus licheniformis CG-B52	179	179	97%	2e-51	99%	WP_017474787.1
endoglucanase [Bacillus licheniformis] >gb|EID46965.1| BglC [Bacillus licheniformis WX-02]	177	177	97%	5e-51	98%	WP_003182338.1
endoglucanase precursor [Bacillus licheniformis]	177	177	97%	6e-51	98%	CAE82178.1
endoglucanase [Bacillus licheniformis]	177	177	97%	8e-51	98%	BAL45504.1
endo-1,4-beta-glucanase [Bacillus licheniformis 9945A] >gb|AGN36513.1| endo-1,4-beta-glucanase [Bacillus licheniformis 9945A]	177	177	97%	8e-51	98%	YP_008078250.1
Chain A, Structure Of A Novel Cbm3 Lacking The Calcium-Binding Site	132	132	100%	3e-37	68%	2L8A_A
beta-glucanase [Bacillus subtilis] >gb|ABW23437.1| beta-glucanase [Bacillus amyloliquefaciens]	128	128	96%	3e-35	68%	ABO37787.1
beta 1,4 endo glucanase, partial [Bacillus subtilis]	128	128	94%	1e-34	69%	AGV05394.1
endoglucanase [Bacillus mojavensis]	132	132	97%	1e-34	66%	WP_010334430.1
endoglucanase [Bacillus subtilis]	132	132	97%	2e-34	67%	WP_019258536.1
cellulase [Bacillus subtilis]	131	131	96%	3e-34	69%	ACR59602.1

Fig. 37: (b) BLAST result showing identity of cellulase from isolate NAST01

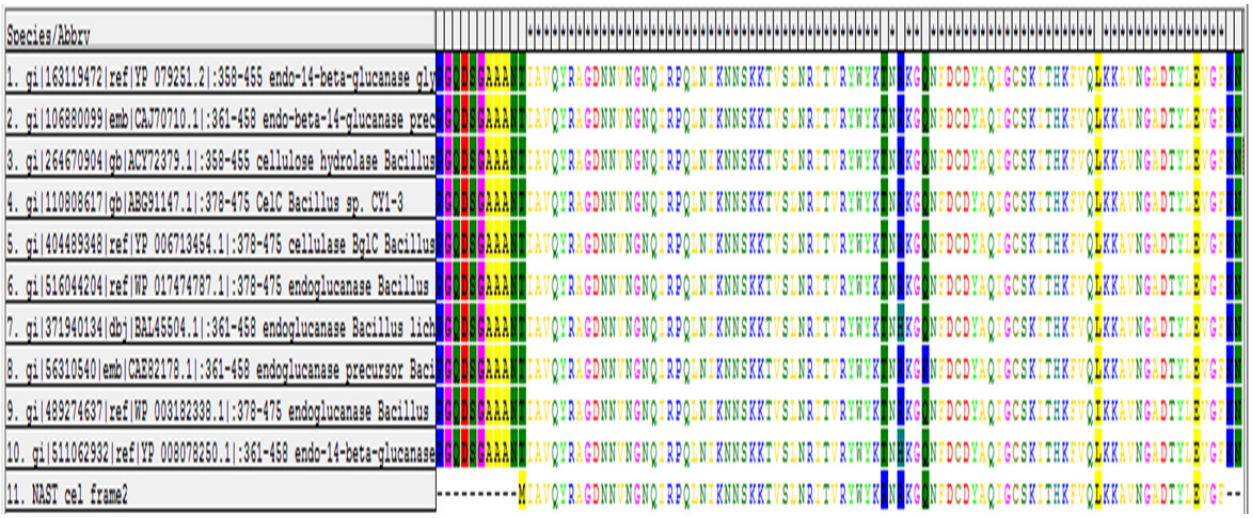


Fig. 38: Multiple sequence alignment of amino acid sequence of cellulase from NAST01 with other endoglucanase with 99 % identity (using Mega 5.2)

4.8.4. Cloning of cellulase gene

Blue and white colonies of transformants were observed on X-gal/IPTG plate with ampicillin (appendix IV). No colonies were observed for competent on the selective media that act as negative control but dense colonies were observed in the LB agar plate. The recombinant plasmid double digested with restriction enzymes *Nde*I and *Hind*III had given a band of size approx 4386 bp in 1.5% agarose gel as shown in figure 39.

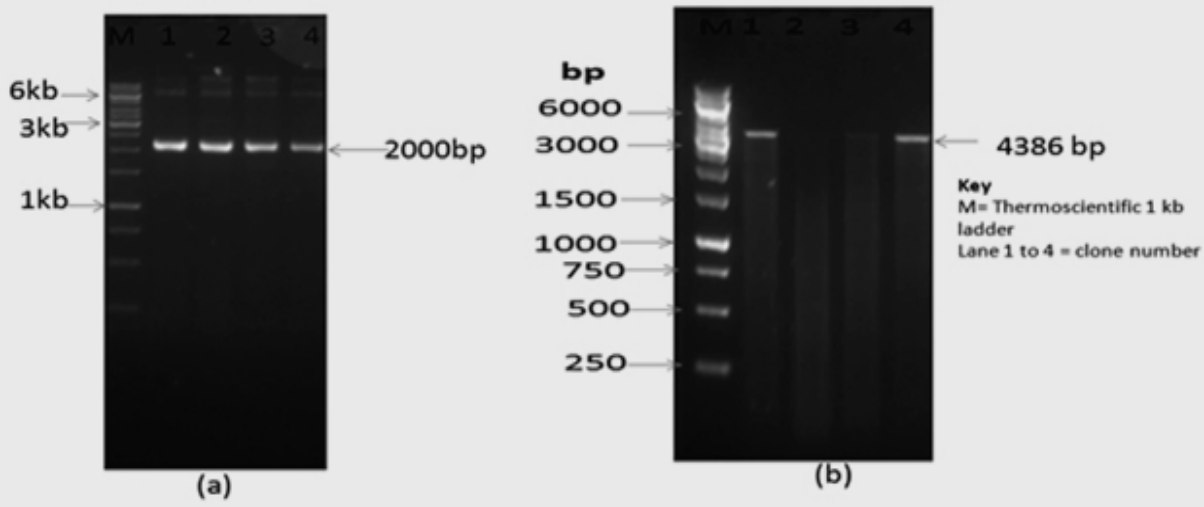


Fig. 39: Banding pattern for clone 1 to 4 on 1.5% agarose gel (a) plasmid circular and (b) *Nde*I/*Hind*III double digested plasmid

CHAPTER 5: DISCUSSION

These days energy crisis in world is leading a great problem and cellulosic ethanol or green biofuel can be a suitable alternative. For cellulosic ethanol to become reality, biotechnological solutions should focus on optimizing the conversion of biomass to sugars. Further, the study of enzyme and enzyme system in microorganism and genetic level study of gene and genetic manipulation can make super enzyme that can degrade the biomass quickly (Amano and Kanda, 2002).

Preliminary screening of 16 bacterial isolates from hot spring water for production of different extracellular enzymes showed that 68.7% isolates were cellulase producer. Besides more than 80% of the isolates screened for different enzymes were protease, amylase, lipase, xylanase, pectinase and caseinase producer. This showed that the bacterial isolates from hot spring water are good source for extracellular enzymes. The isolates producing clearing zones whose diameter ratio change from 1.4 to 2.6, 1.2 to 3.7 , 2 to 4.8, 1.4 to 2.2, 1.8 to 2.9, 1.6 to 4.0 and 2 to 3.6 respectively for cellulase, amylase, lipase, pectinase, protease, caseinase and xylanase. Similar, study was made for production and characterization of thermostable cellulases from *Streptomyces transformants* T3-1 for cellulase, protease, and amylase with clearing zones whose diameter ranged from 1.6 to 2.6, 1.1 to 1.4 respectively (Jang and Chen, 2003). The screening was significant with respect to the enzyme index (EI). The strain showing EI higher than 1.5 were considered as potential producers of cellulase (Florencio *et al*., 2012). In this study, NAST01 was selected for cellulase production among the sixteen isolates because the isolate is active for production of varied enzyme mentioned above and overall enzyme activity is greater than of other isolates. Further, the isolate has clearing zone whose diameter ratio was 2.5 comparable to that of 2.6 in *Streptomyces.* The screening for the strain with highest ratio of clear zone to colony diameter also was obtained for *Bacillus pumilus* EB3 among the 9 strains (Ariffin *et al*., 2006) of bacteria(EB1-EB9) indicated more cellulose degradation on CMC agar plate by a congo red test as in NAST01. Further, the isolate with colony or crude enzyme that showed a solublization of one or more substrates and a corresponding distinct halo or haloes that exceeded the colony diameter or well diameter by a factor of two or more as polysaccharide-degrading enzyme producer (Ten *et al*., 2004).

During the enzyme production, optimization and characterization, it is essential to make phenotypic and genotypic study of organism that make the clarity for the nature hold by the organism. The gram staining of isolate (NAST01) under light microscope revealed the fact that

the organism was rod-shaped and gram-positive and the oxidative nature clarify the need for oxygen for the growth of inoculums. Since the strain was isolated at 50^{o}C, the growth pattern was studied at 50^{o}C with regular incubation at different intervals of time. The strain showed very short lag phase but with longer logarithmic and decline phase followed by dormant phase, possibly due to accumulation of spore at longer time of incubation. The innoculum was prepared at log phase with maximum optimal density (0.15-0.25) compared to other interval of time because earlier study on the enzyme in *Bacillus pumilus* EB3 showed cellulase production during growth phase of bacterium (Ariffin *et al*., 2006).

The genotypic identification of organism that was made based on the 16S rRNA sequence. Analysis of the 16S rRNA gene sequence of NAST01 revealed a high degree of nucleotide sequence similarity 99% and the sequence coverage of more than 99% with *Bacillus licheniformis* strain 55N2-8, 16S ribosomal RNA gene partial sequence. If the compared sequence were 99% identical or above with the database sequence then the isolates were assigned name up to species level and if the sequence identity was between 90% to 98% then the isolates were assigned name up to genus level only (Clarridge, 2004; Matzinger, 2004). From the phylogenetic tree constructed by neighbor joining method also revealed that the strain was most closely related to *Bacillus licheniformis* strains. The main purpose of this study is to produce highly active thermostable cellulase from thermophilic bacteria isolated from hot spring. Microbial enzyme production is highly affected by different factors like pH, incubation time, agitation rate, and temperature of incubation, inorganic and organic nitrogen sources, carbon sources and salt ions. Efficient production of high level of active cellulase can be achieved by improving the culture condition i.e. optimization of culture condition.

pH is one of the major factors which affects the microbial growth. Microorganisms grow at definite pH i.e. optimum pH. Beyond which the microorganisms' do not survive due to the disruption of the plasma membrane or inhibiting the activity of enzyme and membrane transport proteins (Harrison, 1991). pH optimum for *B. licheniformis* NAST01 to produce celullase was 7.0. The optimum pH for cellulase production varies amongst the *B. licheniformis* isolated from different sources. Generally, it is in the range 5.0 to 7.5 (Yin *et al*., 2010; Geng *et al*., 2012).

