Purification and characterization of endoglucanase from *Bacillus licheniformis* NA8

A

DISSERTATION REPORT

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CERTIFICATE

This is to certify that the thesis entitled "**Purification and characterization of endoglucanase from *Bacillus licheniformis* NA8**'' submitted by **Ms. Aanchal** in partial fulfilment of the requirements for the award of degree of Master of Technology in Biotechnology to Thapar University, Patiala, is a record of students's own work carried out by her under my supervision. The report has not been submitted for award of any other degree or certificate in this or any other Univerity or Institute.

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CANDIDATE'S DECLARATION

I, hereby declare that the work presented in dissertation entitled "**Purification and characterization of endoglucanase from *Bacillus licheniformis* NA8**" in partial fulfillment of the requirements for award of degree of Master of Technology, in Biotechnology Department of Biotechnology, Thapar University, Patiala, is an authentic record of my own work during the period of one year from July, 2013-July, 2014, under the supervision of **Dr. Dinesh Goyal**, Professor & Head, Department of Biotechnology, Thapar University. The report has not been submitted for the award of any other degree or certificate in this or any other university.

Date:

Place: Patiala **(Aanchal)**

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Date:

Place: Patiala **(Aanchal)**

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ABBREVIATIONS

CMC	Carboxymethyl cellulose
CMCase	Carboxymethyl cellulase
DNS	3, 5-dinitrosalicylic acid
et al	And others
Etc	And other things
rpm	Rotation per minute
Viz	As follows
$FeSO_4.7H_2O$	Ferrous sulphate heptahydrate
K_2HPO_4	Di potassium hydrogen phosphate
$MgSO_4.5H_2O$	Magnesium sulphate pentahydrate
$CaCl_2$	Calcium chloride
$HgCl_2$	Mercuric chloride
$PbCl_2$	Lead chloride
$(NH_4)_2SO_4$	Ammonium sulphate
$CuCl_2$	Copper chloride
$CdCl_2$	Cadmium chloride
OD	Optical density
g	Gram
g/L	Gram per Liter
H	Hour
L	Litre
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
μg	Microgram

SYMBOLS

β	Beta
α	Alpha
%	Percentage
°C	Celsius

ABSTRACT

Carboxymethyl cellulase activity in the (CMCase) the cell free supernatant of cellulose degrading bacteria *Bacillus licheniformis* NA8 was studied using 2% carboxymethyl cellulose. The protein was precipitated upto 60-80% saturation with ammonium sulphate and further purified using DEAE-Sepharose column. 4.76 fold purification of endoglucanase was achieved from crude extract with a yield of 83.49% through DEAE sepharose column chromatography. The molecular weight of the protein was 24 kDa as determined by SDS-PAGE and further confirmed by zymogram analysis. The endoglucanase was found to be stable between pH 5–7 and temperature between 30−60°C with optimal activity at pH 6.0 and temperature 50°C. The K_m value of the enzyme for the substrate carboxymethyl cellulose was recorded to be 0.25 mg/mL. The purified cellulase was activated by Mn^{2+} and Co^{2+}, but inhibited by Hg^{2+}, Cd^{2+}, Pb^{2+}. The purified endo-1,4-glucanase showed maximum (2.01 ± 0.03 U/mL) substrate specificity for Carboxymethyl cellulose (CMC) followed by Avicel and filterpaper. The high catalytic activity and its stability to temperature, pH, surfactants, and metal ions indicated that the cellulase enzyme from *B. licheniformis* NA8 is good candidate for hydrolysis of lignocellulosic waste and biorefinery processes.

CHAPTER 1
INTRODUCTION

Cellulases have become important commercial enzymes in the contemporary market because of their ubiquitous applications in diverse fields. Presently in context of fossil fuel scarcity, the most important application of cellulases is in hydrolysis of lignocellulosic biomass to fermentable sugars, which could be used for bioalcohol (bioethanol or biobutanol) production (Bhat, 2000; Ranjan et al., 2013). Enormous amounts of agricultural, industrial and municipal cellulosic wastes have been accumulating or used uneffciently due to the high cost of their utilization processes (Kim et al., 2003). Cellulose, a polymer of β-1,4- linked glucose units, is a major polysaccharide constituent of plant cell walls is the most common natural renewable biopolymer, is commonly degraded by the hydrolytic action of a multicomponent enzyme system-the cellulase and represents the key step for biomass conversion. Cellulose microfibrils are insoluble cable-like structures that are typically composed of approximately 36 hydrogen-bonded chains containing 500 to 14,000 β-1,4-linked glucose molecules. Cellulose microfibrils comprise the core component of the cell walls that surround each cell. Roughly one-third of the total mass of many plants is cellulose (Chris Somerville, 2006). Cellulases are inducible enzymes which are synthesized by microorganisms during their growth on cellulosic materials (Lee and Koo, 2001). The complete enzymatic hydrolysis of cellulosic materials needs different types of cellulase; endoglucanase (1,4-1-D-glucan-4-glucanohydrolase; EC 3.2.1.4), exocellobiohydrolase (1,4-1-D-glucan glucohydrolase; EC 3.2.1.74) and 1-glucosidase (1-D-glucoside glucohydrolase; EC 3.2.1.21) (Yi et al., 1999). The endoglucanase randomly hydrolyzes the β-1,4 bonds in the cellulose molecule, and the exocellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity and then at end cellobiose is converted to glucose by glucosidase (Bhat and Bhat, 1997).

Cellulases are widely used in production of animal feed, formulation of detergents, juice clarification, paper industry and wine production. Cellulases contribute to 8% of the worldwide industrial enzyme demands and the demand is expected to increase by 100% within 2014 (Costa et al., 2008). For these processes, thermophilic and or alkalophillic or acidophilic microorganisms as sources of thermostable and wide range of pH stable enzymes are needed, because of their higher stability and activity over a wider range of

temperatures and pH (Bakare et al., 2005). However, bacteria may also serve as a novel source cellulases due to their higher growth rate, more complex glycoside hydrolases providing synergy with higher potency because of organismal diversity of extreme niches. The main challenges to improve the whole process associated with the conversion of cellulosic biomass to ethanol include increasing polysaccharides hydrolysis yield, decreasing loading of hydrolytic enzymes, optimizing or eliminating the pretreatment and developing a consolidated bioprocessing where the enzyme production, the hydrolysis step and fermentation of sugars into ethanol occur in a single process (Amore et al., 2012).

Bacteria has high growth rate as compared to fungi has good potential to be used in cellulase production. Celluloytic property of some bacterial genera such as *Cellulomonas* species, *Pseudomonas* species, *Bacillus* species and *Micrococcus* species were reported (Nakamura and Kappamura., 1982). Cellulase yields appear to depend on a complex relationship involving a variety of factors like inoculum size, pH, temperature, presence of inducers, medium additives, aeration, and growth time (Robson and Chambliss, 1989). Cellulose degradation occurs in three general steps. Over the years, culturable, cellulase-producing bacteria have been isolated from a wide variety of sources such as composting heaps, decaying plant material from forestry or agricultural waste. Screening for cellulase production can be done by enrichment growth on microcrystalline cellulose as a sole source of carbon. Screening for bacterial cellulase activity in microbial isolates is typically performed on carboxymethylcellulose (CMC) containing plates.

Many workers have purified and characterized cellulases isolated from different bacteria viz. *Thermomonospora* sp. (George et al., 2001), *Cellulomonas* sp. YJ5 (Yin et al., 2010), *Melanocarpus* sp. MTCC 3922 (Kaur et al., 2007), *Pseudomonas fluorescens* (Bakare et al., 2005), *Bacillus* sp (Acharya and Chaudhury 2011; Bajaj et al., 2009). In present study CMCase from *Bacillus licheniformis* NA8 was purified and characterised and its activity was optimized for various parameters such as pH, temperature and substrate specificity.

CHAPTER 2
REVIEW OF LITERATURE

Growing concerns regarding the impacts of fossil fuel consumption on global climate change and energy security have led to the demand for accelerated development of biofuels. Lignocellulosic biomass is of great interest as a starting material for biofuel production (Himmel et al., 2007; Schubert, 2006; Stephanopoulos, 2007). Biological routes to ethanol, butanol, and other fuels have advantages over thermochemical routes; however, the cost effective release of fermentable sugars from plant biomass remains a challenge. Approximately 70% of plant biomass is locked up in 5- and 6-carbon sugars. These sugars are found in lignocellulosic biomass comprised of mainly cellulose (a homologous polymer of glucose linked by β-1-4 glycosidic bonds) hydrolyzed by a complex enzyme system named as cellulase (exoglucanase, endoglucanase and β-glucosidase etc.); lesser hemicelluloses (heterologous polymer of 5- and 6-carbon sugars consists of pentoses D-xylose, D-arabinose and hexoses D-mannose, D-glucose, D-galactose with sugar acids); and least of all lignin (a complex aromatic polymer). So, extensive research is being carried out to search of a potential cellulolytic microorganism which can act on lignocellulosic biomass and natulally produce bioethanol, which can further help in mankind development.

Cellulose

Cellulose is the most abundant biomass on the earth. Cellulases are inducible enzymes which are synthesized by large number of microorganisms either cell-bound or extracellular during their growth on cellulosic materials (Lee, 2001). Cellulose a crystalline polymer of D-glucose residues connected by β-1-4 glucosidic linkages (Figure 1), being the primary structural material of plant cell wall, is the most abundant carbohydrate in nature (Saha, 2004). On average, cellulose accounts as 50% of the dry weight of plant biomass. Such plant biomass is the only foreseeable sustainable source of fuels and materials available to humanity. Agricultural residues are a great source of lignocellulosic biomass which is renewable, chiefly unexploited and inexpensive. These renewable resources are leaves, stems, and stalks from sources such as corn fiber, corn stover, sugarcane bagasse, rice straw, rice hulls, woody crops, and forest residues. Besides, there are multiple sources of lignocellulosic waste from industrial and agricultural processes, e.g., citrus peel waste, coconut biomass, sawdust, paper pulp, industrial waste, municipal cellulosic solid waste,

and paper mill sludge (Sadhu et al., 2013). The structure of cellulose with its hydrogen bond makes it insoluble in most solvents and is partly responsible for the resistance of cellulose against microbial degradation (Jorgensen, 2005).