The effect of agitation rate on cellulase production was found increasing almost linearly up to 200 rpm and then constant. Agitation results in to the change in the cell morphology enhancing the cell permeability. At low speed, the bacteria grow slowly and the production of enzymes will be low. As the speed increases there is increase in bacterial growth along with the

production of enzymes. However at high agitation, the production of enzymes remains stationary or decreased due to the shearing of the cell (Ibrahim and Al-Salamah, 2009).

The most important factor regarding the growth of bacteria and production of enzymes is temperature. Every microorganism has their own optimum temperature for growth. Beyond which they cannot survive. At low temperature growth rate was slow and enzyme production was also low. With the increase in temperature, enzyme production found increased. Thermophiles can grow in the temperature range from 45 to 55^{o}C and the enzymes they produce are thermostable and can be active above optimum growth temperature. However the extreme thermophiles can grow beyond that temperature. The isolated strains meet the criteria of thermophilic organism that grow at temperature above 50^{o}C (Perry and Staley, 1997). Souichiro *et al*., 2004 also reported the optimum temperature for the growth and cellulose degradation by *C. straminisolvens* at 50-55^{o}C. Similarly, Immanuel *et al*., 2006 recorded maximum endoglucanase activity in *Cellulomonas, Bacillus and Micrococcus sp*. at 40^{o}C at neutral pH.

Results indicated that the optimum incubation period for cellulase production was 48 hr at 50^{o}C. Similar kind of result was obtained for the industrial scale up of optimization for the production of carboxymethylcellulase from rice bran by a marine bacterium*, Bacillus subtilis* subsp, *subtilis* A-53 (Lee *et al*., 2010). After 48 hr, the production of the enzyme decreases probably may be due to the denaturation, depletion of the nutrients available and the inhibition by other components present in the medium (Ramesh and Lonsane, 1987; Akcan *et al*., 2011).

Acharya and Chaudhary 2012 also reported the optimum pH and temperature value for *Bacillus licheniformis* MVS1 and Bacillus sp. MVS3 isolated from Indian hot spring from 6.5 to 7.0 and 50 to 55^{o}C respectively with most effective cellulase production at 50^{o}C.

The sequential optimization for cellulase production media was done using 0.5% of various inorganic and organic nitrogen sources. With 0.5% each NH_4SO_4, cellulase production was enhanced. Ammonium compounds are proved to be the most favorable inorganic nitrogen source for cellulase synthesis (Spiridonov and Wilson, 1998). Except urea, all the organic nitrogen sources tested were found suitable for cellulase production than inorganic sources. The similar kind of results have been reported in *B. subtilis* and *B. circulans* (Ray *et al*., 2007). Yeast extract was found best for the production of cellulase with CMC as substrate. Furthermore, the concentration of 0.4% yeast extract suitable for extracellular cellulase production.

Besides nitrogen source, the carbon source optimization is also equally affects the production of cellulase. Carbon source regulates the enzyme production (Lynd *et al*., 2002). So, optimization of different carbon source in cellulase production is essential. Different carbon sources like CMC, cellobiose and avicel, have been used and 1% CMC was found to be the best for the production of cellulase from *B. Licheniformis* NAST01 strain. The CMCase assay showed the activity 0.0227 U/ml, 0.017 U/ml, and 0.013 U/ml for respective substrate. There observed a linear relationship of enzyme production with CMC up to 1%. Not much increase in cellulase production observed after 1%. So for the optimization of cellulase 1% CMC was used. The optimization is similar with Lee *et al*., 2010 where the combination of rice bran and yeast extract were best combination for carbon and nitrogen source. The optimization is in agreement with the research made by Jang and Chen, 2003 who also concluded carbon source like CMC was found to be good carbon source to stimulate cellulase production. The researcher made optimum production using media which contained 1 or 1.5% CMC-sodium salt as carbon source with significantly higher activities ranging from 29.6 to 40.3 IU/ml, than those media that contained 0.5% CMC (13.7 to 14.5 IU/ml). Addition of 2% CMC to the culture had no significant stimulating effect.

Generally, NaCl is used as mineral salt for basal medium to stabilize enzyme produced in the production medium. Maximum enzyme activity (0.02432 U/ml) was obtained when the media was supplemented with 0.25% NaCl. Jaradat *et al*., 2008 had also reported that the maximum production of cellulase using CMC as carbon source when 0.2% NaCl was used. Most of the bacterial cellulases were produced at the NaCl concentratin in the range from 0.2-3.0 (Lee and Blackburn, 1975; Mountfort and Asher, 1985). However, the fungal cellulase from *Trichoderma reesei* RUT C30 was maximum when 0.5% NaCl was used (Mekala *et al*., 2008). But in halotolerant the cellulase production can be make even at 25% NaCl (Zhao *et al*., 2012).

The scale up of enzyme is necessary for the production of enzyme in optimized condition to take the product in economical scale and in industrial level. Scale up of enzyme production was done in optimized condition but with dissolved oxygen 25% and aeration rate 0.6 L/min. The activity of produced crude enzyme in 3 L flask (0.0215 U/ml) was comparable to that in Erlenmeyer flask (0.029667U/ml) with 50 ml at 7.0, 50^{o}C, 200 rpm for 2 days. The result also indicate that the production can be taken in industrial scale similar to the optimization made for the production of CMCase from marine bacterium*, Bacillus subtilis subsp*. *subtilis* A-53 (Lee *et al*., 2010).

The bacterial crude cellulase enzymes were found to precipitate at 60 to 80% ammonium sulphate from *Bacillus subtilis* YJ1 (Yin *et al*., 2010). CMCase produced by an alkalothermophilic

actinomycete was precipitated at 30 to 55% ammonium sulphate (George *et al.*, 2001). A sequential purification that was performed for the cellulase isolated from *B. licheniformis* strain NAST01 is in agreement with above study. The enzyme was precipited at 60% ammonium sulphate. The precipitate was dissolved in buffer, dialysed against the same buffer at pH 7.0±0.2 for overnight at 4^{o}C showed increase in specific activity from 0.1029 U/mg to 0.699 U/mg. The removal of salt usually occurs with the use of dialysis bag. The dialyzed enzyme that was loaded to Sephadex G-75 gel-permeation chromatography column and bound cellulase eluted using 0.5M McIlvaine buffer (at pH 7.0±0.2) with 0.1 M NaCl re-precipitated and dialyzed. There was a significant increase in specific activity and purification fold to 1.235 U/mg and 31.21 fold respectively. The trends of increase in specific activity as well as purification fold were similar with the down streaming, enhancing purity of enzyme. But the % yield was in decreasing order as in the study made in cellulase from *Bacillus subtilis* (Yin *et al*., 2010), *Bacillus licheniformis* (Bischoff *et al*., 2006).

The zymogram analysis using 12% SDS-PAGE showed a band of size approximately 55 KDa with respect to Broad Range Protein Molecular Weight Markers (Promega, USA). Furthermore, assuming an average mass of 110 g per amino acid, NAST01 strain cellulase had the predicted chain length of the 55 kDa (based on PCR product of size 1500 bp with approx.500 amino acid). It has been reported that the molecular mass of cellulase is in the range of 31 to 94 kDa (Endo *et al*., 2001). Moreover, CMcellulase proteins with a molecular weight range of 30 to 65 kDa were reported in *B*. *pumilus* EB3 (Ariffin *et al*., 2006). Besides a protein size larger than 37 to 43 kDa of the cellulases from *B*. *licheniformis* (Bischoff *et al*., 2006). The molecular wt. of endo-1,4-β- glucanase from moderate thermophilic strain *Bacillus licheniformis* ATCC 14580 with 52.2 KDa (Aftab *et al*., 2012) and purified cellulase from *Bacillus amyloliquefaciens* utilizing rice hull (Lee *et al*., 2008). Similarly, the putative cellulase from *Bacillus subtilis* with Cel15 and Cel73 showed size of protein band of 54 and 27 kDa respectively (Li *et al*., 2009).

Thus, from the above resemblance, analogies and instances, the enzyme showed character similar to endoglucanase or putative cellulase rather than having complete system of exoglucanase, endoglucanase and β-glucosidase.

The biochemical characterization of partially purified enzyme at various pH from 4.0 to 8.0 at temperature of 50^{o}C shows pH 5.5 is optimum for the enzyme activity. The enzyme is found stable from pH 6.0 to 7.5 for an hour at room temperature. The optimum conditions obtained was similar to that the optimum conditions obtained for two *Bacillus* strains viz. strain CH43 and HR68 producing endo-β-1,4-glucanase at pH 6.0 and pH 5.0 at temperature 50^{o}C respectively. However, the high proteolytic activity in NAST01 strain could be reason for the low

activity of cellulase as fall in production that coincided with rise in proteolytic activity (Mawadza and Zvauya, 1996).

The effect of temperature on enzyme activity also showed the enzyme was active from 40 to 50^{o}C and remains most active at 50^{o}C (0.1330±0.00665U/ml) at pH 5.5. The thermostability of enzyme from 15 to 60 minutes at pH 5.5 also showed enzyme remain stable from 40^{o}C to 50^{o}C for 15 mins and the activity was retained up to 30 min although there was slight decrease in activity. Further, incubation for more than 30 min at the respective temperature result gradual loss of enzyme activity. Unlike at 55^{o}C, the activity at first gradually lost up to 30 min and then remains almost constant. The temperature profile is similar to the enzyme C2, a CMC liquefying enzyme with temperature optimum at 50^{o}c and the enzyme retains full activity at pH 4 to 6.4 upon incubation at 50^{o}C for 30 min (Li and Gao, 1997).