Cellulase degrading enzymes

The enzymes for the degradation of celluloses belong predominantly to the hydrolases which leave glycosidic bonds by hydrolysis; therefore the enzymes that catalyse the breakdown of cellulose are referred to as Cellulases. Cellulase is a family of at least 3 groups of enzymes namely cellobiohydrolases (exoglucanases, EC 3.2.1.91), endoglucanases (EC 3.2.1.4) and β-glucosidase (EC 3.2.1.21) (Kuhad et al., 1997). The exoglucanase (CBH) acts on the ends of the cellulose chain and releases β-cellobiose as the end product; endoglucanase (EG) randomly attacks the internal O-glycosidic bonds, resulting in glucan chains of different lengths; and the β-glucosidases act specifically on the β-cellobiose disaccharides and produce glucose (Bayer et al., 1998). Therefore, crystalline cellulose is efficiently hydrolyzed by the synergistic action of all three types of Cellulases. Some organisms (for example, *Trichoderma* sp.) produce all three types of cellulases and efficiently degrade cellulose by their synergistic effect (Okada et al., 1975). β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose, as both endoglucanase and exoglucanase activities are often inhibited by cellobiose (Harhangi et al., 2002).In most organisms, cellulases are modular enzymes that consist of a catalytic core connected to a cellulose binding domain (CBD) through a flexible and heavily glycosylated linker region. The CBD is responsible for bringing the catalytic domain in an appropriate position for the breakdown of cellulose (Gilkes et al., 1991). Cellulase degrades cellulose using its various subunits including (CBH), endoglucanases (EGs), type1 and type 2 polysaccharide monooxygenases (PMOs) (PMO1 and PMO2, respectively). Cellobiose dehydrogenase (CDH) is a potential electron donor for PMOs. EGs and PMOs cleave internally cellulose chains releasing chain ends that are targeted by CBHs. CBHs generate cellobiose or oxidized cellobiose that are subsequently hydrolyzed by action of β-glucosidase (Figure 2).

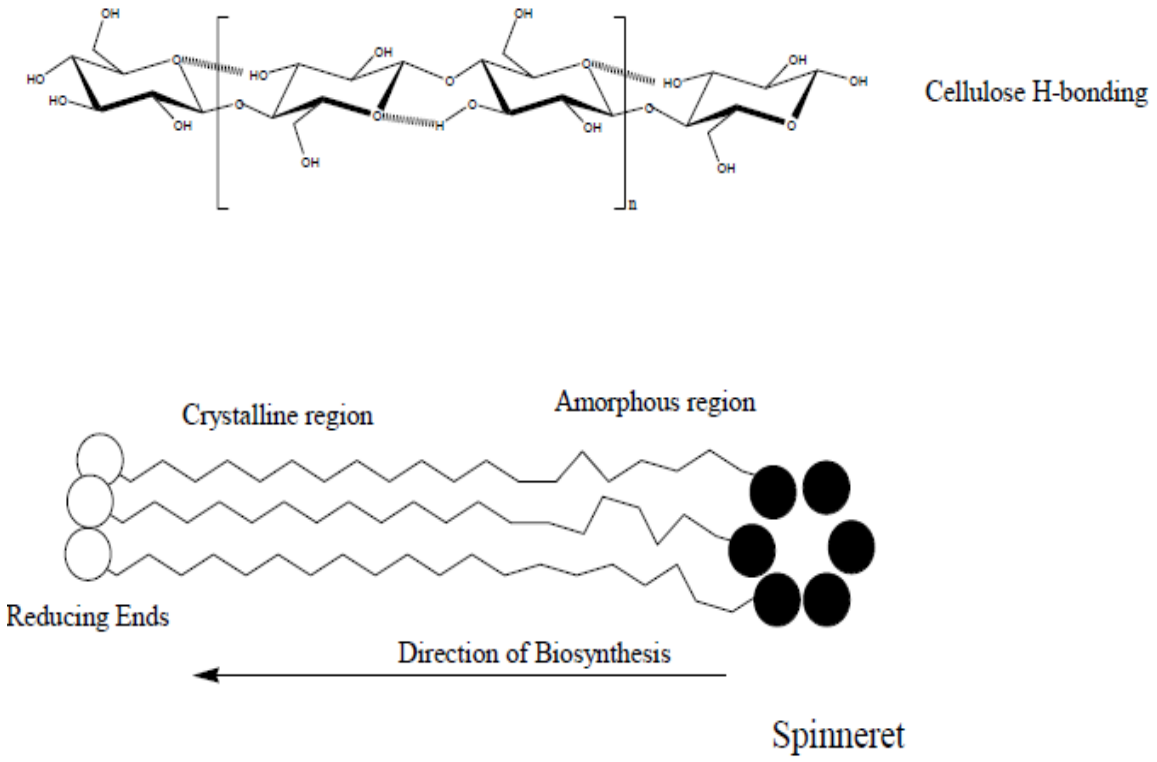

Figure 1. The structure of Cellulose (Source: Humphreys et al., 2010).

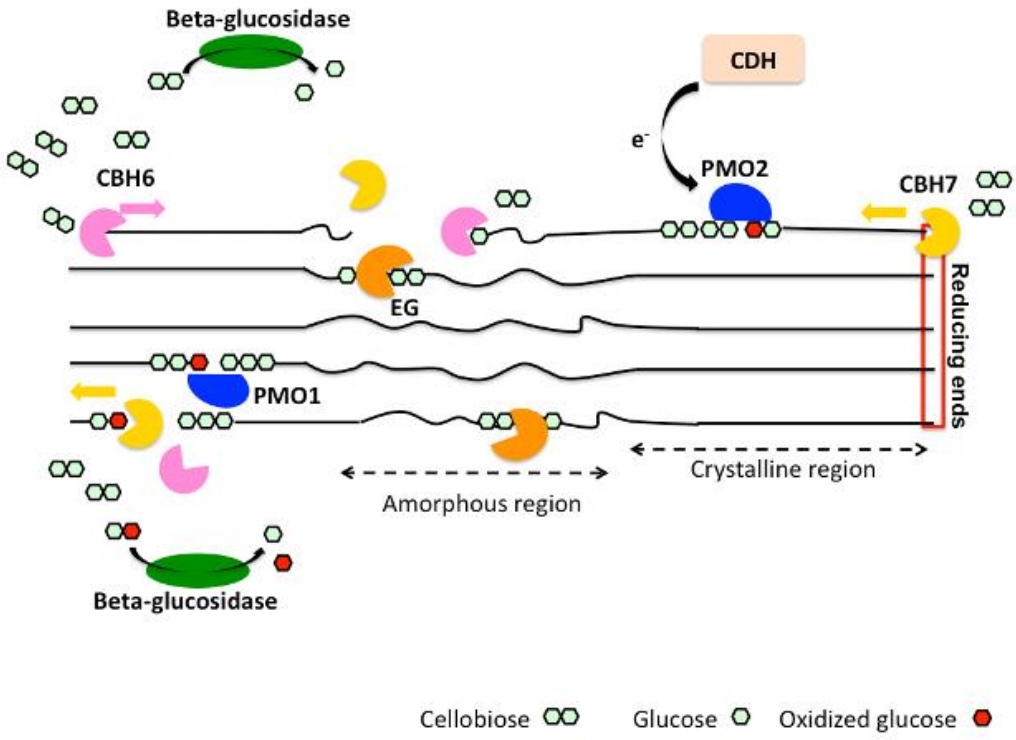

Figure 2. Enzymatic Action of cellulase cellobiohydrolases (Soure: Dimarogona et al., 2012).

Cellulosic substrates

Many types of substrates are used as a carbon source for the growth of the microorganisms: Carboxymethyl cellulose (CMC), filter paper, paranitrophenyl-β–D glucopyranoside (pNPG), cellobiose etc. (Mandels et al., 1976). Carboxymethyl cellulose (CMC) or cellulose gum is a cellulose derivative with carboxymethyl groups (-CH_2-COOH) bound to some of the hydroxyl groups of the glucopyranose monomers that make up the cellulose backbone. It is often used as its sodium salt, sodium carboxymethyl cellulose. The functional properties of CMC depend on the degree of substitution of the cellulose structure as well as the chain length of the cellulose backbone. Filter paper is a semi-permeable paper barrier placed perpendicular to flow of liquid. Filter paper comes in various porosities and grades depending on the applications it is meant for. Cellobiose is a disaccharide which consists of two glucose molecules linked by a β (1→4) bond. It can be hydrolyzed to glucose enzymatically or with acid. Cellobiose has eight free alcohol (OH) groups, one acetal linkage and one hemiacetal linkages, which give rise to strong inter- and intra-molecular hydrogen bonds. It can be obtained by enzymatic or acidic hydrolysis of cellulose and cellulose rich materials such as cotton, jute or paper.

Cellulose degrading microorganisms

The microbial degradation of cellulosic materials is a well-defined process which can lead to the generation of gases and soluble organic compounds within radioactive waste disposal sites. Cellulose is a highly recalcitrant substrate for enzymatic degradability and the capacity to completely hydrolyze the cellulose macromolecule is restricted to a relatively select but diverse group of microorganisms. In a typical cellulose degrading ecosystem, a variety of cellulolytic bacteria (e.g. *Clostridium, Bacillus* sp.) and fungi (e.g. *Penicillium, Aspergillus, Trichoderma* sp.) work in concert with related microorganisms to convert insoluble cellulosic substrates to soluble sugars, primarily glucose, which is then assimilated by the cell (Bayer et al., 1998). Due to excellent ability to produce and secrete a complete set of cellulose degrading enzymes, that makes their production viable at industrial scale, the soft rot fungus *Trichoderma reesei* has been in the focus of cellulase research for decades (Persson et al., 1991). Even though dozens of novel strains with improved characteristics have been engineered and successfully applied in industrial production since the 1980s, the most studied cellulolytic organism to date *T. reesei* RUT C30 (Velkovska et al., 1997) still preserves its leading role as the major test organism in fundamental cellulase research.

Table 1. List of cellulose degrading bacteria.

Oxygen relationship	**Genus**	**Representative species**	**References**
Aerobic	*Bacillus*	*B. polymyxa*	Ivanen et al., 2009
		B. subtilis	Li et al., 2008
		B. subtilis	Shabeb, et al., 2010
	Cellulomonas	*C. flavigena*	Perez-Avalos et al., 2008
	Paenibacillus	*Paenibacillus* sp.	Wang et al., 2008
	Acidothermus	*A. cellulolyticus*	Rignall et al., 2002
	Pseudomonas	*P. fluorescens*	Sethi et al., 2013
Anaerobic	*Clostridium*	*C. thermocellum*	Fan et al., 2009
	Spirochaeta	*S. thermophile*	Bergquist et al., 1999
	Thermotoga	*T. neapolitana*	Bergquist et al., 1999

Characterization of bacterial cellulases

Enzymes show its own pH sensitivity, temperature optima, thermal stability, substrate specificity, kinetics and effect of different chemical towards its activity. The broad range of pH tolerance and thermal stability displayed promising characteristics of cellulases for the biofuel and biobased industry. Many workers have purified and characterized cellulases from different bacteria such as *Cellulomonas* sp. YJ5 (Yin et al., 2010), *Thermomonospora* sp. (George et al., 2001), *Melanocarpus* sp. MTCC 3922 (Kaur et al., 2007), *Pseudomonas fluorescens*, *Pyrococcus horikoshi* (Kang et al., 2007), *Bacillus* sp. C1 (Sadhu et al., 2013), *Bacillus subtilis* AS3 (Deka et al., 2013), *Bacillus licheniformis* WBS1 and *Bacillus* sp. WBS3 (Acharya and Chaudhury, 2011), *Paenibacillus barcinonensis* MG7 (Asha et al., 2011), *Bacillus* sp. M9 (Bajaj et al., 2009), *B. licheniformis* (Bischoff et al., 2006), *Bacillus* sp. HSH-910 (Kim et al., 2005) and *B. sphaericus* JS1 (Singh et al., 2004). Purification of bacterial cellulase by ion-exchange chromatography such as DEAE (diethylaminoethanol) sepharose and sepharyl chromatography has been extensively used at pilot scale for large scale purification. It is also used for direct recovery of proteins and other charged molecules, as the technique is known to have high resolving power, high capacity, simple to operate, highly robust, generic and economical (Abdullah, 2004).

Biodegradation of lignocellulosic biomass

Bioconversion of cellulose into fermentable sugars is a biorefining area that has invested enormous research efforts, as it is a prerequisite for the subsequent production of bioenergy. Sugars and starch comprise the feedstock for 90% of the produced ethanol today, but the most prevalent forms of sugar in nature are cellulose and hemicellulose. Lignocellulosic biomass can be converted to ethanol by hydrolysis and downstream fermentation processing. The process is much more complicated than just fermentation of C6 sugar and is still far from being cost effective as compared to the production of bioethanol from starch or sugar crops. In hydrolysis, the cellulosic part of the biomass is converted into sugars, and fermentation converts these sugars to ethanol. Lignocellulosic biomass consists of 10–25% lignin, which contains no sugar, and therefore impossible to convert into sugars. Lignin is therefore a residue in ethanol production, and it represents a big challenge to convert it into a value-added product. Agricultural and industrial wastes are among the main causes of environmental pollution. Their conversion into useful products may reduce the intensity of the problems caused by them. These wastes include green gram husk, black gram husk, rice bran, wheat bran etc. are underutilized in India. A large quantity is left in farm lands to be decomposed by microorganisms such as bacteria and fungi (Okafor et al., 1987). Economically, the most important industrial material other than food stuffs affected by microorganisms are cellulose and wood products. Proper utilization of these wastes in the environment will eliminate pollution and convert them into useful byproducts (Milala et al., 2005).

Microorganisms have developed well adapted cellular machinery in order to take energy from plant biomass and involved in the production and secretion of carbohydrate-active enzymes by playing saprophytic life style which involves living in dead or decaying organic matter (de Souza, 2013). To initiate the production of industrially important products from cellulosic biomass, bioconversion of the cellulosic components into fermentable sugars are necessary (Kumar et al., 2008). Therefore, the enzymatic conversion of these biomasses into fermentable sugars at pilot scale has potential application in bioethanol industry. Although extensive work has been carried out to meet the future challenges of bioethanol production, there is no self-sufficient process or technology available to convert the lignocellulosic biomass for bioenegy generation. Fungi and some bacteria are mainly accountable for biodegradation of lignocellulosic substrate. Low lignin content biomass is favoured by bacterial degradation owing to low or limited release of lignin degrading enzyme such as lignin peroxidises (LiP), laccase (Lac), manganese

peroxidise (MnP), versatile peroxidise, and H_2O_2 generating enzyme such as glyoxal oxidase (GLOX) and aryl alcohol oxidase. Among fungi, *Phanerochaete chrysosporium* and *Phlebia radiata* are well known a producer of extracellular peroxidises (Lee et al., 1997), as well as *Coriolus tersicolor*, which was shown to produce the intracellular peroxidise (Lobarzewski, 1990). A white-rot basidiomycete, *Rigidoporous lignosus*, is also known to secrete two oxidative enzymes, Lac and MnP, responsible for solubilizing the lignin in a synergistic way (Galliano, 1991). *P. chrysosporium*, *Ceriporiopsis subvermispora*, *Phlebia subserialis*, and *Pleurotus ostreatus*, which are able to metabolize the lignin in a variety of lignocellulosic biomass (Keller et al., 2003).

Screening of cellulase producing bacteria

Screening for bacterial cellulase activity in microbial isolates is typically performed on plates containing crystalline cellulose or microcrystalline cellulose such as Avicel in the agar at a final concentration of 0.1-0.5% (w/v). After incubation of a suitable period, a zone of clearing surrounding the colonies will be indicated that cellulose producer (Kluepfel et al., 1988). The colonies of cellulolytic *Cytophaga* spp. did not shown any clearing zone (Schlegel et al., 1986). So the diameter of the clearing zone may not accurately reflect the true cellulase activity. For a rapid screening of cellulase producing bacteria, after the incubation of the agar medium are containing 0.5% (w/v) carboxymethyl cellulose (CMC) as sole carbon source and flooded with 1% (w/v) Congo red (Teather and Wood, 1982). After 20 min, the dye is decanted and the plates are again flooded with 5 M NaCl which is decanted after 20-30 min. Positive colonies are detected to be surrounded by a pale orange to clear zone against red background. The cellulolytic bacteria can be screened directly on such plate, but replica plating from master plate is preferred for isolation of active colonies as flooded reagent impairing isolation. Plant et al. (1988) has reported a semi-quantitative assay for cellulase activity in bacteria by using cellulose-azure into the upper two layers of agar tubes. The dye released from the substrate is determined densitometrically. Kasana et al. (2008) found that Gram's iodine for plate flooding in place of hexadecyltrimethyl ammonium bromide or Congo red, gave a more rapid and highly discernable results.

Cellulase: Application and future prospects

Cellulases from microorganism have been exploited for a broad range of industrial application such as animal food and feed, textile, detergent, paper, wine and for sustainable production of many chemicals and enzymes. Cellulases have been commercially available for more than 30 years, and these enzymes have represented a target for both academic as well as industrial research (Singh et al., 1999; Singh et al., 2007). The enzyme complex is

the most successful in finishing of cellulose-based textiles (Karmakar and Ray, 2011), can remove partially detached microfibrils from cotton or cotton blended garments and restore a smooth surface and original colour to the garments (Ibrahim et al., 2011). Application of cellulase in paper and pulp industries has increased considerably during last decade. Cellulase along with hemicellulases have also been used for biomodification of fiber properties with the aim of improving drainage and beatability in the paper mills before or after beating of pulp (Dienes et al., 2004). Enzymatic saccharification of lignocellulosic materials such as sugarcane bagasse, corncob, rice straw, *Prosopis juliflora*, *Lantana camara*, switch grass, saw dust, and forest residues by cellulases for biofuel production is perhaps the most popular application being investigated (Gupta et al., 2011). Bioconversion of lignocellulosic materials into useful and higher value products normally requires multistep processes (Kuhad et al., 2010). Technologies are currently available for all steps in the bioconversion of lignocellulosics to ethanol and other chemical products. However these technologies must be improved to produce renewable biofuel and other byproducts at prices, which can compete with more conventional production systems. Not only the recalcitrance of the substrate, but also several other factors limit cellulase efficiency during the hydrolysis process which includes end product inhibition, thermal deactivation of the native protein, nonspecific binding to lignin (Yang and Wyman, 2004) and irreversible adsorption of the enzymes to the heterogeneous substrate. Strategies for recycling and reusage of the enzymes may also be used to reduce enzymatic hydrolysis costs. The recovery of enzymes is largely influenced by adsorption of the enzymes onto the substrate, especially to lignin and enzyme inactivation. There are several reports where the nonspecific and irreversible adsorption of cellulase to lignin has been observed (Yang and Wyman, 2004).

CHAPTER 3
MATERIALS AND METHODS

CMCase of cellulose degrading bacteria *Bacillus licheniformis* NA8 was partially purified using ammonium suphate precipitation and futher purified by coloumn chromatography. The purified endoglucanase was optimized for different parameters such as pH, temperature, thermal stability and metal ions effect. Kinetics and thermostability of purified enzyme was also done to check the efficiency and industrial application of enzyme.

Microorganism: *Bacillus licheniformis* NA8 (GenBank Accession number: KF840405)

Already characterized bacterial strain was isolated previously from compost.

Production and purification of cellulase produced by *Bacillus licheniformis* NA8

The bacterial isolate NA8 was grown in 100 mL enriched media (Appendix I) in 250 mL flask was inoculated with 5% overnight grown culture (O.D. 0.6 at 600 nm) and incubated at 37°C under shaking condition (120 rpm) for 24 h. After 24 h of incubation, cell free supernatant collected after centrifugation (7,000 x g, 20 min) was investigated for CMCase activity.

CMCase activity in cell free supernatant of *Bacillus licheniformis* NA8 (Miller, 1959)

CMCase activity was determined by estimating the amount of reducing sugar using DNS (3, 5-Dinitrosalicyclic acid) method as per Miller, (1959).

Procedure:

The overnight grown culture of *B. licheniformis* NA8 (2%) was inoculated in flask containing Enriched media (Appendix II) supplemented with 0.5% CMC and incubated at 37°C. Two ml of culture from flask having *B. licheniformis* NA8 was taken and centrifuged in cooling centrifuge at 10,000 rpm for 10 min. 500 µL of CMC was dissolved in 50 mM potassium phosphate buffer (pH 7) and incubated in water bath at 50°C for 10 min. After incubation 500 µL of sample supernatant was added and again incubated in water bath at 50°C for 30 min. After incubation 3 mL of DNS reagent (Appendix III) was added and placed in boiling water bath for 10 min. Sample was cooled at Room Temperature and OD was taken at 540 nm

Determination of protein Concentration by Folin-Lowry method:

To study the protein concentrations of bacterial isolate, 1% (v/v) overnight grown bacterial inoculum (0.6 OD at 600 nm) was added to 100 mL media (Appendix III) in 250 mL flask for and incubated at 37ºC under shaking condition (120 rpm) for 36 h. The aliquots were taken at

an interval of 4 h, centrifuged (7,000 rpm, 20 min) and culture supernatants were used for protein estimation by (Lowry et al., 1951).