The metals ions or additives used with the purified enzyme act as cofactor or inhibitor at optimum temperature and pH for the activity of enzyme. Many researchers have studied the effect of metal ions on the activity of CMCases from different organisms. They tested the activity at a single metal ion concentration. Some metal ions activated while others inhibited. The purified control enzyme without additives showed 0.1776±0.0088 U/ml (100% residual activity) at pH 5.5 and temperature 50^{o}C. The increase in activity up to 0.293±0.014, 0.292±0.014 U/ml (approx. 164% residual activity) respectively was significant with 1 mM metal ions in case of $CaCl_2$ and $FeSO_4$. But the metal ions further increase to 5 mM to 10 mM decrease the activity of enzyme. The result of effect of 1 mM metal ions such as Ca^{2+}, Zn^{2+} and Co^{2+} on endoglucanase resemble with that of increase observed in enzyme from *Bacillus* sp VLSH08 strain applying to biomass hydrolysis (Phan *et al*., 2012). Unlike the lower concentration of EDTA up to 1 mM instead of decrease found to increase activity compared to control. But 5 mM and 10 mM EDTA concentration the decrease in activity was observed. $BaCl_2$, $Pb(CH_3COO)_2$ and NaCl were the additives act as inhibitor for the enzyme purified and the inhibiton of $BaCl_2$ and NaCl resemble with that of result of Wang *et al*., 2010. The result of effect of Mn^{2+} seems contrary to the result of Siddiqui *et al*., 1997. The reason could be the lower concentation even then 1 mM could activate the enzyme activity , whereas with the increase in concentration of metal ion decrease the activity showing mixed type inhibition.

The kinetic parameter studied revealed that the V_{max} and K_m obtained as 42.13 µmol per minute and 10.73 mg/ml using non-linear regression model and fitting the data of substrate concentration with respect to enzyme velocity. The 95% confidence interval for V_{max} range from 33.81 to 50.45 µmol/min and K_m from 6.798 to 14.66 mg/ml (R^2=0.9509). The Lineweaver-Burke plot drawn with reciprocal of substrate concentration to that of reciprocal of velocity also had

given K_m and V_{max} 7.041 mg/ml and 33.38 µmol per minute that lies in the range of Michaelis-Menten kinetics non-linear regression model. The K_m value is similar to that of hydrolysis of CMC following a Michaelis-Menten kinetics with a K_m of 9.13 mg/ml but with V_{max}, of 3469 $\mu Mmin^{-1}$ by cellulase from the cellulolytic strain *Streptomyces sp*. G12 isolated from compost (Amore *et al*., 2012). It also resembled to that of CMase from *Paenibacillus polymyxa* (Kumar *et al*., 2012) with K_m of 8.73 mg/ml but V_{max}, with 17.805 mM/ml/min and with data presented by Ko *et al*., 2010 with K_m 11.25 mg/ml and a V_{max} of 1250 µmol/min/mg with CMC as substrate for the characterization and pulp refining activity of a *Paenibacillus campinasensis* cellulase. But the K_m and V_{max} value obtained were slightly more than that from β-1,4-endoglucanase from *Bacillus* sp. with K_m of 4.1 mg/ml and V_{max} 25 µmol/ml/min (Afzal *et al*., 2010). The variation in V_{max} is possibly due to the different in activity of endoglucanase of purified enzyme that differ with optimal condition.

Besides endoglucanase activity, purified enzyme also showed considerable activity of exo-glucanase and β-glucosidase as evident from the hydrolysis of filter paper (4.07±0.203 fpu/ml), avicel (0.034±0.0017U/ml), cellobiose (0.156±0.051U/ml), and pNPG (0.007194±0.0003U/ml) respectively. It implies that possibly there could be β-glucosidase besides the presence of novel endoglucanase with both exo-endoglucanase activities. Similar results have been observed with the cellulase enzyme isolated from *Streptomyces sp*. LX (Li *et al*., 1998). The study also implies the possibility for other component in cellulase system in the strain NAST01 and there is need of further characterization of cellulase enzyme system present by molecular tools and techniques.

Amplification of 1.5 kb of PCR product indicates the correct fragment amplification from genomic DNA of *B. licheniformis* strain NAST01. Similar result was also reported by Aftab *et al*., 2012 on amplification of cellulase gene from *B. licheniformis* ATCC 14580. However, possibly due to the degradation of forward primer only the sequence from reverse primer was obtained. The partial nucleotide sequence showed high homology with the C-terminal sequences of *cel*5 gene of B*. licheniformis* strain GXN151 and the translated amino acid sequence showed the endoglucanase belong to Carbohydrate binding module family 3 (CBM_3) or presence of Carbohydrate binding domain. CBD plays crucial role in the hydrolysis of even crystalline cellulose by binding to the surface of cellulose.

On the basis of amino acid sequence there are 12 groups of families for catalytic domain of cellulase, and 26 families of CBM (http://afmb.cnrs-mrs.fr/~pedro/CAZY/cbm.html). The C-terminal amino acid sequence showed similarity with the CBD domain which is homologous with similar domains from several other bacterial cellulase. The characterization of

endocellulase from NAST01 strain showed some analology with respect to hydrolysis of crystalline as well as amorphous cellulosic substrate i.e. observed in endocellulase from GXN151 with Cel9a and Cel12a. Moreover, Cel9a with both catalytic, a linker domain, and CBM_3 but Cel12a with only catalytic domain (Liu *et al*., 2004). Further, the translated amino acid showed 99% identity with endo-1,4-β-glucanase precursor of *Bacillus licheniformis*, Celc of *Bacillus sp*. CY1-3, glycoside hydrolase family 5 and BglC of *B.Licheniformis* DSM 13=ATCC 14580, and endoglucanases of *B. Licheniformis strain* (figure 37b). The blastx and blastp found a putative conserved domain with 86 conserved amino acid (NAST cel frame2) i.e. similar with that of CBM_3, of 150 amino acids as shown in figure 38. CBM_3 domain with 85 residues starting at 506 and ending at 591 was also reported in *Bacillus pumilus* endoglucanase, EglA (Lima *et al*, 2005). Although the sequence was incomplete, but conserved amino acid pattern was found similar to that mentioned for CBM_3 as shown in figure 40 (Tormo *et al*., 1996). Similarly, the amino acid sequence for 1, 4-endoglucanase corresponding to NASTcel aligned with CipB from *C. thermocellum* strain YS as shown below. The truncated S1 protein encoded by the CipB derivative that bound tightly to cellulose consisted of C-terminal proximal 167 residue sequence which showed complete identity with residues 337-503 of mature S_L from *C. thermocellum* strain ATCC 27405. The cellulose-binding domain interacted with both crystalline and amorphous cellulose, but not with xylan. These results showed amplified product codes for the endocellulase gene.

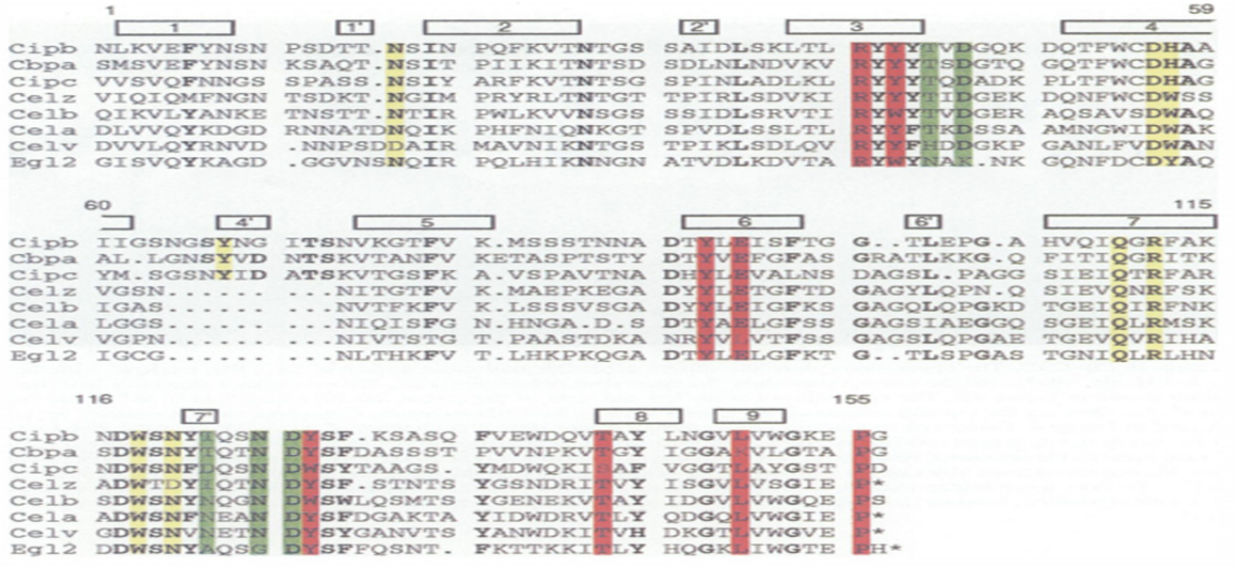


Fig. 40 :(i) Structure-based sequence alignment of selected CBDs from family III

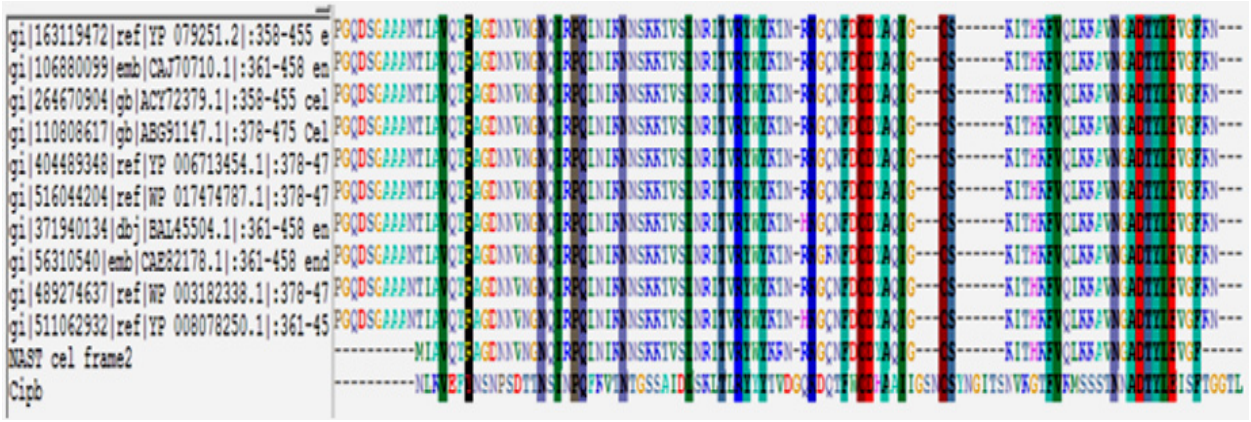


Fig. 40 :(ii) Structure-based sequence alignment of selected multiple aligned endoglucanases from figure 38 using Bioedit with that of *cipB* from *Closteridium* (shaded region represent conserved amino acid)

For the expression of cellulase gene the gene was first cloned to T-vector and the resulted recombinant plasmid restricted with *Nde*I/ *Hind*III enzymes showed a single band at 4380 instead of two fragments with approximate size 1500 bp and 2880 bp as that of cloning strategy as shown in figure 8. This was due to the ligation of cellulase gene reverse to that of the strategy mentioned as shown in the figure 41. This indicates the fragment was successfully cloned. Same T-vector (PTZ57R/T) was used for the cloning of PCR fragment (Aftab *et al.*, 2012). Besides T-vector other cloning vectors have been used prior to the cloning into an expression vectors. For further conformation the plasmid pCelNAST01 was sent for sequencing.