Procedure:

Bovine serum albumin (BSA) fraction V (HiMedia labs, Mumbai, India) standard solutions were prepared in the range of concentrations 0.2, 0.4, 0.6, 0.8 and 1 mg/mL. 200 µL of BSA was pipetted out for standard curve. 5 mL of Reagent C (Appendix III) was added and mixed well with taken BSA sample and incubated for 10 min. 1N Phenol reagent (0.5 ml) was added and mixed at once and incubated for 30 min and then O.D was taken at 750 nm.

Ammonium sulphate precipitation

A loopful of *B. licheniformis* NA8 was inoculated in enriched media (Appendix I) and was incubated for 24 h. Cells were harvested and centrifuged (10,000 rpm for 10 min at 4º C) and its supernatant was then taken and ammonium sulphate was poured slowly into the supernatant over a period of 4-5 h allowing the salt to dissolve slowly. Supernatant was continually stirred at 4°C during dissolution of ammonium sulphate. Precipitates were recovered by centrifugation (10,000 rpm for 10 min at 4º C), and dissolved in 50 mM potassium phosphate buffer (pH 7.0). Further, precipitated fraction of protein was dialyzed overnight at 4°C against the same buffer (1:50 volume). The CMCase activity and protein concentration was measured and specific activity calculated.

Ion-exchange chromatography

The dialyzed ammonium sulphate fractions were applied to a column (16 cm length, 2 cm diameter) of DEAE-Sepharose^{TM} Fast Flow beads (GE, Healthcare). The column was equilibrated with 150 mL 1M NaCl buffer (pH 8.0) and washed with 150 mL of 50 mM potassium phosphate buffer (pH 7) (Appendix II). The proteins were eluted with linear gradient of 100–600 mM NaCl gradient. Eluted fractions 14 each of 2 mL were collected and absorbance was measured at 280 nm. Protein peaks were selected and CMCase assays were performed to measure enzyme activity and further characterization of purified proteins was done.

SDS-PAGE

Sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS–PAGE) was performed in a 12.5% (w/v) polyacrylamide gel (Laemmli, 1970). The Molecular weight of purified endoglucanase was determined on the basis of proteins relative mobility using standard protein marker (Fermentas 14.4–116 kDa). The purified enzyme samples were boiled at 100°C for 5 min and subjected to SDS-PAGE stained with Coomassie Brilliant Blue R-250.

Materials:

RUNNING GEL (12.5%)

1. Distilled water – 3.13 mL
2. 30% Acrylamide & Bisacrylamide – 4.17 mL
3. 4X Separating gel buffer (pH 8.8) – 2.5 mL
4. 10% SDS - 0.1 mL
5. 10% APS (100mg/ml) – 0.1 mL
6. TEMED – 0.01 mL

STACKING GEL (4%)

1. Distilled water – 3.6 mL
2. 30% Acrylamide & Bisacrylamide - 0.66 mL
3. 1M Tris HCl buffer (pH 6.8) – 0.63 mL
4. 10% SDS – 0.05 mL
5. 10% APS (100 mg/mL) – 0.05 mL
6. TEMED - 0.005 mL

Unstained Protein Molecular weight Marker (Fermentas) - 14.4 – 116 kDa

Molecular Weight (kDa)	Protein
116.0	β – galactosidase
66.2	Bovine serum albumin
45	Ovalbumin
35	Lactate dehydrogenase
25	REase BSp 98 I
18.4	β- lactoglobulin
14.4	Lysozyme

Procedure:

Ingredients of running gel were mixed and poured quickly into gel casting stand and some space was leaved for the stacking gel approximately 2 cm below the bottom of the comb. Bubbles were removed by addition of a layer of saturated butanol poured on the top of the gel with water and left for 30 min for the gel to polymerize completely. Butanol layer was discarded and washed with distilled water 3 times followed by pouring ingredients of stacking gel were on the top of the running gel. Comb was inserted in gel after pouring stacking gel and allowed another 1/2 - 1 h for complete polymerization. Gel was clamped in the SDS-PAGE apparatus and buffer chambers were filled with gel running buffer. Purified

protein was taken in 4:1 with the sample buffer and put the sample mixture in boiling water bath for 3 min at 95^0C and then loaded in separate wells in addition to molecular weight marker. Current was applied (80 volt, 25 Ma) and was stopped when sample reaches at 0.5 cm above the end of gel. Separated proteins were visualized using (0.15% w/v) Coomassie Brilliant Blue R-250.

Zymogram analysis

Zymogram with 2% CMC was performed by denaturing enzyme samples with 1% SDS in Tris HCl buffer (0.05 M, pH 6.8) as per protocol of Schwarz et al., (1987). Gel was prepared by including 0.1% CMC before polymerization in the 12.5% SDS-PAGE resolving gel mixture. Gel mixtures were heated at 30°C and CMC was added slowly to prevent aggregation. APS and TEMED were added when CMC was dissolved and gel was allowed to polymerize at room temperature. Commercial grade *Aspergillus niger* cellulase (100 µg/mL) was used as a positive control. Partially purified protein was taken in 4:1 with the sample buffer and loaded in the wells and current supply (80 volt, 25 Ma). Current supply was stopped when sample reaches at 0.5 cm from top of the gel. After electrophoresis, gel was washed five times at room temperature; each wash for 30 min with 50 mL of wash buffer (50 mM potassium phosphate buffer). Remaining CMC in the gel was stained with 0.1% Congo red solution and incubated for 10 min at room temperature. Gel was destained by washing in 50 mL of 1 M NaCl until cellulase bands became visible as clear zones where CMC was degraded due to CMCase activity. After destaining for 20 min, 100 µL of glacial acetic acid was added to the gel for improved band visualization (Waeonukul et al., 2007).

Biochemical characterization of purified CMCase

Biochemical characterization of CMCase in cell free supernatant of *Bacillus licheniformis* NA8 was determined by measuring the amount of reducing sugar released in the medium and thus respective concentration of enzyme in U/mL was determined.

Determination of optimum pH for CMCase activity

The optimum pH of the crude enzyme was determined by incubating the mixture of equal volume of crude enzyme and 2% (w/v) CMC in the presence of appropriate buffers for different pH like 50 mM sodium citrate (pH 4.0, 5.0), 50 mM potassium phosphate (pH 6.0, 7.0) and 50 mM glycine-NaOH buffer (pH 8.0, 9.0). The reaction mixture of various pH buffers (Appendex II) were incubated for 30 min at 50°C. The enzyme assays were carried out by determining the amount of reducing sugars released as described above.

Determination of optimum temperature and thermal stability of CMCase

The optimum temperature for the activity of crude cellulase was determined by assaying the enzyme at various temperatures ranging between 30°C to 80°C in reaction mixture containing 2% CMC dissolved in 50 mM potassium phosphate buffer (pH 7.0) with incubation time of 30 min and the amount of reducing sugar was determined as described above. Thermal stability of cellulase was determined by pre-incubating the enzyme at various temperatures ranging from 30°C-80°C for 30 min. After incubating the enzyme for 30 min, 2% (w/v) CMC dissolved in 50 mM potassium phosphate buffer (pH 7.0) was added followed by incubation time of 30 min and the amount of reducing sugar was determined as described above.

Effect of temperature on CMCase at different time intervals

The 50 mL of enriched medium (Annexure II) was inoculated with *B. licheniformis* NA8 (5% v/v) and incubated at 37°C under shaking condition (120 rpm).

Procedure:

The 24 h grown culture was taken and centrifuged at 12000 rpm for 10 min at 4°C to separate out supernatant out of cell debris. Then the supernatant was taken in eppendorfs and was incubated in water bath at different temperature 40, 50, 60, 70 and 80ºC. At different time intervals the incubated supernatant was collected and enzyme activity was calculated as per discussed earlier.

Effect of Metal ions and other additives on CMCase activity

This experiment was carried out to test the effect of metal ions including Ag^{+} ($Ag(NO)_3$), Ca^{2+}($CaCl_2$), Hg^{2+}($HgCl_2$), Pb^{2+}($PbCl_2$), NH^{4+}($(NH_4)_2SO_4$), Cu^{2+}($CuCl_2$), Cd^{2+}($CdCl_2$), K^{+}(KCl), Na^{+}(NaCl), Mg^{2+} ($MgCl_2$), Mn^{2+}($MnCl_2$), Fe^{2+}($FeCl_2$), Co^{2+}($CoCl_2$), Zn^{+}($ZnCl_2$) and additives like EDTA, SDS at 5 mM concentration in potassium phosphate buffer (50 mM). Samples were assayed in duplicate and the control (without any additive) was taken as having 100% activity.

Substrate specificity studies of CMCase

The hydrolytic ability of cellulase produced from *B. licheniformis* NA8 was checked against 2% (w/v) CMC, avicel, filter paper, xylan, starch and β-Glucan was checked in 50 mM phosphate buffer (pH 7.0) was determined to evaluate the substrate specificity of purified.

Kinetic parameters of *B. licheniformis* NA8 CMCase

The Michaelis-Menten constant (K_m) of purified extracellular CMCase was determined by varying the concentration of CMC. The kinetic parameters were determined from Lineweaver-Burk double reciprocal plot (Lineweaver and Burk, 1934). The initial velocity

measured by quantitatively measuring the amount of one of the product at various time intervals (Robyt and White, 1990).

Materials: CMC stock of 0.1, 0.24, 0.38, 0.52, 0.65, 0.82, 0.95, 1.08 mg/mL in 50 mM potassium phosphate buffer.

Procedure:

The purified endoglucanase eluted out by column was taken in test tube (50 µL) and added to 1 mL CMC of different concentrations i.e 0.1, 0.24, 0.38, 0.52, 0.65, 0.82, 0.95, 1.08 mg/mL and mixture was further dissolved in 50 mM potassium phosphate buffer (950 µL). The above mixture was incubated at 50°C for different time period i.e 8, 10, 13, 16 and 20 min. Then the CMCase activity was calculated as per discussed before. Then graphs of different concentrations were drawn to calculate velocities and then Lineweaver Burk plot (Lineweaver and Burk, 1934) was drawn to calculate V_{max} and k_m.

CHAPTER 4
RESULT AND DISCUSSION

B. licheniformis NA8 was grown harvested and cell free supernatant was checked for its CMCase activity. The precipitation of CMCase from cell–free supernatant was carried out by ammonium sulphate fractionation method followed by ion exchange chromatography using DEAE–Sepharose. Molecular weight of cellulose was determined by SDS-PAGE and confirmed by Zymography. Thermal stability of *B. licheniformis* NA8 was also determined at various temperature ranges. The effect of various metal ions including Ag^{+}, Ca^{2+}, Hg^{2+}, Pb^{2+}, NH^{4+}, Cu^{2+}, Cd^{2+}, K^{+}, Na^{+} etc. and additives like EDTA, SDS at 5 mM concentration was checked. Further Kinetics and thermodynamic studies of purified CMCase was carried out.