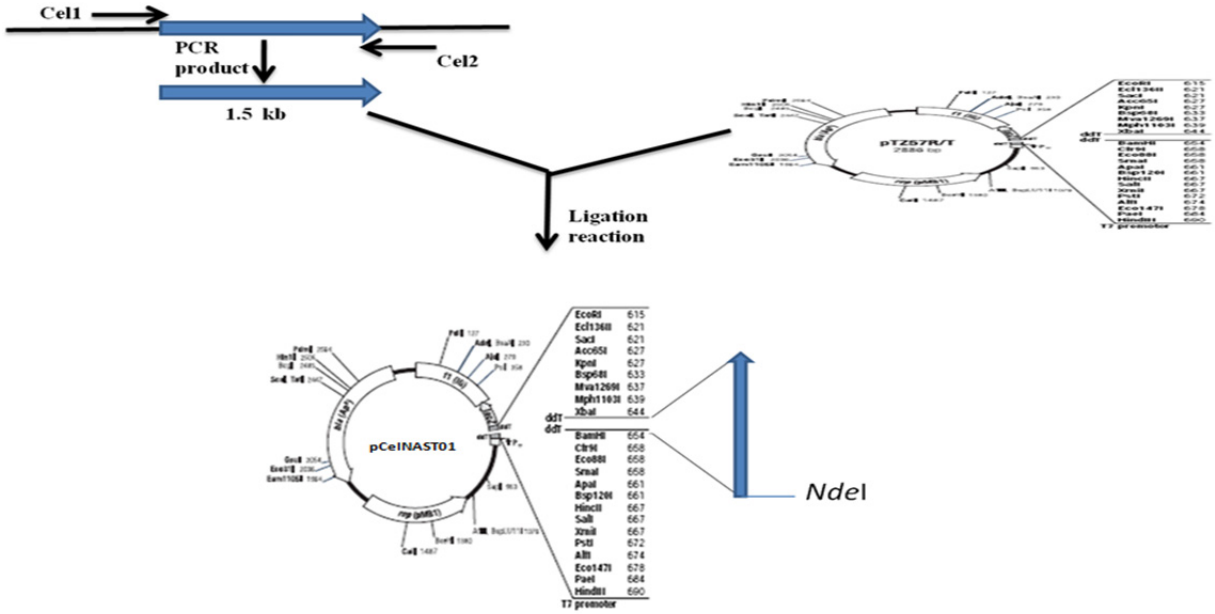


Fig. 41: Recombinant plasmid pCelNAST01

CONCLUSION

The hot spring appeared to be the potential natural source for the isolation and screening of cellulolytic bacteria and varied enzymes. NAST01 was screened for highest cellulase activity based on the hydrolysis of substrate CMC. Further, the isolate was identified as *B. licheniformis* NAST01 strain by 16S rRNA sequence analysis. The production of crude enzyme in flask and scale up in bioreactor at optimum condition showed 0.029677 U/ml and 0.0215 U/ml respectively. The down streaming with salt precipitation, dialysis and column purification showed increase in enzyme specific activity from 0.03956 U/mg to 1.235 U/mg which was almost 31.21 fold higher than crude enzyme. The enzyme production, purification and characterization are helpful for the utilization of enzyme from lab scale to industrial level in bioconversion of easily available cellulosic substrates. The present study showed the enzyme has optimum activity at pH 5.5 and stable at pH 6.0 to 7.5 for an hour at room temperature. Similarly, the optimum cellulase activity was observed at 50^{o}C and stable in the temperature range from 40 to 50^{o}C for 30 min. Metal ions like Fe^{2+} and Ca^{2+} at concentration 1 to 10 mM were found to increase activity but Ba^{2+} and sodium chloride of same concentration decrease the activity. Besides the kinetics of enzyme showed K_m 10.73 mg/ml of CMC and V_{max} 42.13 µmol/ml-min. The MW of the purified enzyme was found to be 55 kDa as revealed from 12% SDS-PAGE. The enzyme from NAST01 also hydrolyzed other substrates such as PNPG, avicel, cellobiose, filter paper besides CMC. The genetic manipulation can further enhance the activity of enzyme so that it could be used for specialized purpose and even in hydrolysis of crystalline substrate. The β-1, 4-endoglucanse specific PCR sequence showed nucleotide similar to *cel*5 gene from Bacillus GXN151 cellulase. The protein blast showed it belongs to CBM_3 domain with sequence similarity of 99% with *Bacillus licheniformis* endoglucanse. The gene was cloned into cloning vector PTZ57R/T so that final expression can be made into the expression vector.

Thus, it can be concluded *Bacillus licheniformis* NAST01 strain as potential isolate with β-1, 4-endoglucanse activity. The bacterial gene can be further manipulated by protein engineering technique and the thermostable cellulase enzyme can be scale up to bioreactor for industrial scale production.

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<http://afmb.cnrs-mrs.fr/~pedro/CAZY/cbm.html>

< **http://web.expasy.org/translate/>**

<http://prosite.expasy.org>

APPENDIX I

Construction of Calibration curve

Table 15: A standard calibration curve of glucose

Concentration of glucose(mg/mL)	Mean absorbance (540)
0.00125	0.100
0.0035	0.177
0.00421	0.2
0.0175	0.33
0.035	0.398
0.042	0.517
0.06	0.62
0.0875	0.924

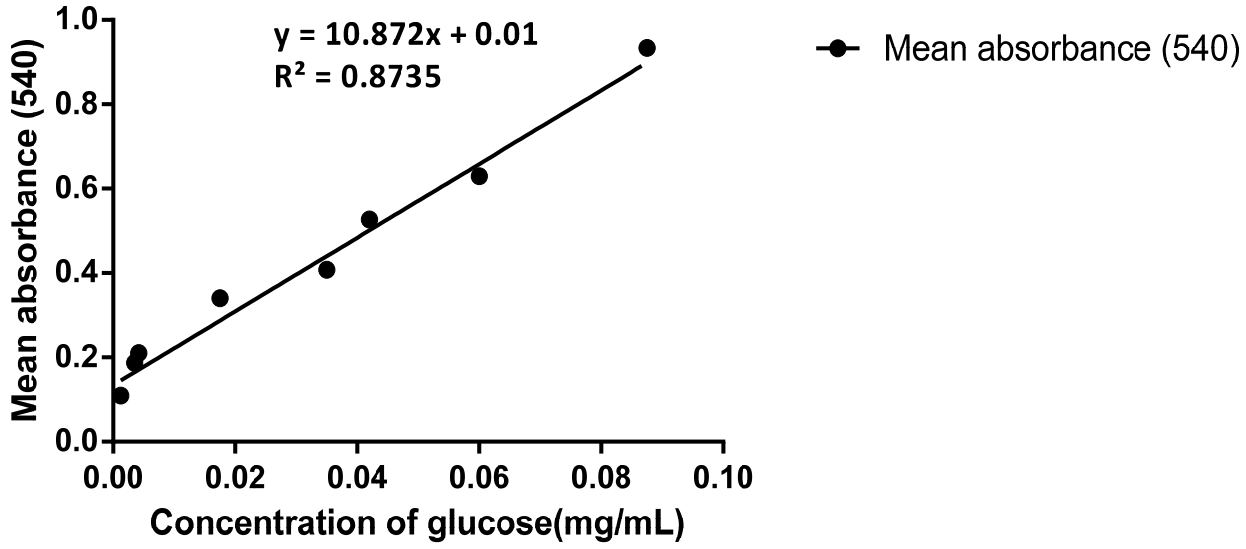


Fig. 42: Standard curve of glucose for DNS assay

Table 16: A standard calibration curve for protein estimation

Concentration of BSA in mg/ml	Mean absorbance (595nm)
0.2	0.100
0.3	0.0450
0.8	0.0460
1.0	0.0630
2.0	0.0740
4.0	0.1360
4.45	0.1550
8.0	0.2630

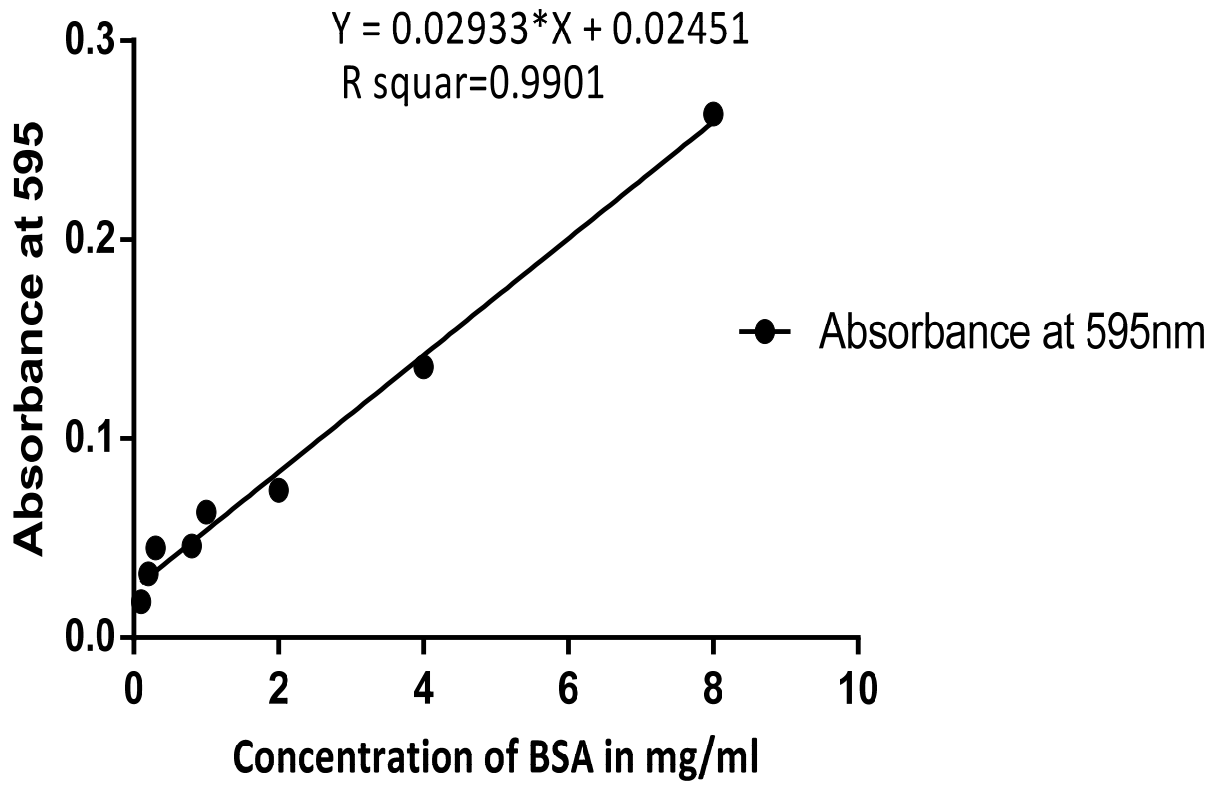


Fig. 43: Standard curve of protein for Bradford assay

Table 17: A standard calibration curve for *p*-nitrophenol estimation

Concentration of *p*-nitrophenol (mg/ml)	Mean absorbance (400nm)
0.0005	0.151
0.001	0.298
0.0014	0.3495
0.002	0.5385
0.004	0.798

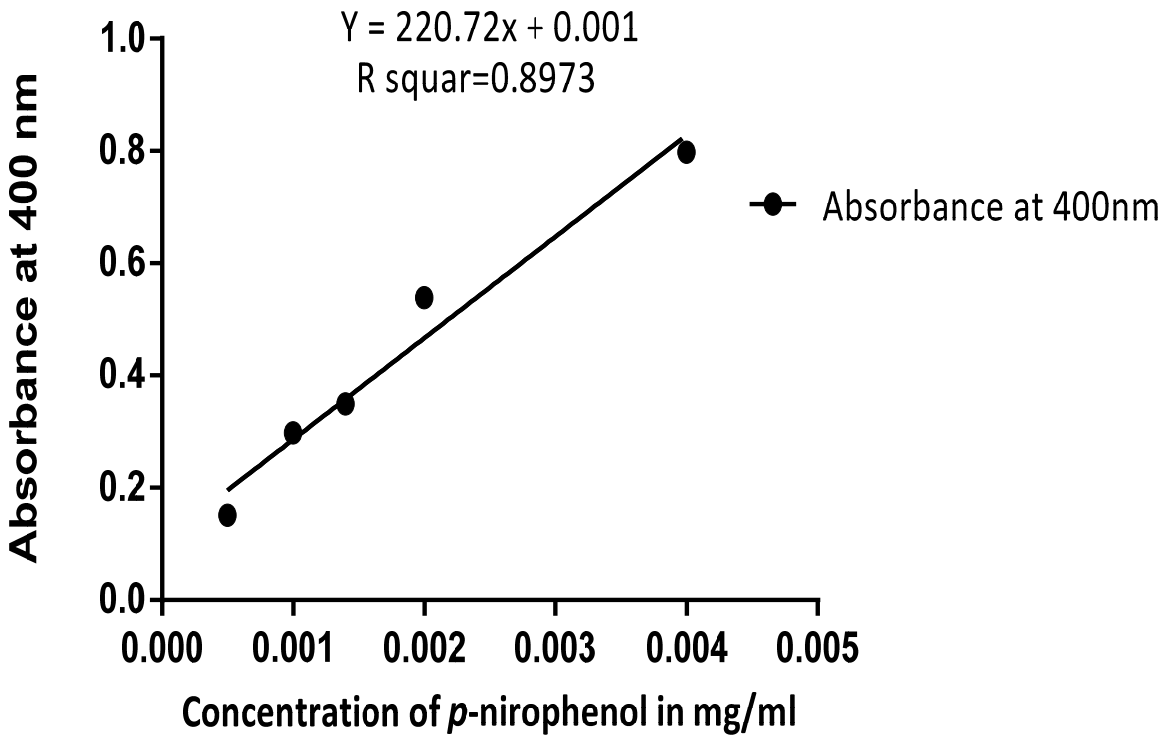


Fig. 44: Standard curve of *p*-nitrophenol for pNPG hydrolase assay

APPENDIX II

Sequence of 16S rRNA specific PCR of ribosomal DNA

>gi|401808243|gb|JX281689.1| Bacillus licheniformis strain NAST01 16S ribosomal RNA gene, partial sequence

TTGCTATACATGCAGTCGAGCGGACCGACGGGAGCTTGCTCCCTTAGGTCAGCGGCGGACGGGTGAGTAA
CACGTGGGTAACCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGCTTGATTG
AACCGCATGGTTCAATTATAAAAGGTGGCTTTCAGCTACCACTTGCAGATGGACCCGCGGCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGG
GACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGA
CGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTAC
CGTTCGAATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGT
CTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGA
GAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCT
CTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGCAGCAAACGCATTAAGCAC
TCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAG
CATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGAT
AGGGCTTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTCAGTTGGGCACTCTAAGGTGAC
TGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACAC
ACGTGCTACAATGGGCAGAACAAAGGGCAGCGAAGCCGCGAGGCTAAGCCAATCCCACAAATCTGTTCTC
AGTTCGGATCGCAGTCTGCAACTCGACTGCGTGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCG
CGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAACACCCGAAGTCG
GTGA

Similarity search of sequence using NCBI nucleotide BLAST for genotypic identification of bacteria

Bacillus licheniformis strain 55N2-8 16S ribosomal RNA gene, partial sequence	2577	2577	99%	0.0	99%	JN366726.1
Bacillus licheniformis strain HNNYDD3 16S ribosomal RNA gene, partial sequence	2575	2575	99%	0.0	99%	JN999851.1
Bacillus sp. 3547BRRJ 16S ribosomal RNA gene, partial sequence	2575	2575	99%	0.0	99%	JF309229.1
Bacillus licheniformis strain DQgbc4 16S ribosomal RNA gene, partial sequence	2575	2575	99%	0.0	99%	GQ470399.1
Bacillus licheniformis strain CICC 10334 16S ribosomal RNA gene, partial sequence	2575	2575	99%	0.0	99%	GQ375243.1
Bacillus licheniformis strain NJ-5 16S ribosomal RNA gene, partial sequence	2575	2575	99%	0.0	99%	FJ435674.1
Bacillus licheniformis strain D50 16S ribosomal RNA gene, partial sequence	2573	2573	99%	0.0	99%	KC441782.1
Bacillus sp. MBEP52 gene for 16S rRNA, partial sequence	2573	2573	99%	0.0	99%	AB733572.1
Bacillus licheniformis strain KVR 16S ribosomal RNA gene, partial sequence	2571	2571	99%	0.0	99%	HM852443.1
Bacillus licheniformis strain BL1 16S ribosomal RNA gene, partial sequence	2571	2571	99%	0.0	99%	HQ709382.1
Bacillus licheniformis strain GD3a 16S ribosomal RNA gene, partial sequence	2571	2571	99%	0.0	99%	HM055601.1
Uncultured Bacillus sp. clone CBR4 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	KC347587.1
Bacillus licheniformis strain TUST020 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	KC456634.1
Bacillus licheniformis strain MX5 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	JX027378.1
Bacillus licheniformis strain 30N1-9 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	JN366720.1
Bacillus licheniformis strain RK 202 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	JQ388689.1
Bacillus licheniformis strain HT12# 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	JN013197.1
Uncultured bacterium clone XJPL-LA-75 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	HM063956.1
Uncultured bacterium clone XJPL-LA-71 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	HM063955.1
Uncultured Bacillus sp. clone DQ328D 16S ribosomal RNA gene, partial sequence	2569	2569	99%	0.0	99%	EU050709.1
Bacillus licheniformis strain B12 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	KC441731.1
Bacillus licheniformis strain BaDB24 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	JX237858.1
Bacillus licheniformis strain KJN-1 16S ribosomal RNA gene, partial sequence >gbJQ894491.1| Bacillus licheniformis strain BBE11-1 16S r	2567	2567	99%	0.0	99%	JQ619622.1
Bacillus licheniformis strain SCC112026 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	JN998723.1
Bacillus licheniformis strain SCD113021 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	JN998718.1
Bacillus licheniformis strain YB-1105 16S ribosomal RNA gene, partial sequence	2567	2567	99%	0.0	99%	JQ292848.1

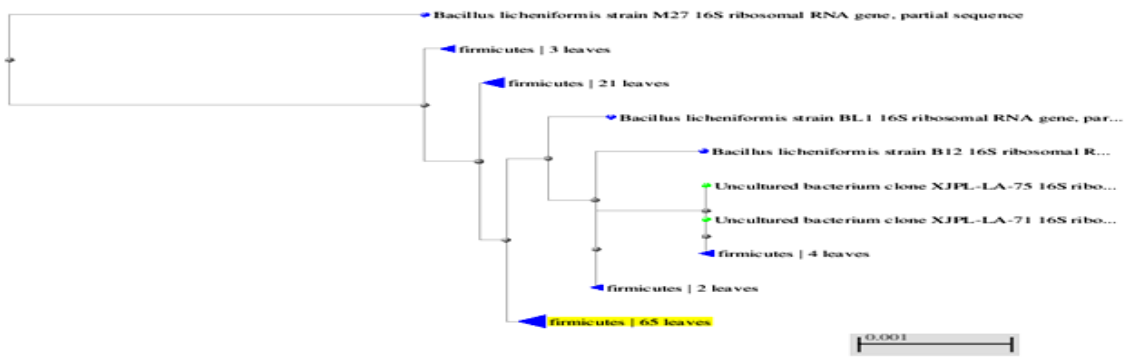


Fig. 45: BLAST tree view of 99 identical sequence using Neighbor joining tree method

Table 12: DNA Quantification for cellulase specific PCR

Sample ID/S.N.	Dilution factor	Absorbance				Ratio 260/280	Ratio 260/230	Concentration	
		230	260	280	320			µg/ml	ng/µl
NAST01/1	10+90	0.199	.0.056	0.032	0.006	1.75	0.31	27.9	27.9
NAST01/2	10+90	0.037	0.048	0.029	0.003	1.64	1.31	24.1	24.1
NAST01/3	10+90	0.049	0.049	0.029	0.003	1.71	1.01	24.7	24.7

The reverse primer based sequence of cellulase gene after sequencing is given below:

CGAGGTAGTACGCAATGGCGCTTTATTTAGGTTCGTTGCCCTGGAAAAAAAAATGGAAGGCAGTGGCCGCTTTATTT
ATTTTTGTTGCCCCCGCCAATGAAAAAAAAGAGCAGACGACCACCTGTTTTTTTTTTTTTTGTTGATGTACCA**TTTT**
AAAACCCTACTTCAAGATACGTGTCTGCTCCGTTTACCGCTTTTTTTAATTGGACAAATTTGTGCGTGATTTTGCTG
CAGCCGATTTGGGCATAGTCGCAGTCAAAATTTTGTCCTTTGCGATTCCTTTTATACCAGTAGCGGACGGTGATTCT
ATTTAAAGAGACGGTTTTTTTGCTGTTGTTTTTAATGTTGAGCTGAGGGCGAATTTGGTTGCCGTTTACATTGTTGT
CCCCCGCTCTGTATTGTACTGCTATCATGTTGGCTGCAACGCCGCTGTCTTGACCTGGTTTATCCTGATCCTTTTTT
AGGTCTATTTGATCTGATCATTCCTAATTGGATCCAAAGTCTCCAGACGAAGCGTTTGGCTTTGCGCGGATGAATCC
CAACGAATATAACTGATGAAGACAGCGACAATTGCGACCCCCCCCCCCCCCTGAATAAACACGTGCCTTGAAAACAC
CACACCCACATAGCTCGGGGTTAGCCCGAACAATTTCCGTATCCGGCTTATATGTTATTGACGATGAAAATATCCCC
CCACCCACCCGAAAACGGAAAGGCACCCTCTCCCTACTCCACAAACCATCACCGCTATCGCCTATGAGAACGAAACG
CGCCCCCTTCTTTTCTCAGAGGAAATGTCGATATTAACCCAACGAAGAGATTCTGCTTTGTGCTATAAAAATAGCGC
CGAAACAATAATATCGCCCTTCTTATCAGACAAGGCCCCCCCTAAGACGAGGAGAGGCCCCGACGTCGTTGTCCCAA
AACGAATTTATTTTAGGGAAATTTTCTCGTGCGCAGCCGGGGCGGGGATTCTCTGGTAGAGTGGAGATTAAATAGCC
CACTGATCGCCAAAAACCCACCCCACGCCGCGTAGAAATACCAAATACGACTGTTCCGCCCTCTCCCCCAAGAAGTC
AGTGCTTATCTGTAAAAAAGCGTTTCCTGTCTCGCCTTATGCCTATCGTATCTGACTTTCCCCTCCCCCCCACCCCA
AAATAAGGGGAACGCCCGAAGGAAAAAGCTCCGATTATTTCAAATTCTTAATTCGTGCGCGTCCCGCTCCGGAGGGA
AACATATATG**12481**

http://web.expasy.org/translate/

Met I A V Q Y R A G D N N V N G N Q I R P Q L N I K N N S K K T V S L N R I T V R Y W Y K R N R K G Q N F D C D Y A Q I G C S K I T H K F V Q L K K A V N G A D T Y L E V G F **Stop**

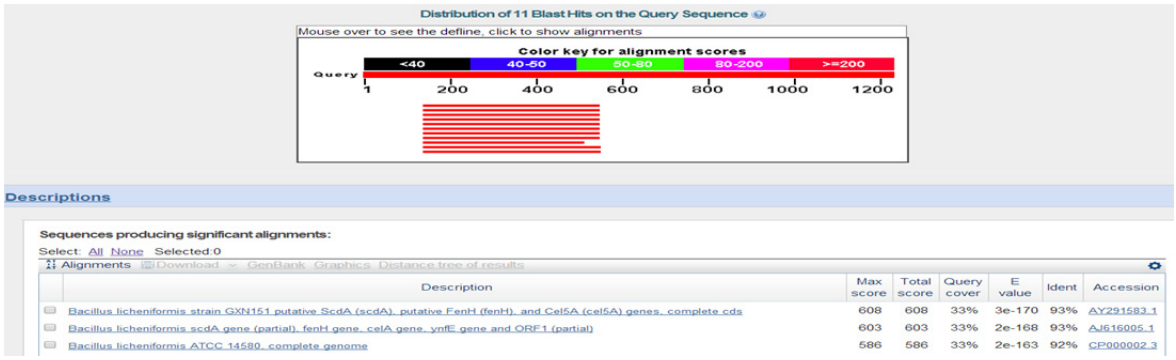
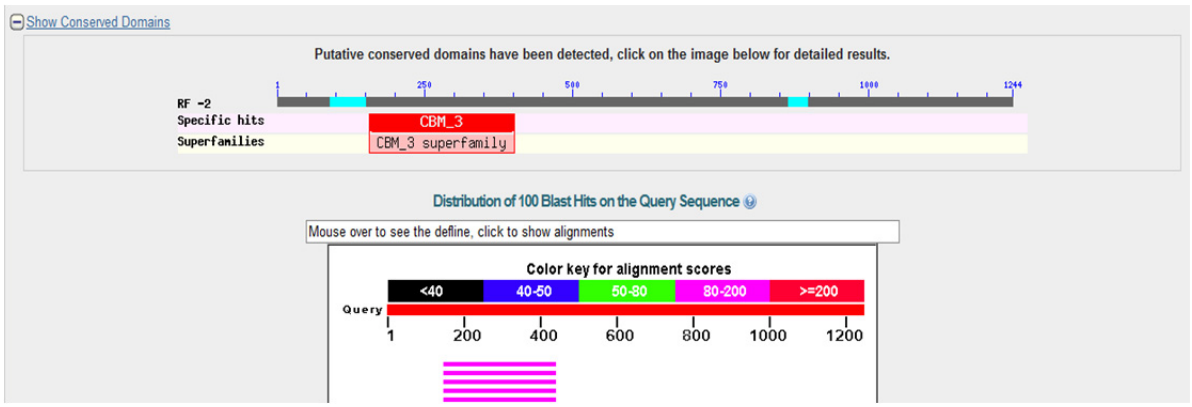


Fig. 46: BLASTn of reverse primer based sequence of cellulase gene showing similiarity



Description	Max score	Total score	Query cover	E value	Ident	Accession
endo-1,4-beta-glucanase, glycoside hydrolase family 5 [Bacillus licheniformis DSM 13 = ATCC 14580] >ref|WP_011198020.1| endoglucanase [Bacillus lic	196	196	23%	3e-53	96%	YP_079251.2
cellulose hydrolase [Bacillus licheniformis]	196	196	23%	3e-53	96%	ACY72379.1
endo-beta-1,4-glucanase precursor [Bacillus licheniformis] >emb|CAJ70714.1| endo-beta-1,4-glucanase precursor [Bacillus licheniformis]	196	196	23%	3e-53	96%	CAJ70710.1
cellulase BglC [Bacillus licheniformis DSM 13 = ATCC 14580] >ref|WP_011201662.1| endoglucanase [Bacillus licheniformis] >gb|AAU40977.1| cellulase	196	196	23%	4e-53	96%	YP_006713454.1
CelC [Bacillus sp. CY1-3]	195	195	23%	4e-53	96%	ABG91147.1
endoglucanase [Bacillus licheniformis] >gb|AAP51020.1| Cel5A [Bacillus licheniformis] >gb|EQM27813.1| endoglucanase [Bacillus licheniformis CG-B52	195	195	23%	6e-53	96%	WP_017474787.1
endoglucanase precursor [Bacillus licheniformis]	194	194	23%	1e-52	95%	CAE82178.1
endo-1,4-beta-glucanase [Bacillus licheniformis 9945A] >gb|AGN36513.1| endo-1,4-beta-glucanase [Bacillus licheniformis 9945A]	194	194	23%	1e-52	95%	YP_008078250.1
endoglucanase [Bacillus licheniformis]	194	194	23%	1e-52	95%	BAL45504.1
endoglucanase [Bacillus licheniformis] >gb|EID46965.1| BglC [Bacillus licheniformis WX-02]	194	194	23%	1e-52	95%	WP_003182338.1
beta-glucanase [Bacillus subtilis] >gb|ABW23437.1| beta-glucanase [Bacillus amyloliquefaciens]	135	135	24%	3e-34	62%	ABO37787.1
endoglucanase [Bacillus mojavensis]	138	138	24%	5e-33	63%	WP_010334430.1
beta 1,4 endo glucanase, partial [Bacillus subtilis]	133	133	23%	7e-33	65%	AGV05394.1
endoglucanase [Bacillus subtilis]	137	137	25%	1e-32	62%	WP_019258536.1
Chain A, Structure Of A Novel Cbm3 Lacking The Calcium-Binding Site	129	129	21%	2e-32	69%	2L8A_A

Fig. 47: Identity of NAST01 cellulase gene with other endoglucanase using BLASTX of NCBI

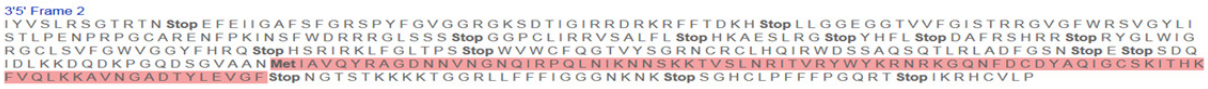


Figure 48: Translated nucleotide of cellulase sequence in 3' to 5' frame 2 (http://web.expasy.org/cgi-bin/translate/dna_aa)

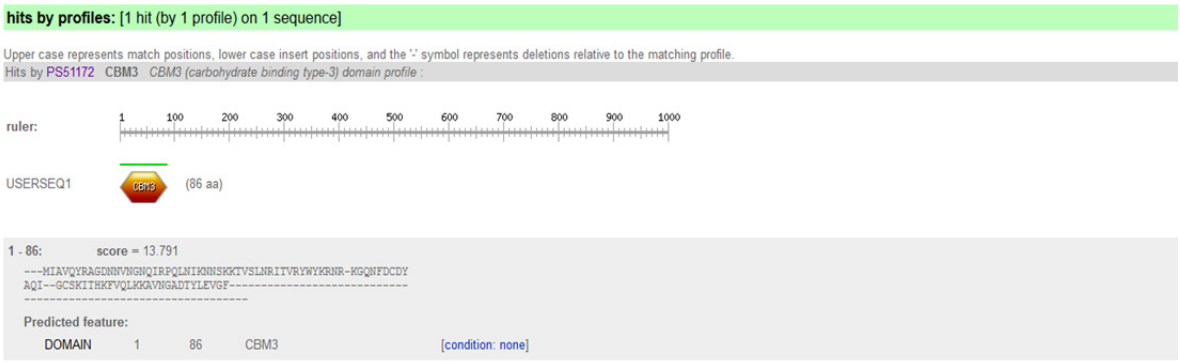


Fig. 49: Finding amino acid conserved region using Prosite (http://prosite.expasy.org)