CMCase activity in cell free supernatant of *B. licheniformis* NA8 (Miller, 1959).

CMCase activity was determined by estimating the amount of reducing sugar using DNS (3,5-dnitrosalicyclic acid) method as per Miller, (1959) using glucose as the standard. Specific CMCase activity of *B. licheniformis* was 1.3 U/mg. The specific enzyme activity reported by thermophilic Cellulase from *Paenibacillus barcinonensis* (Asha et al., 2012) was reported as 1.44 U/mg and 1.77 U/mg of CMCase produced by *Bacillus pumilus* EB3 (Ariffin et al., 2006).

Ammonium sulphate preparation

Ammonium sulphate precipitation of cell free supernatant of 24 h old grown culture was done upto 80% saturation and CMCase assay of partially purified protein was studied by DNS method as described earlier in the presence of 2% CMC dissolved in 50 mM potassium buffer (pH 7.0) at 50^{0}C and was found to be 0.63 U/mL which was 1.52 fold higher than CMCase activity in crude protein. The protein concentration was found to be 0.318 mg/mL (Table 2) in the sample. The thermophilic cellulase from *Penibacillus* sp. strain MG7 was purified through ammonium sulphate fractionation, gel filtration chromatography, and HPLC (Asha et al., 2012).

Coloumn chromatography

The ammonium sulphate precipitated enzyme was dialysed overnight and further purified by DEAE–Sepharose column chromatography. In total 65 fractions were collected, fraction number 17-43 (each 2 mL fraction size) obtained after ion exchange chromatography showed high protein concentration (OD 280 nm) (Figure 3) and were selected for

determination of CMCase activity. Among these, fraction numbers 19–21 showed higher CMCase activities, hence were pooled and selected for further work. The purified enzyme showed specific activity of 6.8 U/mg with 4.76 fold increase in activity (Table 2). The most common method for cellulase purification is by Ion-exchange chromatography. Ion-exchangers have been the most widely used chromatographic technique for the direct recovery of proteins and other charged molecules as the technique is known to have high resolving power, high capacity, simple to operate, highly robust, generic and economical (Abdullah, 2004). Studies and researches on cellulase purification have been widely studied, and most of the work adopted ion-exchange chromatography as the method of purification (Bakare et al., 2005).

Table 2. Summary of purification of CMCase from *B. licheniformis* NA8.

Procedure	**Total activity (U)**	**Volume (mL)**	**Total protein (mg/mL)**	**Specific activity (U/mg)**	**Purification (fold)**	**Yield (%)**
Crude enzyme	112.5	250	0.346	1.3	1	100
Ammonium sulphate precipitation	63	100	0.318	1.98	1.52	56
Ion-Exchange chromatography	52.6	26	0.298	6.8	4.76	83.49

Molecular weight determination and zymogram analysis

SDS-PAGE of partially purified protein showed different bands (Figure 4, Lane L1) which was subjected to Zymography, confirming the protein band of CMCase. In zymogram, a clear band appeared, which shows that cellulase enzyme use the Carboxymethyl cellulose as substrate which was incorporated in the gel prior polymerization and this band was compared with the bands of standard protein ladder and SDS-PAGE which shows that the molecular weight of this cellulase enzyme is 24 kDa (Figure 4). SDS-PAGE was conducted on the crude, Ammonium sulphate precipitate and chromatograpy fractions, followed by zymogram analysis for CMCase activity. The SDS gel (Figure 4) had been divided into 5 different lanes containing protein molecular marker (14.4-116 kDa), cell free supernatant protein, ammonium sulphate precipitated protein, purified CMCase fraction by DEAE-Sepharose and zymogram band. Zymogram analysis of purified CMCase confirmed the

single CMCase activity band of approximate molecular weight of 24 kDa (Figure 4, Lane 4) in CMC.

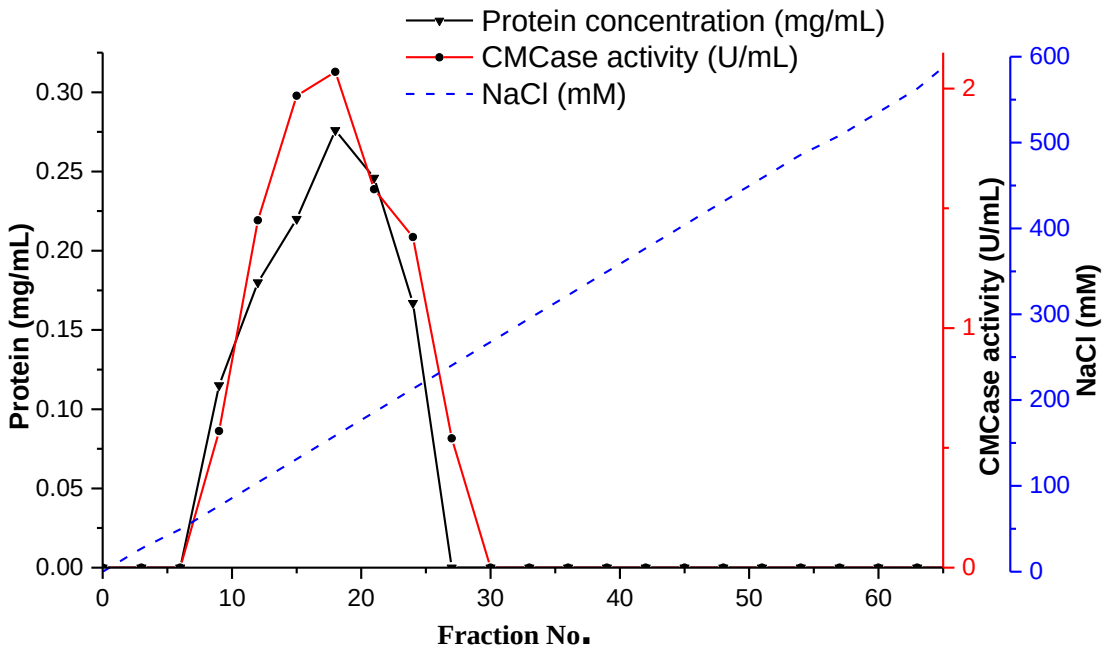

Figure 3. Chromatogram of cellulase from *Bacillus licheniformis* NA8 on DEAE–Sepharose chromatography equilibrated with linear gradient of 0 – 600 mM NaCl.

This result was in accordance with the molecular mass of cellulase from other reported *Bacillus* spp. though there is a wide range of cellulase molecular mass (Table 4). Molecular mass of purified endoglucanase 24 kDa of present study was found to be close to the molecular weight of cellulase from *B. subtilis* GN156 (25 kDa) (Apiraksakorn et al., 2008), *B. licheniformis* (37 kDa) (Bischoff et al., 2006) and *B. subtilis* AS3 (30 kDa) (Deka et al., 2013).

Effect of temperature on CMCase activity and stability

The optimum temperature study for CMCase activity was carried out and found to be 50°C (2.01 ± 0.09a U/mL) (Figure 5, Table 3), and temperature stability range in between 40-50°C. Thermostability studies of purified enzyme revealed residual activity of 70% was retained in temperature range (30-65°C) for 10 min of incubation time. Optimum temperature for CMCase produced by *B. amyloliquefaciens* SS35 is more than that produced by *B. thuringiensis* (40°C) (Lin et al., 2012), and was found synchronous to *Bacillus* sp. (50°C) (Sadhu et al., 2013), *Bacillus* sp. (50°C) (Vijayaraghavan and Vincent,

2012), *B. amyloliquefaciens* DL–3 (50°C) (Lee et al., 2008) and lower than that cellulase produced by *B. subtilis* GN156 (60°C) (Apiraksakorn et al., 2008). The 50 % of residual activity was observed at 69°C at pH 7.

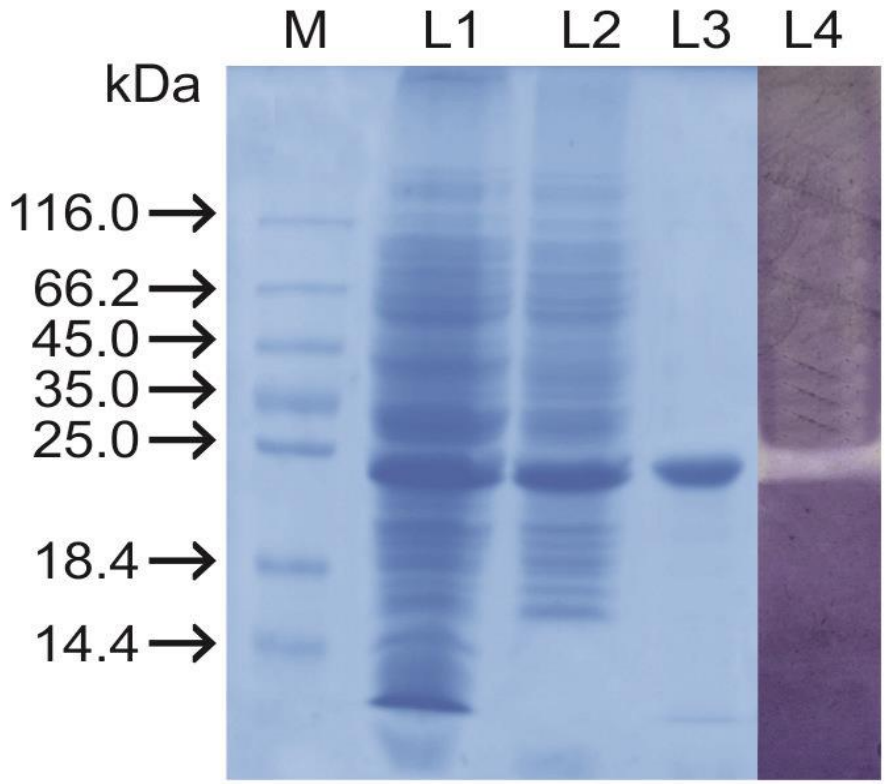

Figure 4. SDS-PAGE of purified cellulase from *B. licheniformis* NA8

Lanes:

M	Molecular Marker (14.4-116 kDa)
L1	Cell free supernatant protein
L2	Ammonium sulphate precipitated protein
L3	Purified CMCase fraction from DEAE-Sepharose
L4	Zymogram of purified CMCase

Table 3. CMCase activity at different temperature

Temperature (°C)	CMCase activity (U/mL)
30	1.41 ± 0.08b
40	1.87 ± 0.11a
50	2.01 ± 0.09a
60	1.60 ± 0.04b
70	0.94 ± 0.03c
80	0.62 ± 0.03d

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

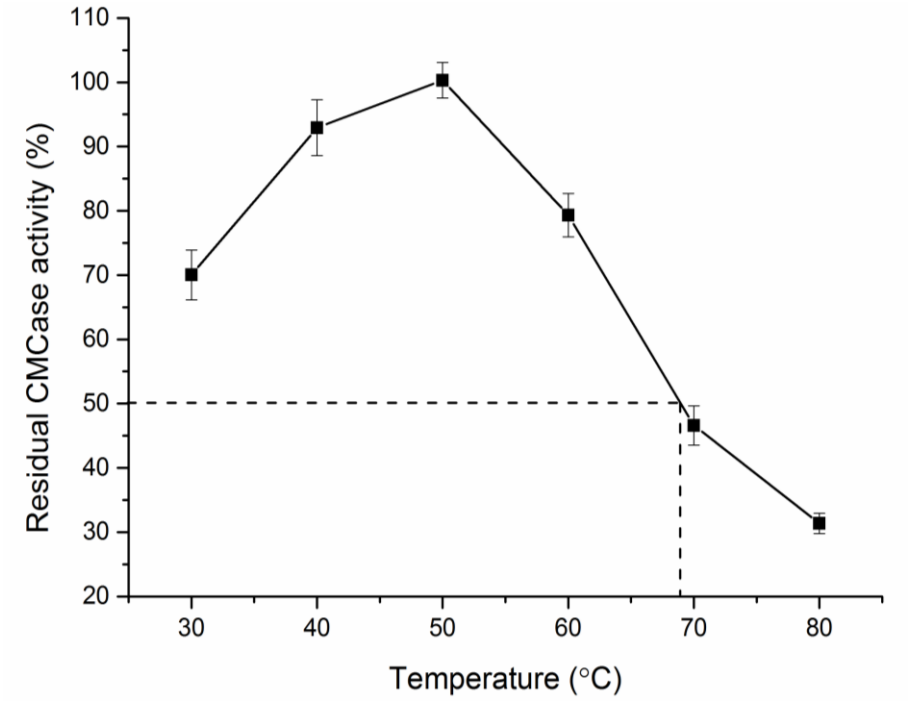

Figure 5. Effect of temperature on CMCase activity

The vertical bars designate the standard errors for the mean of three replicative tests.

Effect of pH on CMCase activity and stability

Most of the *Bacillus* strains used commercially for the production of cellulase have an optimum pH between 6.0 and 9.0 for growth and enzyme production. The optimum pH for the *Bacillus licheniformis* NA8 was found to be 7.0 (1.98 ± 0.12a) (Figure 6, Table 4), which was in accordance with the previous study by (Ray et al., 2007), where pH 5 - 7.0 was more suitable for cellulose produced by *B. subtilis* and *B. circulans*. Yin et al., (2010) also reported cellulase from *B. subtilis* YJ1, which was active at an optimum pH of 6.0 and

stable in the pH range of 6.5–7.5. Cellulases from *B. pumilus* EB3 had an optimum pH of 7.0 and were stable at pH range 6.0 -9.0. This is in contrast with most of the fungal cellulase which showed an optimum pH of 4.0-6.0 (Ariffin et al., 2006) and pH 7 of *Bacillus* sp. C1 (Sadhu et al., 2013). The present study revealed that CMCase of *B. licheniformis* NA8 retained 50% of residual activity when incubated for 10 min at pH range (5-7).

Table 4. CMCase activity at different pH

pH	CMCase activity (U/mL)
4	0.57 ± 0.02d
5	1.52 ± 0.05b
6	1.98 ± .12a
7	1.83 ± 0.14a
8	1.26 ± 07c
9	0.66 ± 024d

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

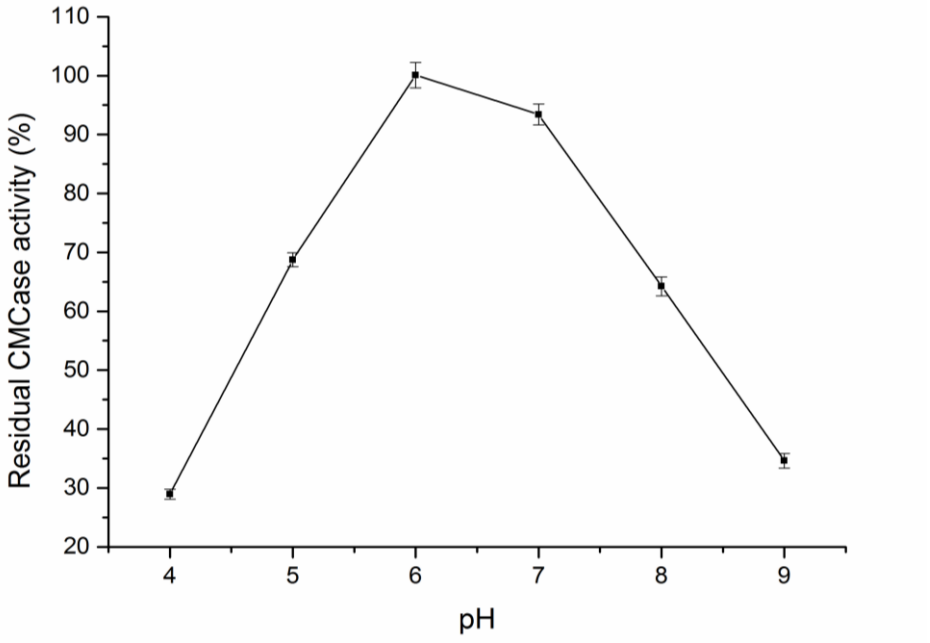

Figure 6. Effect of pH on CMCase activity

The vertical bars designate the standard errors for the mean of three replicative tests.

Thermodynamics of CMCase stability

The purified CMCase of *B. licheniformis* NA8 was incubated in 50 mM potassium-phosphate buffer (pH 7) (Annexure II) at various temperatures from 40-90°C and pseudo-first-order plots were used for determining the extent of thermal inactivation (Figure 7). The enzyme was stable in range of 40-50°C exhibited maximum stability at 50°C. At higher temperatures half-life decreased sharply and remained 25 min at 80°C. The Tm corresponds to the temperature at which the enzyme activity drops down to the 50% of the initial activity. Thermostability of an enzyme molecule is defined as its ability to resist thermal inactivation in the absence of substrate, while the ability of an enzyme to work at elevated temperatures in the presence of substrate is called thermophilicity (Georis et al., 2000).

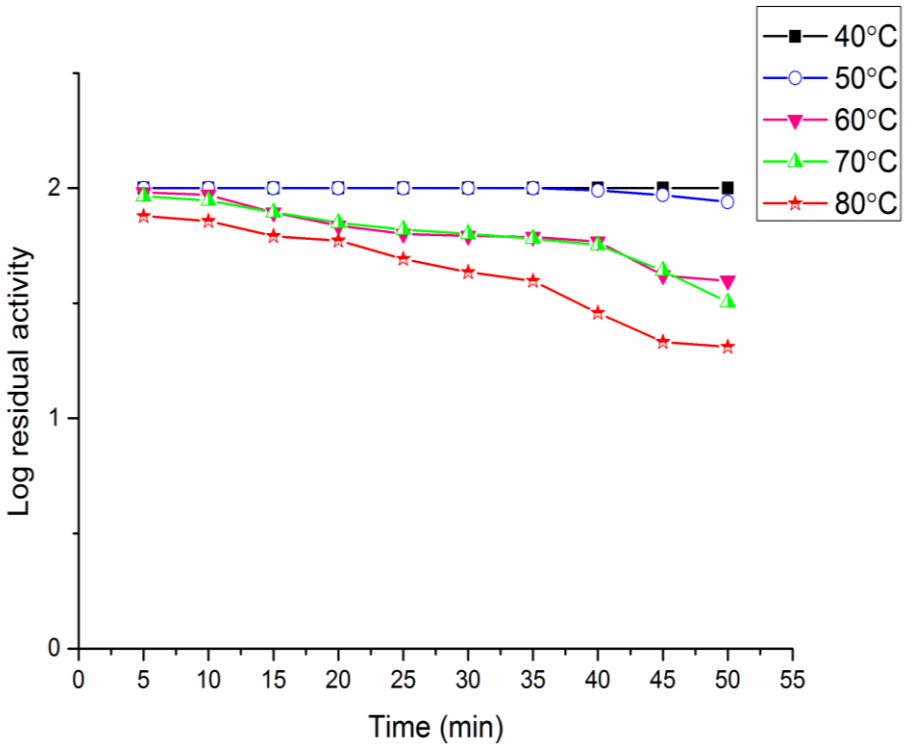

Figure 7. A Pseudo-first-order plots for irreversible thermal denaturation of *B. licheniformis* NA8 CMCase.

Effect of metal ions/additives and chemical denaturants on CMCase stability

The effect of various metal ions was studies and most of metal ions did not show considerable enhancement of CMCase activity except manganese and cobalt ions with percentage residual activities 132.88 ± 5.88 and 115.69 ± 6.26 (Table 6), result was synchronous with 184.0 and 150.0 of activity of both ions respectively (Yin et al., 2010). Hg^{2+} and Fe^{2+} strongly inhibited the CMCase activity with residual activities of 42.93 ±

2.37 and 51.67 ± 2.32 respectively. These results matched fairly well with some already studied characteristics of CMCase from *Bacillus* spp. such as *Bacillus* sp. VG1 (Singh et al., 2004) and *B. flexus* NT (Trivedi et al., 2011), same was observed by Asha et al. 2012, Hg^{2+} (1 mM) completely inhibited CMCase activity in case of cloned *Escherichia coli* with gene of a thermophilic *Bacillus* sp. PDV endo-l,4-glucanase gene (Sharma et al., 1987).This phenomenon further confirmed that the active site of the purified cellulase contained SH group. These results are almost similar to that from *Catharanthus roseus* (Smriti and Sanwal, 1999). According to the studies by Saha, (2004) and Murashima et al., (2002), K^{+}, Na^{+}, NH^{4+} did not affect the cellulase from *Rhizopus oryzae* while Co^{2+} and Mn^{2+} activated that from *Mucor circinelloides* (Saha, 2004) and *Chalara paradoxa* (Lucas et al., 2002), respectively. It was observed that Mn^{2+} enhanced the relative enzyme activity (124%) when compared with the control (100%). Ca^{2+}, Cu^{2+}, Hg^{2+}, Mg^{2+}, Na^{2+} and Zn^{2+} inhibited the enzyme activity and the relative activity was 88%, 47%, 13%, 73%, 56%, and 81%, respectively. One unifying finding of present work was that most of additives (Table 5) inhibited enzyme activity to a certain level only. This indicated robust nature of the enzyme desirable for industrial application. EDTA inhibits the CMCase activity, which was also reported by Lee et al., (2007) at 5mM**.**

Table 5. Effect of different substrates on CMCase activity

Substrate	CMCase activity (U/mL)
CMC	2.01 ± 0.03
Avicel	1.54 ± 0.05
Filter paper	1.44 ± 0.04
β-Glucan	1.06 ± 0.06
Xylan	0.77 ± 0.03
Starch	0.32 ± 0.035

Values are mean ± SD (n =3), bearing different letters in the same column are significant at P<0.05.