Table 13: Plasmid DNA Quantification

Sample ID/S.N.	Dilution factor	Absorbance				Ratio 260/280	Ratio 260/230	Concentration	
		230	260	280	320			µg/ml	ng/µl
Clone 1st	5+95	0.027	0.035	0.021	0.002	1.66	1.29	34.6	34.6
Clone 2nd	5+95	0.011	0.019	0.011	0.000	1.70	1.70	19.3	19.3
Clone 3rd	5+95	0.032	0.072	0.037	0.000	1.93	2.24	72.0	72.0
Clone 4	5+95	0.003	0.017	0.012	0.000	1.43	5.67	17.0	17.0

Table 14: Screening for different enzyme from 16 isolates from hot springs water of Gorkha

Isolates	Amylase	Cellulase	tween 80(L)	Tributyrin (L)	Pectinase	Skim milk (Protease)	Caseinase	Xylanase
NAST01	4.00	**2.55**	1.6	1.80	2.1	**4.00**	2.60	3.30
NAST02	3.83	2.20	1.9	1.60	2.6	2.70	2.90	**3.83**
NAST03	4.64	1.45	**2.1**	1.90	2.6	2.57	2.35	2.57
NAST04	3.92	1.60	2.0	1.66	**2.9**	2.83	2.20	3.66
NAST05	**4.83**	1.92	1.9	1.80	2.5	2.23	2.15	3.46
NAST06	4.33	0.00	1.4	1.60	2.0	1.66	2.80	0.00
NAST07	4.67	1.85	1.6	1.50	2.0	2.16	2.40	2.78
NAST08	4.33	0.00	1.8	1.70	1.9	2.00	2.70	3.66
NAST09	3.83	0.00	2.0	1.90	1.8	1.66	3.00	3.58
NAST10	3.92	2.47	1.9	2.04	1.7	2.40	2.40	1.83
NAST11	0.00	0.00	2.2	1.90	2.1	2.20	2.90	2.08
NAST12	2.84	1.83	2.0	2.00	2.2	0.00	**3.60**	2.15
NAST13	2.00	2.00	**2.1**	**2.25**	2.1	0.00	2.90	2.80
NAST14	0.00	2.25	1.8	1.75	1.9	0.00	2.80	1.36
NAST15	**4.84**	0.00	**2.1**	1.70	2.1	2.40	2.00	1.40
NAST16	0.00	2.30	2.0	2.13	1.8	2.10	2.40	1.20

APPENDIX III

Composition of basal media, reagents and buffers

The composition of bacterial enzyme screening and production media optimization (preliminary media) is based on slight modification of media formulated by (Mandels *et al*. 1962) and pH was adjusted 7.0 using 0.1 M NaOH .

Preliminary screening media

Constituents	g/L
KH_2PO_4	1
K_2HPO_4	1.145
$MgSO_4.7H_2O$	0.4
NH_4SO_4	5
$CaCl_2.2H_2O$	0.005
$FeSO_4.7H_2O$	0.00125
Substrates (CMC, xylan,pectin,skim milk, , casein and starch 0.5% each; tween 80 (2%), tributyrin (2%))	Agar 1.3% was incorporated to solidify the plate and the media was mixed by heating and stirring and autoclaved at 121^{o}C (15 psi pressure) for 15 min and poured in aseptic zone in sterile plate.
For the production media optimization substrate (CMC) 1% without agar was used.	

Nutrient Broth

Ingredients	g/L
Peptic digest of animal tissue	5
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5
Final pH (at 25^{o}C)	7.4±0.2

All the components were mixed uniformly and autoclaved at 121^{o}C at 15 psi pressure for 15 minutes.

Nutrient Agar

Ingredients	**g/L**
Peptic digest of animal tissue	5
Beef extract	1.50
Yeast extract	1.50
Sodium chloride	5
Agar	15
Final pH (at 25^{o}C)	7.4±0.2

All the components were mixed uniformly and autoclaved at 121^{o}C at 15 psi pressure for 15 minutes. The media was poured into sterile bacterial culture plate (Petri plate).

Luria Bertani Broth/Agar

LB broth was prepared by dissolving following constituents:

Ingredients	**g/L**
Bacto Tryptone	10
Bacto Yeast extracts	5
NaCl	5

The ingredients were dissolved in 800 ml of water, and pH was adjusted to 7.0 with NaOH. The volume was adjusted to 1000 ml.

LB agar plate was prepared by dissolving above constituents and adjusting pH. 15g/L agar is added to make final volume. The media was autoclaved at 121^{o}C, 15 psi pressure for 15 mins .

Before pouring into plates, the medium was allowed to cool to 55^{o}C and the media without antibiotics was poured. Similarly, 50 µg/ml ampicillin was mix gently to prepare LB-ampicillin agar plate. The LB agar plate, LB-ampicillin agar plates were allowed to solidify.

LB ampiciilin X-Gal/IPTG plates were also prepared by drying plates with LB-ampicillin under UV light at room temperature for 30 min followed by addition of 40 µl of X-Gal stock solution (20

mg/ml) and 40 µl of IPTG(from 100 mM stock solution) and spread evenly with sterile L-shaped glass spreader. The speeded X-Gal/IPTG solution was allowed to absorb at room temperature.

Composition of reagents and buffer

DNS assay and Bradford assay

The reagents for DNS and Bradford assay were prepared as

Reagents

- Dinitrosalicylic Acid Reagent Solution, 1%
    - Dinitrosalicylic acid: 10 g
    - Phenol: 2 g
    - Sodium sulfite: 0.5 g
    - Sodium hydroxide: 10 g
    - Water added to: 1 liter
- Potassium sodium tartrate solution, 40%

Procedures

Test was done using 0.5 ml of substrate (1% CMC) taken and mixed with 0.5 ml Mcllvaine buffer (pH 7.0). Mcllvaine buffer was prepared using 0.2M Na_2HPO_4 (16.47ml) and 0.1 M citric acid (3.53ml). 0.5 ml of supernatant enzyme was mixed in a test tube. Similarly, the boiled enzyme along with substrate and buffer was prepared parallel as enzyme blank. Substrate blank was prepared in similar manner without enzyme and instead 0.5ml water was used. Test, enzyme blank and substrate blank were incubated at 50°C for 15 minutes and 3 ml DNS was added and boiled for 10 minutes and cool down. Absorbance was taken at first in scanning mode from 500-600 and then λ_{max} (540 nm), the wavelength in which maximum absorbance observed was taken as standard wavelength for all assay after subtracting blank.

Reagents

- Commasie Brilliant Blue G(25 mg)
    - 12.5ml ethanol
    - 21.25 ml KH_2PO_4(HCl was added to make pH 2.0) and volume was made upto 50 mL

- Solution was made upto 200 ml
- 50 mL orthophosphoric(H_3PO_4) acid was added
- The reagent was filtered and stored at 4◦C in a dark bottle.

Procedure:

In 0.8 ml broth from production media, 0.2 ml Bradford reagent was added. Blank was prepared with production media without growth and 0.2 ml Bradford reagent was added. Absorbance was taken at 595 nm after 5 minutes and blank was subtracted. Total protein content after a day and two days was measured to optimize different parameters for the production media.

SDS-PAGE

The components for preparing resolving and stacking gels for Tris-glycine SDS-Polyacrylamide gel electrophoresis are:

Resolving gel (12%):

Solution components	**5 ml**	**10 ml**	**15 ml**	**20 ml**
H_2O	1.6	3.3	4.9	6.6
30% Acrylamide	2.0	4.0	6.0	8.0
1.5 M Tris(pH 8.8)	1.3	2.5	3.8	5.0
10% SDS	0.05	0.1	0.15	0.2
10% $(NH_4)S_2O_8$	0.05	0.1	0.15	0.2
TEMED	0.002	0.004	0.006	0.008

The resolving gel was prepared in 10 ml volume with above components in order shown. It was casted after proper mixing upto 2/3rd portion. The top layer was overlaid with TDW gently and was left vertically for 45 min for polymerization.

Stacking gel (12%):

Solution components	**1 ml**	**2 ml**	**3 ml**	**4 ml**
H_2O	0.68	1.4	2.1	2.7
30% Acrylamide	0.17	0.33	0.5	0.67

0.5 M Tris(pH 6.8)	0.13	0.25	0.38	0.5
10% SDS	0.05	0.1	0.15	0.2
10% $(NH_4)S_2O_8$	0.01	0.02	0.03	0.04
TEMED	0.001	0.002	0.003	0.004

After polymerization of resolving gel, the overlay water was poured off and top of the gel was washed several times with deionized water. The stacking gel prepared above was poured over the resolving gel and immediately a Teflon comb was inserted slowly and left for polymerization for 20 min. After polymerization, the comb was removed and the wells were washed with TDW 3 to 4 times. The slab was mounted in the electrophoresis device and tris-glycerine electrophoresis buffer (running buffer) was added in both the tanks.

The protein sample 20 µl was mixed with equal volume of sample loading buffer in an eppendorf tube. The mixture was boiled for 5 min in order to denature the protein. The prepared samples along with protein marker were loaded in each well according to the predetermined order. SDS-PAGE was run with constant current supply of 40 mA for 4 hr. The glass plates were removed when the bromophenol blue reached bottom of the gel. The gel was stained with staining solution Comassie Brilliant Blue (0.1% w/v CBB) for an hour. It was fixed in fixing solution for 20 min followed by destain in destaining solution for 4 hr to overnight.