Substrate specificity studies of CMCase

The purified enzyme showed highest activity against CMC i.e 2.01 ± 0.03 U/mL (Figure 8, Table 5). Result concluded that endoglucanase of *B. licheniformis* was having highest substarte specificity towards CMC following avicel (1.54 ± 0.05 U/mL), filterpaper (1.43 ± 0.05 U/mL), β-Glucan (1.06 ± 0.06 U/mL), Xylan (0.76 ± 0.03 U/mL) and minimum was for starch (0.32 ± 0.03 U/mL). Most of the endoglucanases reported from *Bacillus* degrade

only CMC. Few *Paenibacillus* spp. showed endoglucanase activity with a carbohydrate binding domain, which can degrade CMC as well as crystalline cellulose [Ogawa et al., 2007]. Other endoglucanases with avicelase activity have been reported in *B. circulans* and *Bacillus*. sp. D04 (Kim, 1995).

Table 6. Effect of various modulators on CMCase activity.

Metal ions/Additives	Residual activity (%) (5mM)
Control	100
Ag^{+}	071.52 ± 3.62f
Ca^{2+}	088.59 ± 4.43de
Hg^{2+}	042.93 ± 2.37g
Pb^{2+}	051.67 ± 2.32g
NH_4^{+}	106.92 ± 4.95bc
Cu^{2+}	111.25 ± 6.75bc
Cd^{2+}	049.23 ± 3.09g
K^{+}	100.49 ± 5.32cd
Na^{+}	105.35 ± 6.38bc
Mg^{2+}	108.27 ± 6.57bc
Mn^{2+}	132.88 ± 5.88a
Fe^{2+}	087.53 ± 5.47de
Co^{2+}	115.69 ± 6.26b
Zn^{+}	077.60 ± 6.05ef
EDTA	079.02 ± 4.31ef
SDS	078.45 ± 2.18ef

Values are mean ± SD (n =3), bearing different letters in the same column are significant at $P<0.05$.

Enzyme kinetics of purified CMCase

Michelis-Menton constant (K_m) and maximum velocity of reaction (V_{max}) of purified extracellular cellulase was determined by Lineweaver Burk double reciprocal plot at varying substrate (CMC) concentration and was found to be 0.12 mg/mL and 41.66 µmoL/min/mL respectively (Figure 9). The K_m value denotes the amount of substrate needed to achieve half the maximal initial reaction velocity (Tong et al., 1980) and is a measure of the apparent affinity of an enzyme for its substrate. It was observed that activity of endoglucanase from *B. licheniformis* NA8 was greatly influenced by the substrate concentration. The K_m value of cellulase was found 3.6 mg/mL in *Pseudomonas fluorescens* (Bakare et al., 2005), as 4.97 mg/mL in *Actinobacteria anitratus* and 7.90 mg/mL in *Branhamella* sp. (Ekperigin 2007). It is difficult to explain difference of K_m

values of insoluble substrate like cellulose, however, it may be due to the isolates are of different source. The present kinetic study of CMCase revealed that enzyme have good substrate specificity and efficiency to carry out forward reaction, the K_m for different CMCase producing bacterial species have been compiled in Table 7.

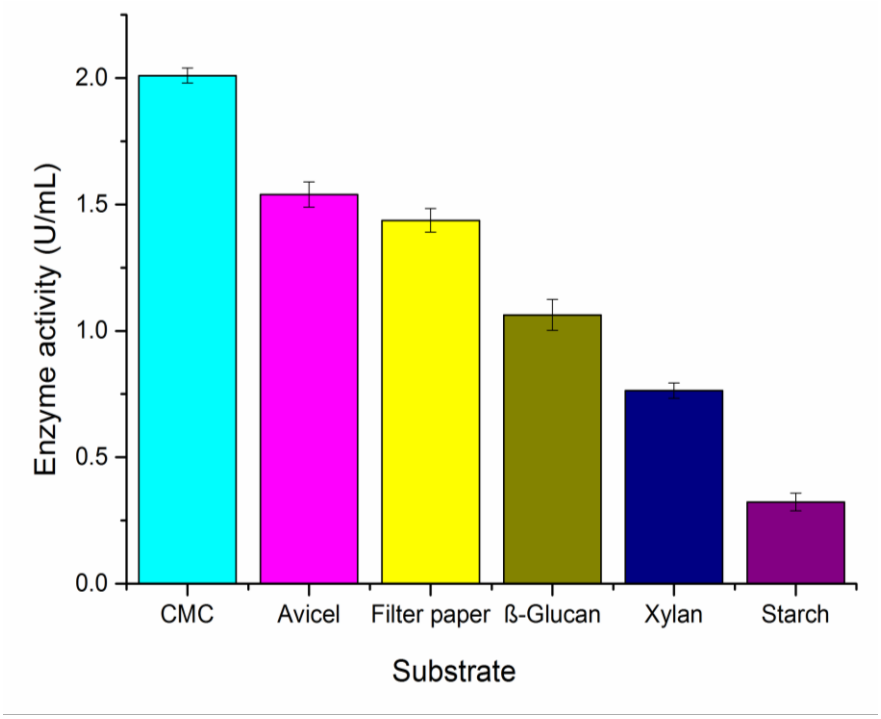

Figure 8. Substarte specificity of purified enzyme produced by of *Bacillus licheniformis* NA8 with different substrates

The vertical bars designate the standard errors for the mean of three replicative tests.

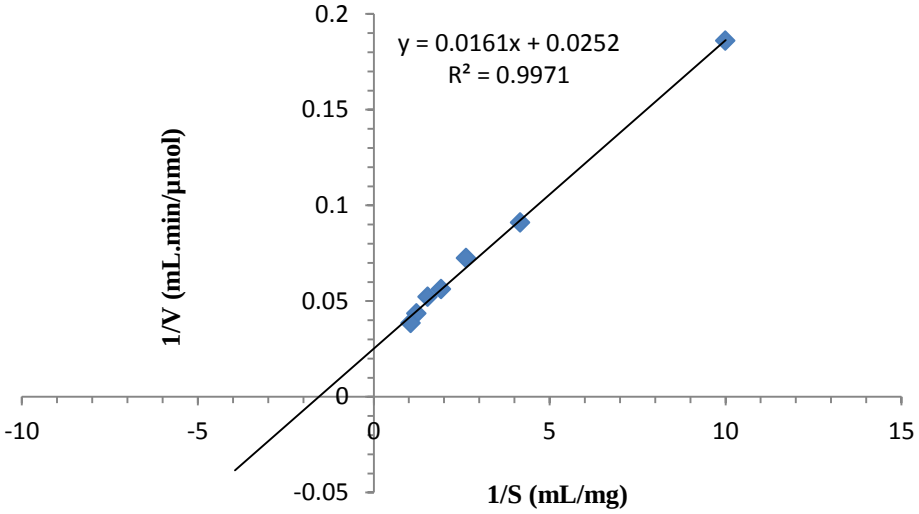

Figure 9. Lineweaver-Burk double reciprocal plot for the determination of Michaelis–Menten constants purified *Bacillus licheniformis* CMCase.

Table 7. Molecular weight, K_m *and* V_{max} of endoglucanase produced by bacterial species.

Bacteria	**Molecular mass (kDa)**	**K_m (mg/mL)**	**V_{max} (µmoles/ mg/min)**	**Reference**
B. licheniformis NA8	24	0.11	2.02	Present study
B. amyloliquefaciens DL-3	54	ND	ND	Lee et al., 2008
Bacillus sp. M–9	54	ND	ND	Bajaj et al., 2009
B. licheniformis	37	ND	ND	Bischoff et al., 2006
Bacillus sp. PDV	33	0.588	ND	Sharma et al., 1990
Bacillus sp. CH43	40	1.5	0.00093	Mawadza et al., 2000
Bacillus sp. HR68	40	1.7	0.0017	Mawadza et al., 2000
B. subtilis AS3	30	0.13	3.38	Deka et al., 2013
B. subtilis GN156	25	1.53	0.0085*	Apiraksakorn et al., 2008
Bacillus sp.	97	0.25	20#	Sadhu et al., 2013
Bacillus sp. DUSELR13	ND	3.11	0.56*	Rastogi, et al., 2010
B. flexus NT	97	6.18	370.17	Trivedi et al., 2011

µmoles/mL/min
* U/mL

SUMMARY

1. A potential cellulase producing *Bacillus licheniformis* NA8 was checked for its CMCase activity in cell free supernatant which was optimum at pH 6.0 and temperature of 50°C.
2. Cellulase produced by *B. licheniformis* NA8 was thermally stable up to 50°C and activity decreased gradually beyond 50°C and negligible at 80°C.
3. Cell free supernatant was subjected to precipitation using ammonium sulphate upto 80% saturation followed by dialysis and column chromatography. 4.76 fold increase in CMCase activity of purified enzyme was observed with respect to crude enzyme in cell free supernatant.
4. Crude, partially purified and purified enzyme was separated on SDS-PAGE and different bands were seen, purified protein was subjected to Zymography for affinity staining. A clear band of purified CMCase of 24 kDa was observed.
5. Enhancement of CMCase activity was observed in presence of manganese and cobalt ions, whereas Hg^{2+} and Fe^{2+} strongly inhibited the CMCase activity. One unifying finding of present work was that most of additives ()inhibited enzyme activity to a certain level only, indicating robust nature of the enzyme desirable for industrial application.
6. Endoglucanase of *B. licheniformis* NA 8 was having highest substrate specificity towards CMC (2.01 ± 0.03 U/mL) followed by avicel, filterpaper, β-Glucan, Xylan and minimum for starch.
7. Kinetic studies of purified enzyme was done to determine Michelis-Menton constant (K_m) and maximum velocity (V_{max}) of the reaction, which was found to be 0.12 mg/mL and 41.66 µmoL/min/mL respectively. Low K_m and comparatively high V_{max} justify for good substrate affinity of the purified enzyme.

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Appendix I

(A) ENRICHED MEDIA

Ingredients	Quantity (g/L)
Peptone	5.0
Yeast extract	5.0
K_2HPO_4	1.0
$MgSO_4.5H_2O$	0.25
$FeSO_4.7H_2O$	0.25
CMC	5.0

(B) NUTRIENT BROTH

Ingredients	Quantity (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5

(C) NUTRIENT AGAR

Ingredients	Quantity (g/L)
Peptic digest of animal tissue	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Agar	15

Appendix II

(A) BUFFERS

1. (50 mM) Sodium citrate buffer (pH 4.0, 5.0 and 6.0)

Stock solutions: A: 0.1 M citric acid; B: 0.1 M sodium citrate.

Use x mL A+ y mL B and dilute to 100 mL with 50 mL distilled water.

A (mL)	B (mL)	pH
33.0	17.0	4
20.5	29.5	5
9.5	41.5	6

2. (50 m M) Potassium phosphate buffer (pH 7.0 and 6.0).

Stock solutions: A: 1M K_2HPO_4 ; B: 1 M KH_2PO_4

Use x mL A+ y mL B and dilute to 1000 mL with 995 mL distilled water.

A (mL)	B (mL)	pH
3.075	1.925	7
0.66	4.34	6

3. Glycine-NaOH buffer (pH 8.0, 9.0 and 10.0)

Stock solutions: 0.2 M glycine; 0.2 M NaOH.

Combine 25 mL glycine stock with x mL 0.2 M NaOH and dilute with distilled water to make 100 mL solution.

0.2 M NaOH (mL)	pH
2.0	8
6.0	9
19.3	10

APPENDIX III

Standard curve of Glucose

CMCase activity was measured by the reducing sugars formed in a modified dinitrosalicylic acid (DNS) method (Miller, 1959) using xylose as the standard.

Materials:

a. Stock: 2 mg/mL glucose

b. DNS reagent

Distiiled Water	1415 mL
3, 5-Dinitrosalicyclic acid	10.5 g
NaOH	19.8g
Dissolve the above and then add:	
Rochelle salts (Na-K tartatate)	306 g
Phenol (melt at 50°)	7.6 mL
Sodium metabisulphite	8.3 g

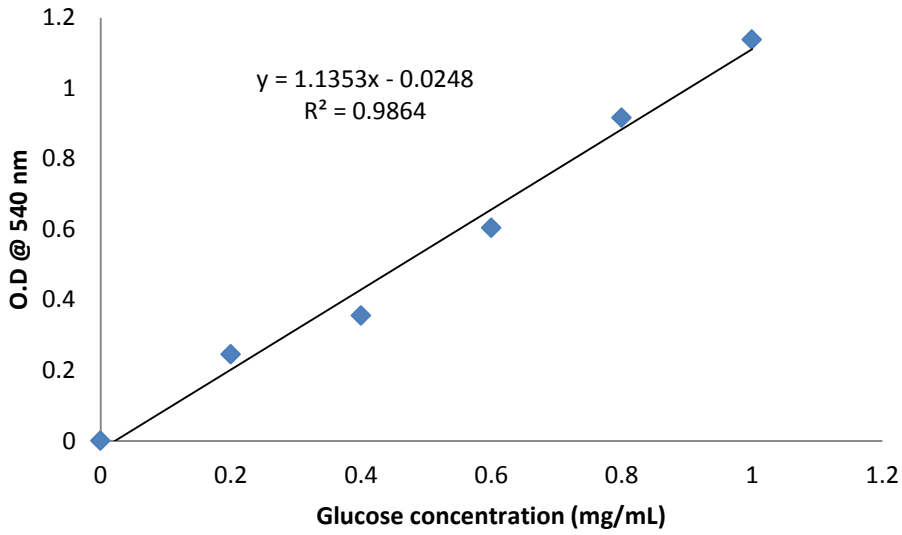

Figure 10: Standard curve of glucose

Protein determination method

Protein concentration was determined by Folin's protein determination method (Lowry et al., 1951), standard curve was drawn using BSA as standard.

Procedure:

Reagent A	20 g Na_2CO_3
	4 g NaOH
	Add distilled water to make 1000 mL
Reagent B-1	1 g $CuSO_4.5H_20$
	Add distilled water to make 100 mL
Reagent B-2	2 g Na-K Tartarate
	Add distilled water to make 100 mL
Reagent C	(Keep only one day)
	1 mL Reagent B-1
	1 mL Reagent B-2
	100 mL Reagent A
	Mix in this order

(1 N) Phenol Reagent - Dilute Folin Ciocalteau Reagent (2 N) with an equal volume Trichloroacetic acid (10%) in water.

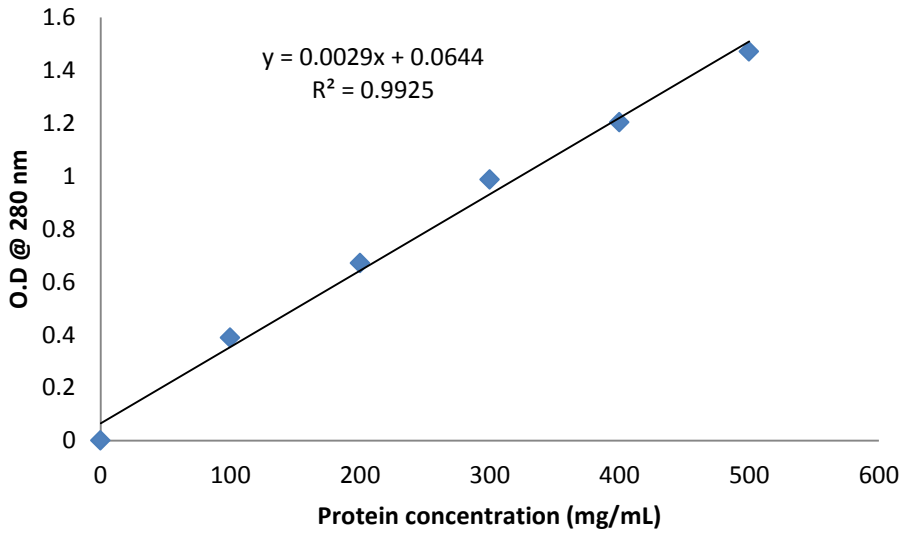

Figure 11: Standard curve of Protein concentration

APPENDIX IV

Analysis of proteins were done through sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using the method as developed by Laemmli, (1970). All solutions for SDS-PAGE were made as follows:

1. 10 x SDS-PAGE running buffer

30.3 g Tris (Hydroxymethyl) Aminomethane

144.0 g Glycine

10.0 g Sodium dodecyl sulphate (SDS)

These compounds were dissolved in 1 L distilled water and stored at room temperature until required. When required for electrophoresis, the running buffer was diluted 10 times with distilled water to obtain the required volume.

2. 10% SDS stock solution

10 g SDS was dissolved in 100 mL distilled water.

3. 10% Ammonium persulphate (APS) solution

0.1 g APS was dissolved in 1 mL distilled water

This solution was prepared freshly prior to preparing a gel.

4. 30% acrylamide stock solution

29.8 g acrylamide

0.2 g bis-acrylamide

These compounds were dissolved in distilled water and then made up to 100 mL volume. The bottle was wrapped in aluminium foil to protect it against light and stored at 4°C.

5. Stacking gel buffer (0.5 M Tris-HCl, pH 6.8)

6 g Tris (Hydroxymethyl) Aminomethane

This was dissolved in distilled water and the pH was adjusted with hydrochloric acid (HCl) to a pH of 6.8 before the solution was made up to 100 mL. This buffer was stored at 4°C.

6. Resolving gel buffer (1.5 M Tris-HCl, pH 8.8)

18.15 g Tris (Hydroxymethyl) Aminomethane

This was dissolved in distilled water and the pH was adjusted with hydrochloric acid (HCl) to a pH of 8.8 before the solution was made up to 100 mL. This buffer was stored at 4°C.

7. SDS sample buffer (5x)

2.5 mL distilled water

1 mL 0.5 M Tris-HCl buffer (pH 6.8)

3 mL glycerol

2 mL 10% SDS stock solution (w/v)

1 mL 1% bromophenol blue (w/v)

The sample buffer was stored at room temperature. Prior to preparing samples for electrophoresis, 5 µL β-mercaptoethanol was added to 95 µL of SDS sample buffer. SDS sample buffer (3 µL) was added to 15 µL of protein samples before boiling and electrophoresis.

8. Coomassie Brilliant Blue protein staining solution

The staining solution was prepared by dissolving 0.075% (w/v) Coomassie Brilliant Blue R250 in a solution of 30% methanol and 10% glacial acetic acid. Staining of polyacrylamide gels was done for a minimum of 1 h or overnight on a platform shaker.

9. Coomassie destain solution

Destaining solution was prepared by mixing 30% methanol, 60% distilled water and 10% glacial acetic acid. Gels were placed in destain solution on a platform shaker until protein bands appeared against a clear background. Gels were then placed in distilled water.

10. Preparation of SDS-PAGE gels

Resolving gel

The 12.5% SDS-PAGE resolving gels were prepared in a small beaker by adding the following solutions in sequence:

3.13 mL distilled water

2.5 mL 1.5 M Tris-HCl buffer (pH 8.8)

4.17 mL 30% acrylamide stock solution

0.1 mL 10% SDS stock solution

0.1 mL 10% APS solution

0.05 mL TEMED

The solution was lightly mixed by swirling the beaker before pouring the gel. Gels were then covered with about 50-100 µL of isopropanol and allowed to set. For zymograms, 0.5 mL of a 2% (w/v) stock solution of birchwood xylan, CMC or locust bean gum, or 1.5 mL of a 2% (w/v) pectin solution, was added to the resolving gel solution and the distilled water reduced by the same volume. In the case of birchwood xylan, CMC and pectin, the solution was added to the resolving gel solution prior to the APS solution. However, in the case of locust bean gum, the substrate was first dissolved in the correct volume of boiled distilled water. As the locust bean gum is highly viscous, this ensured a homogeneous solution of the substrate in the gel.

The 4% stacking gels were prepared in a small beaker by adding the following solutions in sequence:

3.6 mL distilled water

0.63 mL 0.5 M Tris-HCl buffer (pH 6.8)

0.66 mL 30% acrylamide stock solution

0.05 mL 10% SDS stock solution

0.01 mL 10% APS solution

0.005 mL TEMED

The isopropanol was removed from the resolving gel with some filter paper. The stacking gel solution was poured on top of the resolving gel before plastic combs were inserted to form the wells.