Reagents for SDS-PAGE

A. Monomer (30%) Acrylamide solution

Acrylamide, C_3H_5NO (MW: 71.08)		29g
Bis-Acrylamide (N, N Methylene Bisacrylamide, $C_7H_{10}N_2O_2$, MW: 154.17)	1g	
TDW	up to	100ml

B. Sodium Dodecyl Sulphate (10%), $CH_3(CH_2)_{10}$-$CH_2OSO_3^-Na^+$

SDS		10g
TDW	up to	100ml

C. Lower Tris (pH 8.8)

1.5 M Tris (Tris base)		18.17g
TDW	up to	100ml

D. Upper Tris (pH 6.8)

0.5 M Tris (Tris base)		3.03g
TDW	up to	50ml

E. Ammonium persulphate (10%), $(NH_4)_2S_2O_8$ (MW: 228.2)

APS		50mg
TDW	upto	500µl

F. Loading/ Sample buffer

Upper Tris		1.25ml
10% SDS		3.0ml
Glycerol		4.75ml
β-mercaptoethanol		0.5ml
0.1% Bromophenol Blue dye		0.5ml

G. Comassie Brilliant Blue (CBB) staining solution (0.1% w/v)

CBB G-250		50mg
Glacial acetic acid		2.5ml
Methanol		25.0ml
TDW	upto	50ml

H. Fixing solution

7.5% glacial acetic acid		18.7ml
5% methanol		12.5ml
TDW	upto	250ml

I. Destain Solution

7.5% glacial acetic acid		50ml
5% methanol		250ml
TDW	upto	500ml

J. Running Buffer (Electrode Buffer)

39 mM Tris		4.724g
48mM Glycine		3.603g
0.1% SDS		0.37g
pH		8.4
TDW	up to	1000ml

Tris EDTA (pH 8.0)

A. Tris-Cl (1 M), pH 8.0

Tris base		121.1g
Concentrated HCl		42ml
DDW	up to	1000ml

B. EDTA solution (0.5 M) pH 8.0

Disodium EDTA.2H_2O	186.1g
Stirred vigorously and pH adjusted using NaOH pellets to	8.0
DDW	up to 1000ml

C. 10X Tris EDTA(TE), pH 8.0

100mM Tris-Cl (pH 8.0)

10mM EDTA (pH 8.0)

For dissolving DNA 10 x TE is diluted to 1 x.

Electrophoresis Buffer

Buffer	Working solution	Stock solution/L
TAE	1 x	50x
	40 mM Tris-acetate	242 g of Tris base
	1mM EDTA	57.1 ml glacial acetic acid
		100 ml, 0.5 M EDTA (pH 8)
		dd H_2O upto 1000 ml

The 10x stock solution of electrophoresis buffer was diluted with ddH_2O to generate 1 x working solution immediately before use.

6 x (DNA) gel loading buffer

0.25% w/v bromophenol blue

40% w/v sucrose in H_2O (It was stored at 4^oC).

McIlvaine buffer

0.1M buffer of respective pH was made from stock solution of 0.1M Na_2HPO_4 and 0.1M Citric acid as shown below:

pH	0.1M Na_2HPO_4(ml)	0.1M Citric acid (ml)
4.0	7.71	11.12
4.2	8.28	11.72

4.4	8.82	11.18
4.6	9.35	10.65
4.8	9.86	10.14
5.0	10.30	9.70
5.2	10.72	9.28
5.4	11.15	8.85
5.6	11.60	8.40
5.8	12.09	7.91
6.0	12.63	7.37
6.2	13.22	6.78
6.4	13.85	6.15
6.6	14.55	5.45
6.8	15.45	4.55
7.0	16.47	3.53
7.2	17.39	2.61
7.4	18.17	1.83
7.6	18.73	1.27
7.8	19.15	0.85
8.0	19.45	0.55

Preparation of 0.01 M sodium phosphate buffer (pH 7.0±0.2)

1 M NaH_2PO_4 (monobasic; MW = 138) and 1 M Na_2HPO_4 (dibasic; MW = 142) stock solutions were prepared by dissolving 13.8 g in 100 ml and 14.2 g in 100 ml . 42.3 ml of 1 M NaH_2PO_4 and 57.7 ml of Na_2HPO_4 was mixed to make 0.1 M buffer of pH 7.0.The buffer so prepared was diluted to give concentration 0.01M.

Purification of endocellulase specific gene amplicon

The volume of DNA sample (amplicon) was measured followed by the addition of $1/10^{th}$ volume of 3 M sodium acetate, pH 5.2 (final concentration of 0.3 M). It was mixed well by inverting microfuge tube. The volume of cold 100% ethanol i.e. calculated after salt addition, was added up to 2.5 volumes. It was mixed well and place at -20◦C for 30 minutes. Then the mixture was centrifuged at 14000 rpm for 10 to 15 minutes followed by collection of supernatant. To the supernatant 1 ml of 70% ethanol was added followed by centrifugation at 14000 rpm for 2 minutes. Carefully, the supernatant was decanted by inverting into the tissue paper and the pellet was obtained. The pellet was air dried to remove traces of ethanol. Finally, it was suspend in TE buffer (30µl).

Appendix IV

Color plates

Plate 1: Plate showing screening, production, characterization and assay of enzymes (a= production of cellulase in bioreactor, b= screening for different enzyme from 16 bacterial isolate, c = protease, d and g= casseinase screening , e and f= lipase screening tween-80 and tributyrin plate, h=pectinase, i=xylanase, j and l = cellulase, k= screening enzyme activity at different pH of NAST01 strain, m = salt precipitation of crude enzyme in ice cold water, n and o=Blue white screening, p=DNS and Bradford assay, q=12% SDS-PAGE of crude and partially purified enzyme)

Plate: (i) Cellulase enzyme production in bioreactor

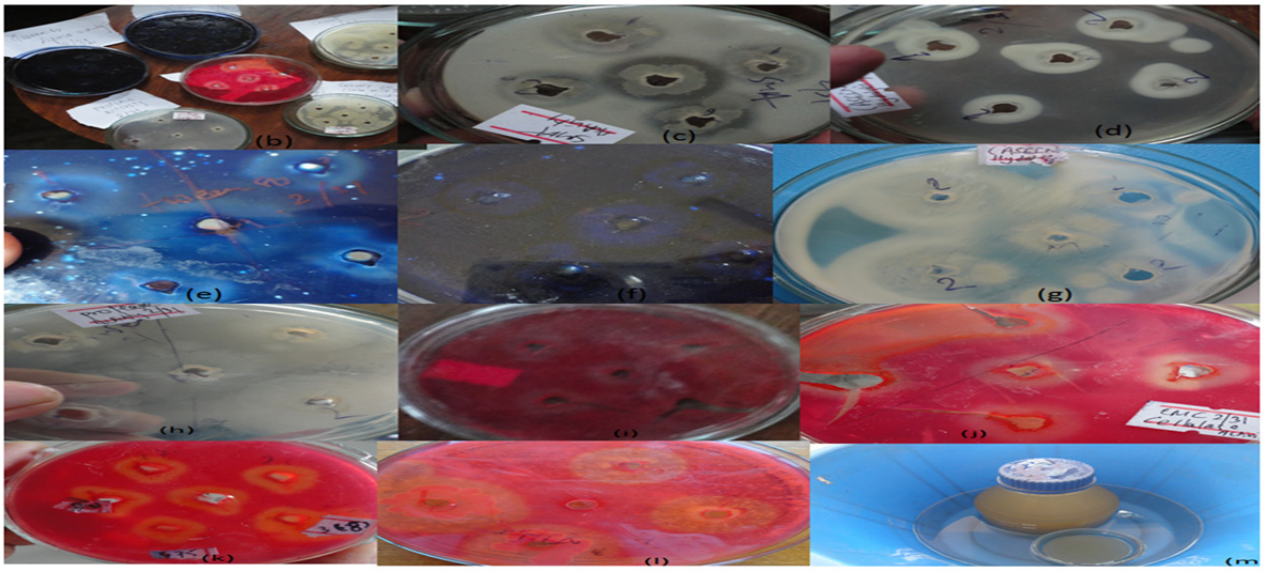


Plate: (ii) Enzymatic screening using different substrates along salt precipitation of crude enzyme

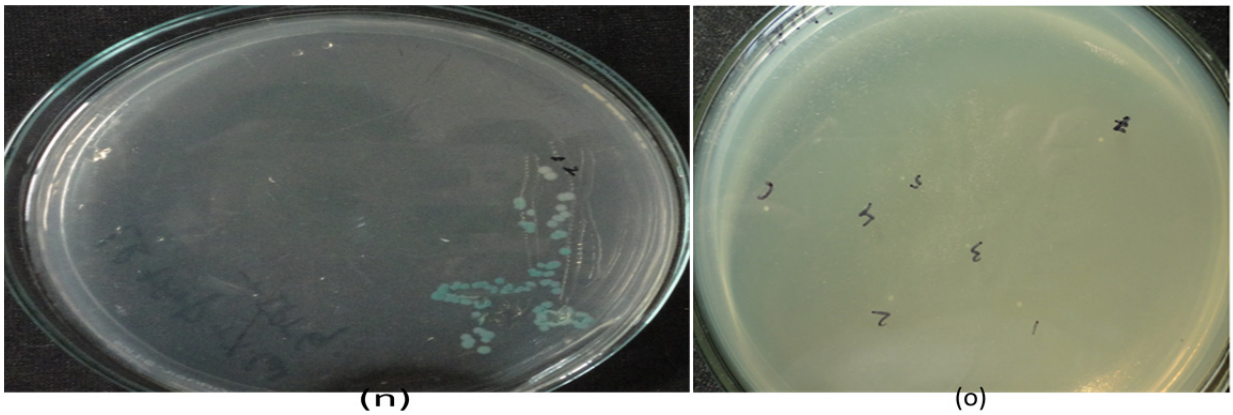


Plate: (iii) Blue-white screening in x- gal/IPTG plate with ampicillin

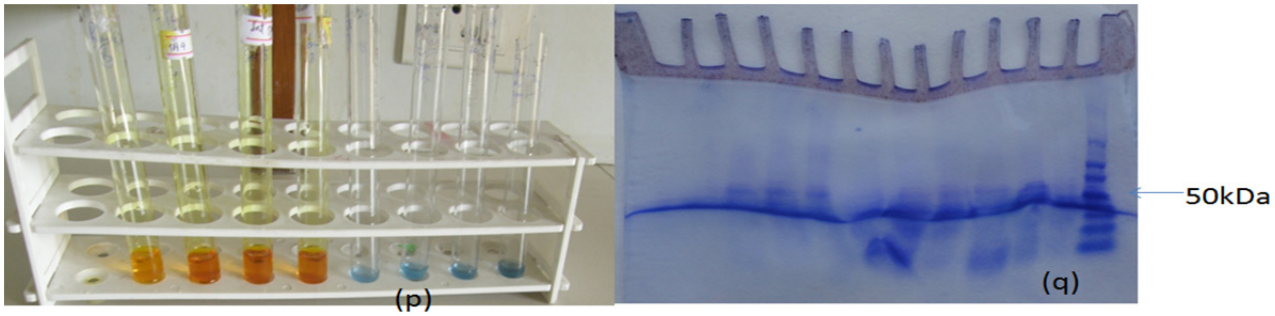


Plate: (iv) Enzyme assay (DNS and Bradford) and 12% SDS- PAGE

Plate 2: Transferring innoculum for enzyme production and in aseptic condition